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Induction of Prdx1 and Prdx6 in Liver Cells by Serum and TPA

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Abstract

Peroxiredoxins are thiol-specific antioxidants that protect cells from oxidative damage and have proliferative and anti-apoptotic activity. We investigated the effect of serum and phorbol ester treatment on expression of Prdx1 and Prdx6 in H2.35 cells, and the possible role of Sp1 on Prdx6 induction. Serum stimulation induced a 30% increase in Prdx1 mRNA and a three-fold increase in Prdx6 mRNA. We showed a similar effect of phorbol ester treatment, which led to a 30% increase in Prdx1 mRNA, and over a two-fold increase in Prdx6 expression. Analysis of the Prdx6 proximal promoter sequence revealed four consensus Sp1 sites. Inhibition of Sp1 with mithramycin A blocked Prdx6 induction by TPA and inhibited the serum-induced transcriptional activity of the Prdx6 proximal promoter. These data suggest an important role for Prdx6 in the cellular response to serum and TPA, and implicate Sp1 as a possible mediator of Prdx6 regulation.

Keywords: Peroxiredoxin, Prdx1, Prdx6, H2.35, Antioxidant, TPA, Serum

1. Introduction

Reactive oxygen species (ROS) are unstable free radical derivates of oxygen formed as byproducts of many biochemical processes in the cell. ROS serve critical functions for many types of cells (Halliwell and Gutteridge, 1999), and recently have been implicated as key regulators of signal transduction (Immenschuh and Baumgart-Vogt, 2005). However, when concentrations of ROS become too high, macromolecules can become oxidized, causing detrimental cellular effects such as cell death (Halliwell and Gutteridge, 1999). These consequences have pathophysiological relevance, as oxidative stress is linked to the development of a number of diseases including cancer and neurodegenerative disease (Cross et al., 1987; Sun and Chen, 1998; Gius and Spitz, 2006).

Peroxiredoxin proteins are thiol-specific antioxidant enzymes that reduce various cellular peroxide substrates using cysteine-containing active sites (Hofmann et al., 2002; Rhee et al., 2005). Mammals possess six members of the peroxiredoxin gene family, and the encoded proteins are classified into three groupings based on the number and position of active site cysteines: the 2-Cys type (*Prdx1-4*), the atypical 2-Cys Prdx (*Prdx5*), and the 1-cys member (*Prdx6*). In addition to their structural differences, these proteins vary in peroxide substrate specificity, cellular localization, tissue expression, and additional enzymatic activities (Hofmann et al., 2002; Rhee et al., 2005). Based on their functions,

peroxiredoxins can protect cells from ROS-induced cell damage and apoptosis, and regulate redox sensitive cell signaling involved in processes such as cell proliferation, differentiation, and transformation (Hofmann et al., 2002; Immenschuh and Baumgart-Vogt, 2005; Rhee et al., 2005). They now have been implicated in the modulation of disease states such as neurodegenerative diseases and cancer (Karihtala et al., 2003; Kinnula et al., 2004; Arner and Holmgren, 2006).

Prdx6 is the only peroxireodoxin that contains a single conserved cysteine residue, and it utilizes glutathione as a reductant (Kang et al., 1998; Hofmann et al., 2002; Rhee et al., 2005). Unlike other peroxiredoxins, Prdx6 reduces both aqueous and lipid peroxides and possesses distinct phospholipase A2 activity (Chen et al., 2000; Manevich et al., 2004; Manevich and Fisher, 2005). As a result of these functions, Prdx6 appears to be involved in phospholipid metabolism and membrane turnover (Manevich et al., 2002; Manevich and Fisher, 2005), and we and others have demonstrated that Prdx6 protects cells from ROS-mediated membrane damage and apoptosis (Manevich et al., 2002; Pak et al., 2002; Phelan et al., 2003; Wang et al., 2003; Wang et al., 2004; Wang et al., 2006; Wang et al., 2006). Prior studies from our lab have reported an abundance of Prdx6 in liver (Sparling and Phelan, 2003; Wang et al., 2003), and its transcriptional regulation by various oxidative stresses in H2.35 cells, a virally transformed mouse hepatocyte cell line (Sparling and Phelan, 2003; Simeone and Phelan, 2005; Gallagher and Phelan, 2007). A recent report demonstrated that Prdx6-knockout mice exhibit a significant increase in hepatocellular injury (Eismann et al., 2009), confirming an important antioxidant role for this protein in liver. Prdx1, the most ubiquitous peroxiredoxin, is also highly expressed in liver (Ishii et al., 1993; Ishii et al., 1995; Ishii et al., 2000), although its regulation has largely been studied in non-liver tissues. Originally named PAG (proliferation associated gene) in human (Prospéri et al., 1993), Prdx1 is overexpressed during cell proliferation and is transcriptionally induced by serum in human mammary epithelial cells (Prospéri et al., 1993). Prdx1 is also induced by the tumor promoter phorbol-12-myristate-13-acetate (TPA) in liver tissue macrophages (Hess et al., 2003). Since liver tissue is susceptible to high levels of ROS associated with normal cellular physiology and acute and chronic injury (Novo and M, 2008), we sought to compare Prdx1 and Prdx6 induction by serum and phorbol ester in H2.35 cells. Given that these proteins represent two distinct members of the peroxiredoxin family expressed in liver, understanding their regulation in hepatocytes can provide important insight into their relative roles in the cellular response to stress in this tissue.

2. Materials & Methods

2.1 Cells, Inducers & Inhibitors

The H2.35 hepatocyte cell line (ATCC) was cultured in DMEM with 4% FCS and 0.2μM dexamethasone, and grown at 33°C in a 10% CO_2 atmosphere. Phorbol-12-myristate-13-acetate (TPA) (Cell Signaling Technologies) was supplied as a 200 μM stock in DMSO. Mitrhamycin A (Tocris) was dissolved in DMSO to 10 mM, and subsequently diluted to 10 μM in PBS.

2.2 Real-Time PCR

For real-time PCR studies, H2.35 cells were plated into 96-well plates at a cell density of approximately 20%, and cultured under appropriate experimental conditions. At the time of harvest, media was removed and adherent cells were rinsed with cold 1X PBS. Cell lysates and reverse transcriptase reactions were performed using the Cells-to-CT Kit (Applied Biosystems). Briefly, cells were lysed in Cell Lysis solution containing DNAseI for five minutes, followed by two-minute incubation with the stop solution. Cell lysates were immediately used for RT reactions, or were stored temporarily at -20 degrees, to be used within 2 weeks. Fifty-microliter reverse transcription reactions were performed using 10 μl of each cell lysate, according to the manufacturer's suggestions. Twenty-μl PCR reactions were performed in triplicate for each target gene using 2 μl RT reaction, 7 μl water, 10 μl of 2X Taqman PCR buffer (Cells-to-CT kit), and 1 μl of the appropriate mouse TaqMan assay: Prdx1 (Mm01621996_s1), Prdx6 (Mm00725435_s1), beta-actin endogenous control (4352933E), or 18s control (4333760F). To ensure that PCR amplification was not due to genomic DNA contamination, parallel RT-reactions were performed in the absence of reverse transcriptase for each experiment. In all cases, reactions from RTs lacking reverse transcriptase amplified no product, or resulted in cycle threshold (Ct) values beyond 35 and seven CTs higher than the positive reactions, representing negligible genomic DNA contamination. Relative expression was calculated using the C_T method (Livak and Schmittgen, 2001), with each target gene normalized against beta-actin levels for the same sample. An appropriate calibrator was chosen for each analysis, and all other samples were calibrated against that sample. All treatments were done in triplicate wells, and experiments were repeated.

2.3 Western Blotting

H2.35 cells were cultured in 60 mm dishes to near confluence under appropriate experimental conditions. Media was discarded, and cells were washed with 2 mL of cold 1X PBS. Cells were lysed in 100 μl of M-PER reagent (Thermo Scientific) at room temperature rocking for 10 minutes, and lysates were collected, spun to remove cell debris, and supernatants transferred to tubes. Total protein was quantified using the Biorad Protein Assay (Biorad) according to manufacturer's recommendations. Twenty-five μg of protein from each sample were separated on 10% tris-HCL gel (Biorad) under denaturing conditions and transferred to PVDF membranes. Membranes were blocked for 15 minutes in

4% skim milk in TBST, and incubated overnight at 4°C with 1:1,000 dilution of Prdx6 antibody (Biosensis, R-169-100) and 1:5,000 dilution of beta-actin antibody (Sigma, A5441) diluted in 1% dry milk in TBST. Blots were rinsed 3 x15 minutes in TBST and incubated for one hour at room temperature with alkaline phosphatase-linked secondary antibodies (Sigma) diluted in 1% dry milk in TBST. Blots were again rinsed 3 x 15 minutes in TBST, followed by 5 minutes in TBS, and then BCIP/NBT substrate was added until band appearance. Reactions were stopped with distilled water.

2.4 Synthesis of Deletion Constructs & Transfections

Various deletions of the *Prdx6* mouse promoter had previously been cloned into the pSEAP2-Basic vector (Clontech) by our laboratory, as previously described (Gallagher and Phelan, 2007). These included constructs containing the mouse *Prdx6* promoter fragment that extends to nucleotide position -184 (relative to the +1 transcription start site). Primers used to amplify the -184 fragment were: Prom-184 forward (5'-CGCTCGAGTACAAGTCCCCGCAATTCTC-3') and PromReverse (5'- CGAAGCTTGTGGTGACGC TGAGAACAAGG-3'). The PCR products were amplified and cloned into the pSEAP2-Basic reporter vector (Clontech) as previously described. H2.35 cells were seeded into 48-well cell culture plates and grown overnight. For each transfection, 0.4 μg of the -184-SEAP2 construct and 0.1 μg of the pGluc-control plasmid were mixed with lipofectamine 2000 reagent (Invitrogen) according to manufacturer's suggestions. Cell media in each well was replaced with either normal growth media or serum free/dexamethasone free media and transfection mixes were added. For Sp1-inhibition studies, mithramycin A was added two hours after transfection. Transfected cells were incubated under normal growth conditions for five days and media collected and spun to remove debris.

2.5 Reporter Assays

The QUANTI-Blue Reagent (Invivogen) was used to measure SEAP levels in cell supernatants. The reagent was freshly prepared from the supplied powder by dissolving one packet in 100 ml sterile water, and filtering through a 0.2 μm membrane. For each assay, 20 μl of sample media were added to 200 μl of QUANTI-Blue reagent, and incubated at 37°C for 24 hours. The absorbance at 655 nM was measured for each sample, and blanked against reagent plus 20 μl appropriate media. For luciferase expression, the Gaussia Luciferase Assay was used (New England Biolabs). Briefly, 15 μl of each media sample was added to a separate well of a 96 well flat bottom white plate. Using the Tecan Infinite F200 microplate reader (Tecan), 50 μl of reconstituted luciferase substrate was auto-injected into each well, mixed for 2 seconds, and integrated for 5 seconds to measure luminescence. All SEAP levels were normalized against relative luciferase levels.

2.6 Sequence Analysis

The proximal 184 nucleotides of the mouse Prdx6 promoter were analyzed for putative transcription factor binding sites using TFSEARCH version 1.3 (Heinemeyer et al., 1998).

3. Results

3.1 Induction of Prdx1 and Prdx6 mRNA by Serum and TPA

We previously determined that Prdx6 expression is significantly inhibited in H2.35 cells upon serum deprivation (Gallagher and Phelan, 2007). To quantify this effect and compare this response to that of Prdx1, we examined the expression of both genes in the presence and absence of serum. As shown in Figure 1A, Prdx1 expression is moderately, but significantly, increased (by approximately 30%) in complete growth media as compared to serum-deprived conditions ($p<0.05$). In contrast, Prdx6 expression is elevated three-fold in the presence of serum ($p<0.001$). These data indicate that Prdx6 is more highly elevated in proliferating vs. quiescent cells as compared to Prdx1. Since Prdx1 is regulated by TPA in other cells (Hess et al., 2003), we also examined the regulation of Prdx1 and Prdx6 by TPA in H2.35 cells. As shown in Figure 1B, TPA also induced the expression of both genes, but with a much greater effect on Prdx6. Prdx1 was upregulated by TPA by approximately 30% after 8 hours ($p<0.005$). After 8 hours of TPA treatment, Prdx6 was induced approximately two-fold ($p<0.005$). These data suggest that both genes may be involved in the cellular response to these stimuli, but possibly to different degrees.

3.2 Induction of Prdx1 and Prdx6 Protein by Serum and TPA

To determine if the significant increase in Prdx6 mRNA in response to serum and TPA resulted in a corresponding protein induction, we used western blotting to measure Prdx6 protein levels before and after stimulation. As shown in Figure 2A, the addition of serum to serum-deprived H2.35 cells for eight hours led to an increase in Prdx6 protein expression. Likewise, eight hours of TPA stimulation also resulted in an increase in Prdx6 protein levels. This data suggests that serum and TPA result in a marked increase in the amount of Prdx6 protein in H2.35 liver cells.

3.3 Identification of Sp1 Sites in Prdx6 Promoter

Based on the marked upregulation of Prdx6 in growing cells and in response to TPA, we were interested in identifying potential transcriptional regulators of Prdx6 that may mediate its induction by one or both of these stimuli. We previously

synthesized several deletion constructs of the mouse Prdx6 promoter, linked to the pSEAP2-basic reporter gene (Gallagher and Phelan, 2007). We analyzed the smallest of these fragments, containing the proximal 184 nucleotides of the mouse Prdx6 promoter, for consensus transcription factor binding sites against the TransFac4.0 database (Heinemeyer et al., 1998). We found three putative Sp1 sites in this sequence, shown in Figure 3 (with the location of the consensus Sp1 sites indicated). In addition, we identified consensus E-box sequences which are putative binding sites for Upstream Stimulatory Factor (USF) and Myc.

3.4 Effect of Mithramycin A on Prdx6 Induction

To test whether Sp1 is involved in the regulation of Prdx6 in growing H2.35 cells, we compared SEAP reporter expression from the SEAP-184 construct in the presence and absence of Mithramycin A, a potent Sp1 inhibitor. As shown in Figure 4A, SEAP expression driven by this proximal promoter is almost completely inhibited by Mithramycin A ($p<0.05$). There is no significant difference between serum-stimulated reporter expression in the presence of mithramycin A and levels found in the absence of serum, suggesting that Sp1 sites in the proximal promoter are largely responsible for the transcriptional activity of this region in growing cells. We also wanted to test whether Sp1 is also involved in the induction of Prdx6 in response to TPA. Experiments using TPA induction of the SEAP-184 construct resulted in no induction (data not shown) suggesting that this proximal promoter is not sufficient to drive induction by TPA. Therefore, we measured Prdx6 expression by real time PCR in serum-deprived cells treated with and without mithramycin A. As shown in Figure 4B, Mithramycin A treatment partially inhibited the TPA-stimulated induction of Prdx6 ($p<0.05$). Although the level of Prdx6 expression in the presence of mithramycin A was significantly suppressed, it was not as low as serum-deprived levels (data not shown).

4. Discussion

4.1 Summary

The mammalian peroxiredoxin family of proteins includes structurally and functionally distinct members that protect different cell types from oxidative stress-induced damage. However, the precise role for these proteins in different cells, and the mechanism by which they are transcriptionally regulated in response to oxidative insult, remains unclear. In the present study, we compared the regulation of Prdx1 and Prdx6 in the H2.35 cell line using real time PCR. We showed that both genes are induced at the mRNA level in response to serum and TPA, with a much more robust effect on Prdx6. Prdx6 induction translated into a corresponding increase in Prdx6 protein expression. We further showed that treatment with mithramycin A resulted in a partial block of TPA-stimulated induction of Prdx6. Examination of the proximal Prdx6 promoter containing four Sp1 sites further showed complete inhibition of serum-stimulated promoter activity upon mithramycin A treatment. Together, these data suggest that Prdx6 is particularly responsive to serum and TPA stimulation in H2.35 cells, and implicate Sp1 as a possible mediator of Prdx6 induction.

4.2 Regulation of Peroxiredoxins by Serum

The cellular regulation of peroxiredoxin activity is known to occur at many levels, suggesting that tight control of this family is critical (Rhee et al., 2005). A wide variety of oxidative stress-inducing agents, including growth factors and cytokines, stimulate peroxiredoxin expression at the level of gene expression. Prdx1 mRNA is upregulated by hypoxia, H_20_2, and tert-BOOH (Prosperi et al., 1998; Li et al., 2002; Rhee et al., 2005; Kim et al., 2007), as well as serum (Prosperi et al., 1998). Likewise, Prdx6 expression is induced by hyperoxia, H_20_2, and nitric oxide (Kim et al., 2002; Kim et al., 2003; Sparling and Phelan, 2003; Simeone and Phelan, 2005; Chowdhury et al., 2007; Diet et al., 2007; Gallagher and Phelan, 2007; Chowdhury et al., 2009), and also by growth stimuli including keratinocyte growth factor (KGF) (Frank and Werner, 1997; Munz et al., 1997; Sparling and Phelan, 2003; Gallagher and Phelan, 2007), lens epithelium-derived growth factor (LEDGF) (Fatma et al., 2001), and TNF-α (Kubo et al., 2006; Gallagher and Phelan, 2007). One of the most significant differences we observed between Prdx1 and Prdx6 in the present study is their expression in growing vs. quiescent cells. The observation that Prdx6 is three times higher in growing H2.35 cells vs. serum deprived cells suggests an important role for Prdx6 in this growth response. We previously reported the upregulation of Prdx6 in H2.35 cells by serum (Sparling and Phelan, 2003; Simeone and Phelan, 2005; Gallagher and Phelan, 2007), and others have demonstrated increased Prdx6 in hyperproliferative epithelium of wounded and psoriatic skin (Kumin et al., 2006). In addition, Prdx6 levels seem to be inversely correlated with TGF-β levels in the lens (Kubo et al., 2006). Together, these data implicate Prdx6 in the maintenance of cellular homeostasis in proliferating cells.

4.3 Regulation of Peroxiredoxins by TPA

TPA is both a known tumor promoter and inducer of apoptosis in various cell types. Although the mechanism by which it elicits these two effects is unclear, the requirement of ROS in TPA-induced toxicity has been demonstrated (Lin et al., 2006). Induction of Prdx1 by TPA in H2.35 cells is not surprising given a prior study reporting Prdx1 induction by TPA in rat liver tissue macrophages and monocytic cells through a PKC/Ras/p38MAPK pathway (Hess et al., 2003). While this study reported nearly eight-fold induction by six hours, our results suggest a more modest effect on Prdx1 in H2.35 cells. The more dramatic induction of Prdx6 by TPA may represent the relative importance of these proteins in these cells, or

may simply suggest a more critical role for Prdx6 in the TPA response, as prior studies have not examined induction of Prdx6 by TPA in any cell line.

4.4 Potential Role of Sp1 in Prdx6 Regulation by Serum

Previous studies from our lab identified the proximal -184 bp of the mouse Prdx6 promoter as sufficient to drive reporter expression in growing H2.35 cells. Our present observations that mithramycin A inhibits this induction to a level comparable to that observed in serum-derived cells implicates Sp1 as a possible regulator of this activity. Sp1 is a redox-regulated transcription factor that has been implicated in the control of cell-cycle genes in normal cell proliferation and cancer (Safe and Abdelrahim, 2005). A prior study demonstrated that the middle Sp1 site bound to Sp1 in gel shift assays (Lee et al., 1999), although the other sites were not examined and corresponding transcriptional activity was never reported. Given its known role in proliferation, it is possible that Sp1 activity is upregulated in growing H2.35 cells and induces Prdx6 as a mechanism of balancing the increased cellular ROS. Since mithramycin A binds to many G-C rich DNA sequences, it is also possible that one or more other DNA binding proteins are responsible for, or contribute to, the serum-stimulated Prdx6 promoter activity seen in H2.35 cells. In addition to the Sp1 sites, the proximal promoter includes two consensus E-box sequences for Upstream Stimulatory Factor (USF) and Myc (shown in Figure 3). Interestingly, Prdx1 binds to the myc protein and suppresses its function (Mu et al., 2002), and a recent study found a myc-dependent regulation of Prdx5 by Prdx1 (Graves et al., 2009), demonstrating myc as a peroxiredoxin regulator. Mithramycin A is also known to suppress myc DNA binding activity, so the inhibitory effect of mithramycin A on Prdx6 induction by serum and TPA may be attributed to suppression of myc, rather than Sp1. This will require further investigation.

4.5 Other Possible Mechanisms of Prdx6 Regulation

The inhibitory effect of Mithramycin A on Prdx6 stimulation by TPA suggests that Sp1 may be involved in mediating part of this response. TPA has been shown to increase transcription of another antioxidant gene, manganese superoxide dismutase, through activation of Sp1 (Porntadavity et al., 2001). Despite a possible role for Sp1 in this activation, the inability of the proximal promoter to respond to TPA suggests that Prdx6 upregulation by TPA likely requires additional upstream regulatory elements and other transcription factors. A recent study revealed that the human Prdx6 gene is transcriptionally regulated through an antioxidant response element (Chowdhury et al., 2009), although a similar element is not found in the mouse promoter. In addition to the previously mentioned consensus sequences, two putative AP1 sites are found between 340 and 470 nucleotides upstream of the transcription start site. It is worth noting that the TPA-dependent induction of Prdx1 reported by Hess et al. was mediated by two proximal AP-1 sites targeted by c-Jun (Hess et al., 2003). The investigation of these potential regulatory elements and the corresponding DNA binding proteins will provide greater insight into the transcriptional regulation of Prdx6. Together with additional functional analyses these studies will help to reveal the precise role for this protein in liver cell physiology and stress responses.

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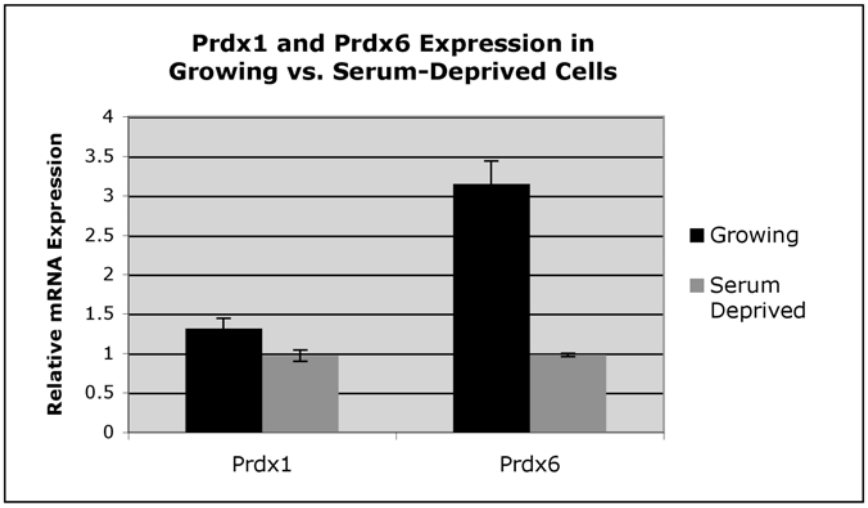
A



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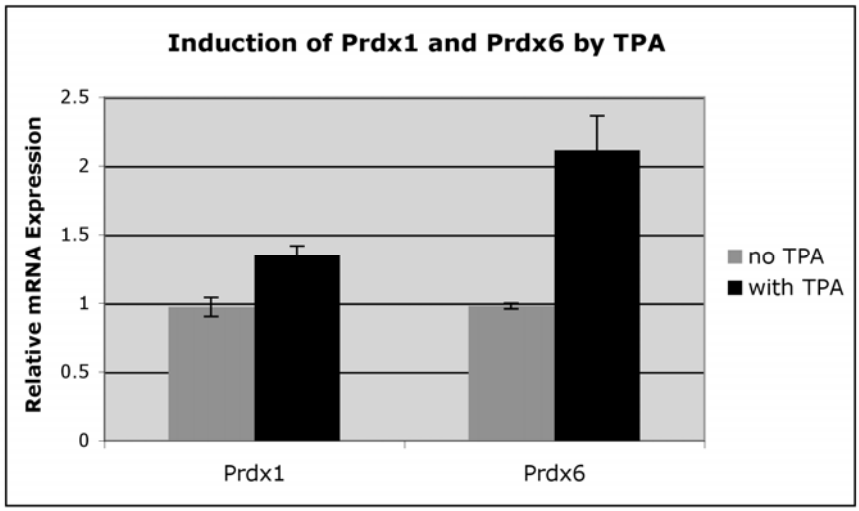
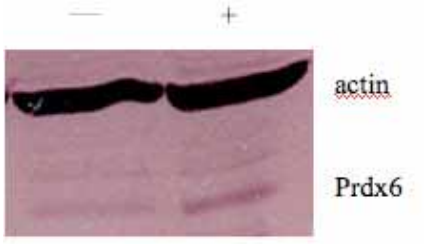


Figure 1. Induction of Prdx1 and Prdx6 by Serum and TPA

H2.35 cells were cultured in complete growth media or serum-free/dexamethasone-free media for 24 hours (A) and serum-free/dexamethasone-free treated cells were subsequently treated with or without 0.5 μM TPA for an additional eight hours (B). All cells were lysed, RT-reactions were performed, and *Prdx1*, *Prdx6*, and *beta-actin* mRNA levels were quantified by Real-Time PCR as described in Materials & Methods. Relative expression was calculated using the C_T method, normalizing to actin and calibrating against the serum-deprived expression level for each gene. Averages of three independent replicates are shown (+/- SDM).

A



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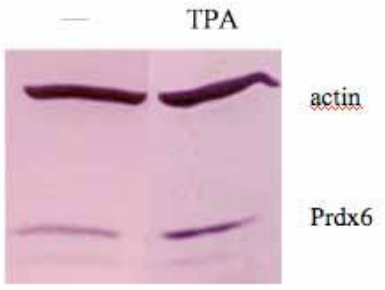


Figure 2. Prdx6 Protein Induction by Serum and TPA

(A) Western blot of Prdx6 and beta-actin in H2.35 cells under serum deprived and growing conditions. (B) Western blot of Prdx6 and beta-actin in serum deprived H2.35 cells in the absence or presence of 0.5 μM TPA for eight hours.

TACAAGTCCCCGCAATTCTCGGTCTTGCGCTTCCTTCCTATTGCACGTGGCCTTCTTTAGCTCCAGAATC
USF / MYC

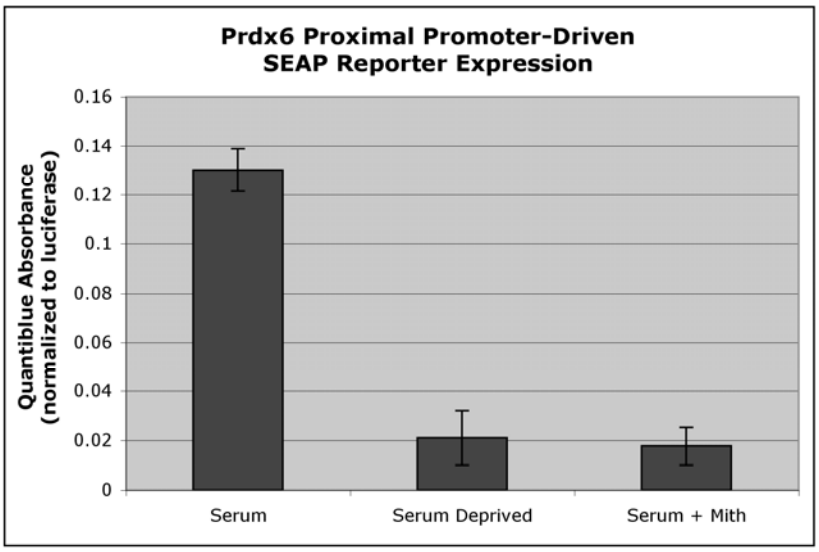
TCCCTTGCCTGCAAGCTCCACGTGAAGAACCGCAGCCGCCAGACTCGCGGTCGCCCCACCCCGCCCCA
USF / MYC Sp1

GCCCCGCCCCAGCCCCGCCCACTCGGCCAGCACTGATCTAGGTCTC
Sp1 Sp1

Figure 3. Prdx6 Proximal Promoter Sequence

Nucleotide sequence of the proximal 184 nucleotides of the mouse Prdx6 promoter. Nucleotide number is relative to the transcription start site for the gene. This sequence corresponds to the region used in the SEAP reporter analysis.

A



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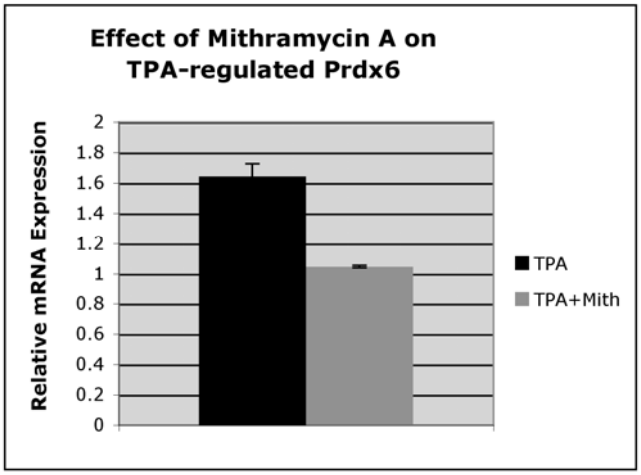

Figure 4. Effect of Mithramycin A on Prdx6 Regulation.

(A) H2.35 cells were transfected with pGluc control vector and SEAP2-184 reporter construct in the indicated media. Two hours after transfection, cells were treated with or without 100 nM mithramycin A, and subsequently cultured under the indicated conditions for five days. SEAP and luciferase activity were measured as described in Materials and Methods. Averages of normalized SEAP reporter expression for three independent replicates are shown (+/- SDM). (B) H2.35 cells were cultured in serum-free/dexamethasone-free media and treated with 0.5 μM TPA for eight hours in the presence or absence of 100 nM mithramycin A. All cells were lysed, RT-reactions were performed, and *Prdx1*, *Prdx6*, and *18S* mRNA levels were quantified by Real-Time PCR as described in Materials & Methods. Relative expression was calculated using the C_T method, normalizing to 18s. Averages of three independent replicates are shown (+/- SDM).

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Localization of Guanylate Cyclase Receptor, Inositol Trisphosphate Receptor, and Calmodulin in Boar Spermatozoa

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Abstract

Several proteins present on the sperm membranes serve as signal transducers facilitating sperm maturation, capacitation, acrosome reaction and fertilization. Among them, guanylate cyclase (GC), the inositol trisphosphate receptor (IP_3R) and calmodulin have been reported rather extensively. Although biochemical data clearly suggest their roles in the fertilization process, the precise location of these proteins in the sperm membranes have not been adequately investigated and need clarification. Using immunolocalization procedures we report that guanylate cyclase receptors (GCR) are localized on the apical region of the acrosome and on the postacrosomal plasmalemma, while IP_3Rs are present in the neck region of the sperm and at lower density along the axonemal membrane. In contrast, calmodulin is restricted to the postacrosomal, basal nuclear membrane and the neck region that corresponds to the redundant nuclear envelope and ER vesicles, with some reaction along the middle piece and tail membranes. Colocalization of these proteins on the sperm membrane together with biochemical data as reported previously provide further insight into their role in the spermatozoan motility and possibly the fertilization process.

Keywords: Boar sperm, Calmodulin, Inositol trisphosphate receptor, Guanylate cyclase

1. Introduction

It is well established that a complex set of biochemical events in mammalian spermatozoa is triggered by several proteins, present on sperm membranes, that are involved in capacitation, acrosome reaction, and fertilization (deKretser et al., 1998). Receptors for cyclic guanosine monophosphate (cGMP) [Bentley and Garbers, 1986, Revelli et al., 2001, 2002, Willipinski–Stapelfeld 2004], cyclic adenosine monophosphate (cAMP) [Kurose et al., 1987, Chang et al., 1990, Harrison and Miller 2000], and inositol 1,4,5-trisphosphate (IP3), [Schilling et al., 1992, Walensky and Snyder, 1995] have been implicated in these processes. The actual mediator of signal transduction appear to be an increase in intracellular Ca^{2+} through voltage gated calcium channels into the sperm membranes (Bentley, et al., 1987, Arab et al., 1990, Verma et al., 1992). cGMP appears to be essential in transporting calcium through ion gated channels into the sperm membranes

(Ramaroa and Garbers. 1985, Wedel and Garbers 2001, Revelli et al., 2002, Sharma 2002), and guanylate cyclase, the ubiquitous enzyme, catalyzes the conversion of GTP to cGMP mediated by nitric oxide (NO) [Bentley et al., 1986]. The peptide mediated protein phosphorylation in spermatozoan membranes appears to be due to enhanced formation of cGMP (Bentley et al., 1986). In capacitated spermatozoa it is known that the acrosome reaction (AR) can be induced by Ca^{2+} ionosphere A23187, an essential step in mammalian fertilization (Jaiswal et al 1998). IP3 has been implicated in the regulation of Ca^{2+} from intracellular membrane stores during hyperactivated motility in several sperm species (Suarez and Osman, 1987, Ho and Suarez, 2001, 2003). IP3 may also be implicated in the acrosome reactions in several species (Bird et al.; 1991). Increase in intracellular Ca^{2+} through IP3-gated channels may therefore be associated with a signaling cascade leading to cell motility, the acrosome reaction, and capacitation (Brass and Joseph, 1985).

The localization GCR, IP3R and calmodiulin on the sperm membranes in boar sperm will give us further insight into their role in the process leading to the various sperm functions.

2. Methods and Materials

2.1 Preparation of Boar Sperm

Boar semen was obtained commercially (Zierke Morris MN.). Only those samples with 90% or more sperm motility were used for the study. Sperm were isolated from semen using PBS-Percoll gradient solution. The gradient was prepared by layering 10 ml of 35% Percoll over 10 ml of 70 % Percoll solution. Approximately 4.5 x 10^8 sperm in 10ml Tyrode's solution was layered over 35% Percoll solution, in a 50 ml centrifuge tube and centrifuged at 2,000 x g for 5 min followed by 4,000 x g for 10 min. The fluffy mass of sperm near the middle of 70% Percoll layer was aspirated using a pipette and transferred to a clean centrifuge tube. The sperm were resuspended in PBS and recentrifuged at 400 x g for 15 min.

2.2 Preparation of anti ROS–GC1 Antibody

The anti-ROS-GC1 antibody was a gift from Dr. Teresa Duda. (**Penn College of Optometry, Elkine Park, PA**). The antibody was prepared in rabbit against c-terminal portion of the protein amino acid 1016-1054. Ca^{2+} ROS-GS1antibody has been reported in the testes (Jankowska et al., 2007**)**. It is known that Cyclic GMP produced by ROS-GC1 regulates the activity of nucleotide gated channel which is present in the testes (Sharma 2002**).**

2.3 Immunocytochemical Procedures

Sperm were fixed in 4% paraformaldehyde in PBS for one hour, washed with PBS and blocked in PBS containing 1% nonfat dry milk. The sperm were washed three times with PBS and incubated with the rabbit monoclonal antibodies at 1:500 dilution, GC (guanylate cyclase ROS-GC1), rabbit monoclonal antibodies against IP_3R1 (Abcam Inc, Cambridge, MA), and mouse anti Calmodulin (Abcam Inc., Cambridge, MA) antibodies at 1:500 dilution for one hour. Sperm were washed three times with PBS and incubated with secondary goat and rabbit antibodies conjugated to Alexa fluorophores were obtained from Invitrogen (Molecular Probes). 4',6'-Diamidino-2-phenylindole (DAPI) [Ted Pella, Redding, CA] was used as counter staining for the nucleus, and images were obtained in a Zeiss 10Z0 fluorescence microscope

2.4 Transmission Electron Microscopy and Immunolabeling

Washed sperm isolates were centrifuged at 4000 X g for 5 minutes, pelleted and fixed by immersion in 2% paraformaldehyde-glutaraldehyde fixative in 0.1 M sodium cacodylate buffer (pH 7.2) for 1hour. The specimen was postfixed in 2% OsO_4 for 1hr, dehydrated in a graded series of ethanol to 100%, and infiltrated using 1:1 alcohol and Spurrs (Ted Pella, Redding, CA**)** for 2 hrs and embedded in Spurrs. Ultrathin sections (~ 90 nm) were cut using a diamond knife and stained with 1% uranyl acetate and 1% lead citrate. For immunolabeling the sections on nickel grids were blocked in 1% (w/v) bovine serum albumin for 30 min and washed three times with water before floated onto affinity-purified primary antibodies (1:500 in blocking buffer) for 1 hour. The grids were washed 5 times (10 min each wash) with water and incubated in secondary antibody 1:500 (15nm Protein A-gold colloidal particles conjugated-anti-rabbit antibody **(**E-Y Labs, Inc., San Mateo, CA) for 45 min and washed 5 times with water. The grids were stained briefly with 1% uranyl acetate (aqueous) and lead citrate and then rinsed in three changes in water and examined by electron microscopy.

3. Results

3.1 Localization of Guanylate Cyclase Receptors

To determine the localization of the components of the ROS-GC1 in boar sperm, immunofluorescence analysis was carried out with specific antibodies against ROS-GC1. Staining for ROS-GC1 was consistently observed in the neck region of the sperm, with weaker staining along the middle piece and at the apical surface of the acrosome (Fig. 1b). Control specimens revealed no labeling (Fig. 1a).

This localization was confirmed by electron microscopy**.** A high density of Protein A-gold particles indicating the presence of GCR was observed on the plasmalemma over the middle piece of the sperm (Fig. 2A). Control specimens showed no labeling by the immunogold procedure (Fig. 2B).

3.2 Localization of Inositol Trisphosphate Receptors

Immunofluorescence analyses were carried out with specific antibodies against IP_3R. IP_3Rs were observed predominantly in the postnuclear neck region, with light staining of the middle piece region (Fig. 3). Control specimens showed no reaction. Regions stained for IP_3R corresponded to clusters of vesicles and nuclear membrane folds also observed in this region (Fig. 3 C, D).

3.3 Calmodulin

Fluorescence staining consistently revealed calmodulin in the neck region and surrounding the basal surface of the nucleus (Fig. 4A, C). Control specimens showed no fluorescence (Fig. 4B)

4. Discussion

The major finding of the study is that there is a colocalization of signal transduction proteins on the sperm membranes. Close association of IP_3R and GCR in sperm membrane supports the suggestion made by Suarez and coworkers that IP3 mediated release of Ca^{2+} from calmodulin stores is related to the control of gated calcium channels during sperm motility and hyperactivated motility (Ho and Suarez, 2001, 2003). These processes may also be operational during fusion of sperm with oocyte membranes at the time of fertilization (Liu and Barker 1997, Revelli et al., 2002). The role of GC is well defined and several studies confirm that GC regulates not only the gated Ca^{2+} channel but it also plays an important role as a chemotactic agent during sperm oocyte fusion in sea urchin (Santos-Sacchi and Gordon 1980, Bentley et al**.,** 1986**,** Cohen-Dayag et al., 1995). It is suggested that initiation of the IP_3/cGMP signal transduction events control the release of Ca^{2+} from calmodulin bound calcium which results in activation of protein kinase C (pKC) causing phosphorylation of enzymes involved in spermatozoan movement.

Depending upon the species of sperm and reaction conditions, signal transduction proteins and receptors appear to have different structural localizations. For instance the IP3 signaling system is localized on the anterior acrosomal region of the mouse, hamster, dog and bull sperm and the acrosomal cap of rat sperm where it might be related to IP3-gated Ca^{2+} channels (Walensky and Snyder, 1995; Ho and Suarez, 2001). A focus of IP_3R is localized at the proximal middle piece of the rat sperm, suggesting that an IP_3-gated calcium store at the proximal middle piece may function in regulation of sperm motility. In contrast, IP_3R was not observed in the proximal middle piece of the mouse, hamster or dog sperm (Walensky and Snyder, 1995). A recent study by Suarez and co-workers (Ho and Suarez, 2003) found IP_3R to be present primarily in the neck region of the bull sperm that corresponds to a plethora of vesicles, mostly associated with the nuclear envelope. Localization of IP_3R at these sites relates to Ca^{2+} storage in the neck region of sperm. Our results using boar sperm clearly corroborates Suarez's findings, but shows vesicular clusters that might be related to the endoplasmic reticulum (ER).

A phosphotidyl inositol signaling pathway has been observed in sperm membranes where hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates IP_3 and diacyl glycerol (DAG), leading to the activation of pKC. Indeed, IP_3 activated Ca^{2+} release from nuclear membrane and ER vesicles may be related to activation of plasma membrane localized pKC, and subsequent phosphorylation of intracellular proteins necessary for sperm function.

Numerous studies have demonstrated that extracellular Ca^{2+} has a regulatory role in the control of motility in sperm of several species (Breitbart, 2002). Sperm membrane-associated Ca^{2+} stores, a Ca^{2+} ATPase or ATP Ca^{2+} pump may be involved in maintaining motility of spermatozoa. Immunocytochemical studies by Suarez and coworkers (Ho and Suarez, 2003) show strong presence of calcium-storage proteins (calmodulin, calreticulin) in the neck region of hyperactivated bull sperm. Our results confirm previous findings showing intense labeling for calmodulin in the neck and in regions surrounding the basal region of the boar sperm nucleus. These results also suggest the interrelatedness of IP3Rs at postnuclear vesicular and ER membranous sites and calmodulin storage sites. Other studies by Camatini and coworkers (1986) demonstrate calmodulin in the acrosomal content and in the equatorial and postacrosomal regions of boar sperm.

Our localization study and other studies described earlier suggest that membrane borne GC, primarily in the neck region of the boar sperm, modulates the influx of extracellular Ca^{2+}, the synthesis of IP_3 that result in release of Ca^{2+} from postnuclear membranous and ER sites and possible activation of pKC, Ca^{2+} pumps and Ca^{2+}-activated ATPases to control axonemal and mitochondrial reactions leading to spermatozoan motility. The question that still remains to be answered is whether colocalization of proteins identified in the sperm membranes act independent of each other or synchronize their functions in unison to regulate spermatozoan motility, capacitation, acrosome reaction and fusion with the oocyte**.** This idea is speculative and needs further investigation.

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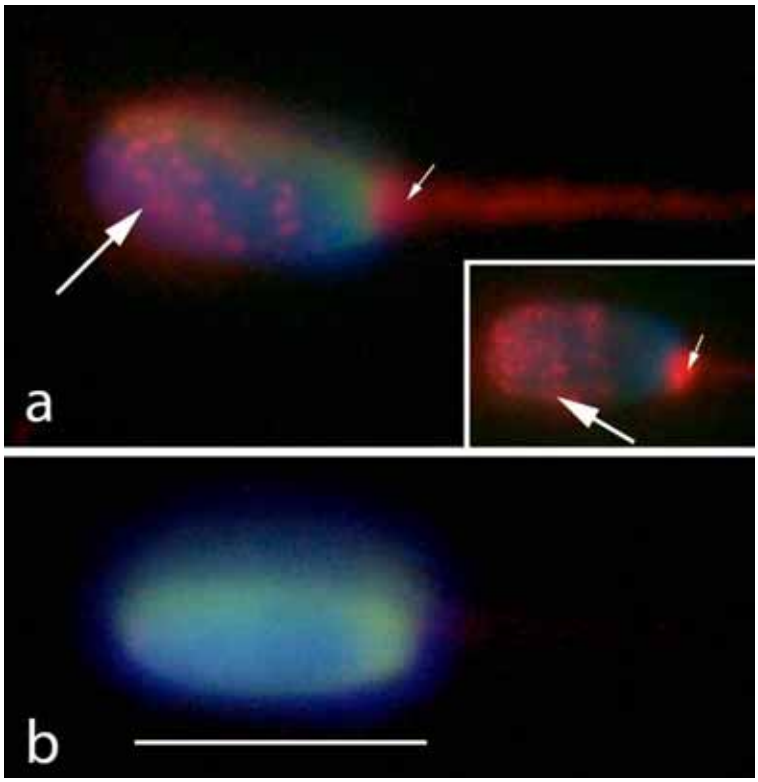

Figure 1. Localization of GCR in mature boar sperm. (a) Sperm stained with DAPI (blue) and antibody to GCR (red) showing the enzyme in the postnuclear neck region, (smaller arrows) and on the plasmalemma at the apical surface of the acrosome, (larger arrows). (b) DAPI-stained control sperm show no reaction. Scale bar = 10 µm.

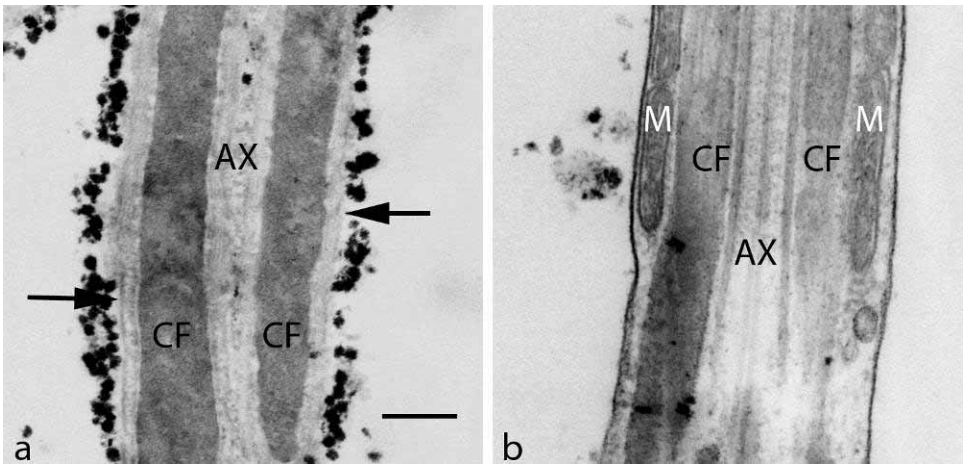

Figure 2. The ultrastructure of the middle piece and tail region of boar sperm are illustrated (**a**) Electron microscope micrographs showing localization of GCR by immunogold labeling on the membrane around the middle piece (arrows). AX = flagellar axoneme; M = mitochondrion; CF = coarse fibers. Control specimen (b) shows no labeling. Scale bar = 0.5µm.

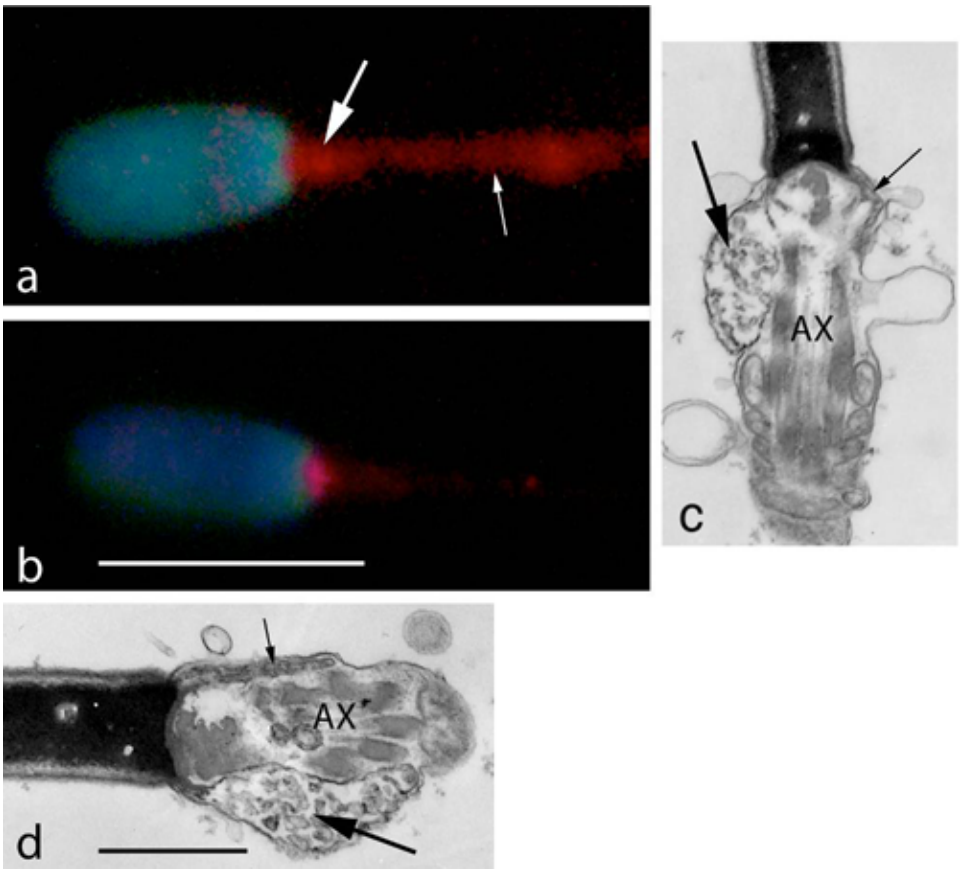

Figure 3. Localization of IP_3R in mature sperm. Boar sperm were probed with antibody to IP_3R. (a,b) Sperm show IP_3R staining in the neck region (larger arrows), with some staining in the middle piece (smaller arrow). Scale bar = 10 μm. (c,d) Portions of the redundant nuclear envelope (smaller arrows) and membrane-bound vesicles (larger arrows) abound in the region. AX = flagellar axoneme. Scale bar = 1 μm.

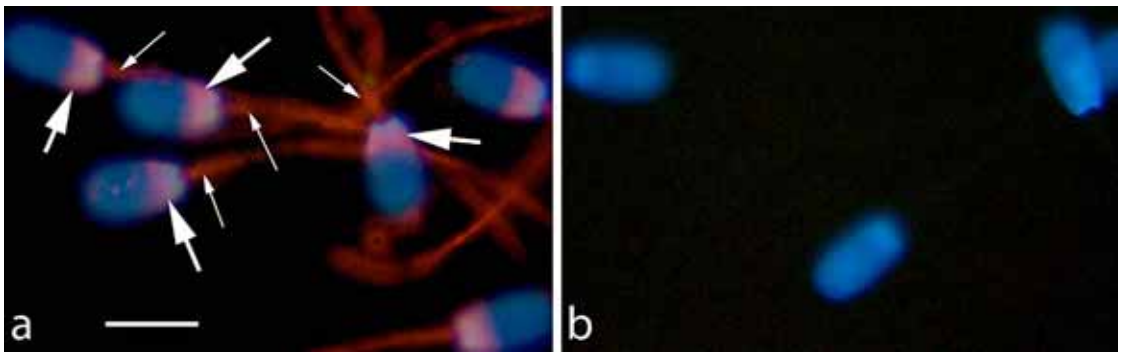

Figure 4. Localization of calmodulin in mature sperm. Fluorescence staining consistently revealed that calmodulin was localized in the neck (larger arrows) and postacrosomal (smaller arrows) regions (a). Control specimens (b) showed no labeling. Scale bar = 10 μm.

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Population Genetics of *Drosophila ananassae*: Evidence for Population Sub-Structuring at the Level of Inversion Polymorphism in Indian Natural Populations

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Abstract

Drosophila ananassae is a cosmopolitan and domestic species distributed in the tropical, subtropical and mildly temperate regions. Population structure analysis in forty-five Indian natural populations of *D. ananassae* was performed employing three cosmopolitan inversions as markers. Pairwise F_{ST} analysis and genetic distance (D) values showed strong genetic differentiation. Though, lowermost values correspond to geographically closest populations, we did not find any significant 'isolation by distance' effect. Values of gene flow based on F_{ST} estimates are very low ($Nm < 5$). All these findings, viz. strong genetic differentiation and minimal gene flow indicate strong sub-structuring in Indian natural populations of *D. ananassae* at the level of inversion polymorphism. This finding is particularly intriguing in case of *D. ananassae* as it is frequently transported via human traffic. Given limited gene flow, populations are expected to diverge genetically due to drift. Low level of gene flow coupled with high degree of genetic differentiation might have occurred historically and is maintained currently. Demographic properties, historical and contemporary events and other factors are more important in shaping the patterns of population sub-structuring, genetic differentiation and gene flow than mere terrestrial habitat characteristics (un) favorable for migration.

Keywords: *Drosophila ananassae,* Natural populations, Chromosome inversions, Gene flow, Population sub-structuring

1. Introduction

Inferring the origin, population structure, and demographic history of a species is a major objective of population genetics. Natural population displays geographic population sub-structure, which is due to differences in allele and genotype frequencies from one geographic region to the other. Population subdivision is centrally important for evolution and affects estimation of all evolutionary parameters from natural and domestic populations (Hartl & Clark, 2007). Chromosomal inversion polymorphism is one of the best studied systems in population genetical studies in *Drosophila.* Chromosomal analyses could be used in evolutionary studies as genetic markers (Powell, 1997) in which chromosome inversions are considered as alleles and are utilized to examine gene flow and other population genetic parameters. In natural populations of *Drosophila,* chromosomal polymorphism due to inversions is common and is an adaptive trait (Da Cunha, 1960; Dobzhansky, 1970; Sperlich & Pfriem, 1986). *Drosophila ananassae* is a member of the *ananassae* species complex of the *ananassae* subgroup of the *melanogaster* species group (Bock & Wheeler, 1972). It shows high degree of chromosomal polymorphism (Singh, 1996). It occupies a unique status in the whole genus of *Drosophila* due to certain peculiarities in its genetical behavior (Singh, 2000). *D. ananassae* harbors a large number of inversions in its natural populations. Of these only three inversions namely, Alpha (AL) in 2L, Delta (DE) in 3L and Eta (ET) in 3R are cosmopolitan in distribution (Singh, 1998). Population genetics of chromosomal polymorphism in Indian natural populations of *D. ananassae* has been extensively studied and the results have clearly shown that there is geographic differentiation of inversion polymorphism (for references see reviews by Singh, 1998; Singh & Singh, 2008).

D. ananassae displays high population sub-structure across the whole distribution range (Stephan, 1989; Stephan & Langely, 1989; Stephan & Mitchell, 1992; Stephan et al., 1998; Das, 2005; Schug et al., 2007). It exhibits more population structure than both *D. melanogaster* and *D. simulans* (Vogl et al., 2003; Das, 2005). It is a cosmopolitan and domestic species, largely circumtropical in distribution. In tropical and subtropical regions of the world, *D. ananassae* is one of the most common *Drosophila* species, especially in and around human habitations. Although populations are separated by major geographical barriers such as mountains and oceans, recurrent transportation by human activity may lead to genetic

exchange. It exists in many semi-isolated populations around the equator, particularly in mainland South-east Asia and on the Islands of the Pacific Ocean (Tobari, 1993). *D. ananassae* is thought to have originated in South-east Asia where most of its relatives occur (Das et al., 2004; Das, 2005).

Forty-five natural populations from different eco-geographic regions of the country (covering the regions from Jammu in north to Kanniyakumari in south and Dwarka in west to Deemapur in east) were analysed for chromosomal inversions and data has been given elsewhere (Singh & Singh, 2007). In the present communication, same quantitative data on the frequencies of three cosmopolitan inversions in Indian natural populations of *D. ananassae* (Singh & Singh, 2007) have been used to arrive at genetic variability estimates, F-statistics and gene flow. The present communication represents the most comprehensive analysis of Indian natural populations of *D. ananassae* using traditional genetic approach and employing chromosomal markers (inversions), genetic variability parameters (H_o, H_e, F) and F-statistics to infer the population structure and gene flow among *D. ananassae* populations from different parts of India.

2. Materials and methods

2.1 Drosophila strains

D. ananassae flies were collected from forty-five different eco-geographical localities of India ranging from Jammu in north to Kanniyakumari in south and Dwarka in west to Deemapur in east (see Singh & Singh, 2007 for details of collection along with their geographical locations).

2.2 Population genetic analysis

Quantitative data on inversion frequencies in forty-five natural populations of Indian *D.ananassae* (Singh & Singh, 2007) was utilized to arrive at genetic variability estimates, F-statistics and gene flow. This is the first time that inversions as chromosomal markers have been employed for population structure analysis. We have used three cosmopolitan inversions of *D. ananassae*, since they have worldwide distribution due to their adaptive role; are integral part of the genetic endowment of the fly and could be treated as alleles at single locus. Since, population sub-structuring or subdivision is acquiring different allele or genotype frequencies from one geographic region to other leading to population differentiation, chromosomal inversions could be utilised for such studies.

Genetic variability was recorded as mean observed (H_o) and expected (H_E) heterozygosity. Population inbreeding coefficient (F) was calculated to deduce the level of inbreeding due to population sub-structuring and also the departure of H_o from HWE. Population structure analysis was done using traditional F-statistics following Wright (1951).

Gene flow between populations was estimated as the number of migrants exchanged between populations per generation (Nm). Nm values were derived from one approach using F_{ST} values, following the island model of Wright (1951) with a small level of migration, whereby:

$$Nm = (1- F_{ST}) / 4F_{ST}$$

Genetic distance (D) approach was also utilized to determine the pattern of geographic variation among Indian natural populations of *D. ananassae*. It was calculated from Nei's (1972) genetic identity (I) using the formula (D=1-I). To test 'isolation by distance' effect genetic distance and geographic distance were correlated.

3. Results

Estimates of genetic variability are given in Table 1. Mean observed heterozygosity (H_o) ranges from 0.15 (AD) to 0.61 (PC), similarly, mean expected heterozygosity (H_E) ranges from 0.15 (ML) to 0.45 (VD). The value of population inbreeding coefficient (F) ranges from – 0.09 (AB) to 0.47 (PU). The overall structure among populations based on F-statistics was higher. As given in Table 1, values of F_{IS} range from – 0.53 (ML) to 0.47 (PU). The values of F_{ST} across each population range from 0.04 (PC) to 0.64 (ML). The values of F_{IT}, which is the most inclusive inbreeding coefficient, range from – 0.41 (PC) to 0.68 (AD).

Pairwise F_{ST} values among populations range from 0.054 (GY vs UJ) to 0.617 (AD vs ML) showing that Indian populations of *D. ananassae* are not homogeneous and exhibit high level of genetic differentiation (Table 2). Table 3, shows F_{ST} based estimates of gene flow ranging from 0.155 (PN-ML, AD-ML) to 4.379 (GY-AD). The gene flow measured as Nm (using F_{ST} values) probably does not represent the real flow and is not the best estimation of this geographic parameter, but this measure permitted us to observe the overall trend.

As given in the Table 4, pairwise genetic distance (D) values among populations range from 0.000 (UJ vs IN and KL vs SD) to 0.436 (LK vs GK). The lowermost D values correspond to geographically closest populations. Genetic distance and geographic distance were insignificantly correlated ($r = 0.200$; $p > 0.05$).

4. Discussion

Due to its extensive population structure, *D. ananassae* could be an appropriate model to analyze the effect of population subdivision on genetic variation.

4.1 Genetic variability

Values of population inbreeding coefficient show the range of - 0.093 to 0.47. In most of the cases H_o is almost similar to H_E (indicating populations are in HWE), only in few cases $H_o < H_E$ (indicating inbreeding), in these cases too, H_o is almost similar to H_E and in rest of the populations $H_o > H_E$ (indicating outbreeding) as is the case in most of the natural populations. Reduction in heterozygosity resulting from population sub-structuring is intimately related to the reduction in heterozygosity caused by inbreeding. This could be understood by interpreting each subpopulation as sort of "extended family" or set of interconnected pedigrees. Organisms in the same population often share one or more recent or remote common ancestors, and so mating between organisms in the same subpopulation will often be mating between relatives (Hartl & Clark, 2007).

4.2 F- statistics

Values of F_{IS} (- 0.53 to 0.47) in most of the natural populations are close to zero indicating random mating in subpopulations. Values of F_{IT}, the most inclusive measure of inbreeding (- 0.41 to 0.68) is found close to zero in most of the cases. Values of F_{ST} show the range of 0.04 to 0.64. So, range wise population subdivision, possibly due to drift accounts for approximately 4% to 64% of the total genetic variation. Presumably, values of F_{ST} are influenced by the size of subpopulations, which is the major determinant of the magnitude of random changes in allele frequency (Hartl & Clark, 2007).

4.3 Genetic differentiation and north-south trends

Pairwise F_{ST} values and genetic distance estimates reveal strong differentiation among the populations particularly between northern and southern regions within the study area, hence showing north-south trends. However, no clinal pattern with respect to inversion frequencies has been found in Indian natural populations of *D. ananassae.* Latitudinal clines have been detected in Indian natural populations of *D. melanogaster* (Das & Singh, 1991; Singh & Das, 1992). These north-south trends have also been reported with respect to inversion frequencies in natural populations of *D. ananassae* (Singh & Singh, 2007). These trends have persisted because of diversifying selection that acts to impede homogenization as a result of gene flow, as is commonly the case among naturally occurring clines (Endler, 1977; Arnold, 1997). Further, strong genetic differentiation observed among *D. ananassae* populations from different eco-geographic regions could be due to geo-climatic heterogeneity and this difference is canalized via rigid polymorphic system in *D. ananassae*. It is because of this, it resists any change brought through any agency or homogenizing force. Further, localized differentiation or similarity among populations could be due to selection and local dispersal or both.

Since, *D. ananassae* flies are domestic and co-habit the human dwellings, which are capable of sustaining the residents, they have inherently poor dispersal capacity. It may be likely that when these are subjected to forced dispersal, these flies may show aversion to mating and hence maintaining the differentiation. Forister (2004) found the similar happening in *Hesperia comma*, a holarctic skipper with very high dispersal potential.

4.4 Gene flow

Our low pairwise gene flow values (only slightly above the range shown by rat snakes, Lougheed et al., 1999) and conversely high F_{ST} values relative to other studies involving non *Drosophila* models (Carmichael et al., 2001; Nice & Shapiro, 2001; Schwartz et al., 2002; Bargelloni et al., 2003; Rueness et al., 2003; Mcrae et al., 2005) are surprising because *Drosophila* by itself has poor dispersal capacity but since it is a human commensal and is co-transported via agency of human travel along with fruits and vegetables so geographic barriers or habitat discontinuity of any kind hardly hinders its movement. Despite this it maintains very high level of genetic differentiation and exists as structured semi-isolated populations. Gene flow between these flies may be restricted despite their being nearly adjacent in some of the localities. So sympatric divergence could also be the explanation for these data as has been hypothesized for host races in butterfly genus *Mitoura* (Nice & Shapiro, 2001).

Habitat barriers play a role in structuring populations (Walker, 2000; Mcrae et al., 2005), so that where habitat barriers are present, we should have found greater population structure, but in case of our model these are insignificant as flies are transported via fruits and vegetables to different parts of the country, and possibilities are that, small number of founders might have started their colony afresh and during that precarious bottleneck period random genetic drift might have played its role in causing differentiation. Further, significant genetic differentiation in nearly all pairwise comparisons (based on the values of genetic distance and pairwise F_{ST} values) across the country suggests that considerable population structuring can occur regardless of distance (in some cases) or any other similarity inducing factors. Evidence for substantial structuring also means that populations within some states may not be connected by frequent dispersal and this would define populations with allele frequencies divergent enough to indicate functional independence (Moritz, 1994; Mcrae et al., 2005). Isolation by distance (Wright, 1943) effect was not conformed statistically as genetic distance and geographic distances are insignificantly correlated in *D. ananassae* populations (Figure 1). It is evident from D values that populations separated by greater geographic distance have higher genetic dissimilarity than those situated close to each other in most of the cases.

D. ananassae does not show temporal divergence (Singh & Singh, 2007). This again conforms lesser intermixing between the populations of *D. ananassae* in terms of migration or gene flow across geological time scale, otherwise there could have been all genetically similar populations of *D. ananassae* throughout its range. It is this strong genetic differentiation that keeps *D. ananassae* populations highly sub-structured and in semi-isolated fashion.

Similar studies (Vogl et al., 2003; Das et al., 2004; Schug et al., 2007) done earlier at molecular level in *D. ananassae* have arrived on the similar conclusion. These studies have shown that F_{ST} values of the order of 0.1 (much lower than our F_{ST} estimates) could be applied to Indian populations. This difference could be attributed to the markers used. Compared to allozymes and molecular markers, the picture of geographic differentiation appears to be different for chromosome inversions, which are more variable and more differentiated even over short distances. This could be partly due to the fact that allozymes and molecular markers are in general more "neutral" than chromosome inversions.

We could therefore conclude, at least in our reasonably broad spatio-temporal study covering the representative eco-geographical regions of the country, that, populations of *D. ananassae* show strong sub-structuring due to genetic differentiation of their natural populations. Given limited gene flow, populations are expected to diverge genetically due to drift. Low level of gene flow coupled with high degree of genetic differentiation might have occurred historically and is maintained currently. Demographic properties, historical and contemporary events and other factors are more important in shaping the patterns of population sub-structuring, genetic differentiation and gene flow than mere terrestrial habitat characteristics (un) favorable for migration.

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Table 1. Estimates of genetic diversity and F-statistics in Indian natural populations of *D. ananassae*

Populations	H_O	H_E	F	F_{IS}	F_{ST}	F_{IT}
Jammu (JU)	0.30	0.33	0.090	0.090	0.214	0.285
Dharamshala (DH)	0.31	0.32	0.031	0.031	0.200	0.225
Kangra (KG)	0.29	0.34	0.147	0.151	0.250	0.363
Dehradun (DN)	0.31	0.35	0.114	0.088	0.227	0.295
Haridwar (HD)	0.27	0.34	0.205	-0.294	0.150	-0.100
Mansa Devi (MD)	0.36	0.39	0.076	0.076	0.187	0.250
Gangtok (GT)	0.23	0.25	0.080	0.120	0.468	0.531
Lucknow (LK)	0.24	0.27	0.111	0.148	0.341	0.439
Guwahati (GU)	0.25	0.25	0.000	0.000	0.446	0.468
Raidopur (RP)	0.25	0.28	0.107	0.107	0.263	0.342
Chowk (CW)	0.29	0.31	0.064	0.096	0.138	0.222
Deemapur (DM)	0.26	0.27	0.037	0.037	0.425	0.446
Shillong (SH)	0.23	0.24	0.041	0.041	0.500	0.520
Patna (PN)	0.18	0.18	0.000	0.000	0.595	0.617
Allahabad (AB)	0.35	0.32	-0.093	-0.093	0.219	0.146
Imphal (IM)	0.31	0.32	0.0310	0.031	0.319	0.340
Gaya (GY)	0.24	0.23	-0.043	-0.043	0.080	0.040
Ujjain (UJ)	0.28	0.37	0.243	0.243	0.195	0.391
Bhopal (BP)	0.25	0.29	0.137	0.142	0.300	0.400
Indore (IN)	0.38	0.37	-0.027	-0.027	0.177	0.155
Jamnagar (JM)	0.23	0.28	0.178	0.148	0.425	0.510
Howarah (HW)	0.25	0.28	0.107	0.074	0.400	0.440
Sealdah (SD)	0.18	0.32	0.430	0.410	0.311	0.600
Kolkata (KL)	0.30	0.33	0.090	0.062	0.319	0.361
Rajkot (RJ)	0.28	0.29	0.034	0.034	0.382	0.404
Dwarka (DW)	0.20	0.23	0.130	0.130	0.510	0.570
Ahemdabad (AD)	0.15	0.19	0.210	0.210	0.595	0.680
Paradeep (PA)	0.25	0.36	0.305	0.330	0.230	0.480
Bhubneswar (BN)	0.22	0.30	0.260	0.300	0.360	0.553
Puri (PU)	0.18	0.34	0.470	0.470	0.276	0.617
Shirdi (SI)	0.19	0.21	0.095	0.100	0.545	0.59
Nashik (NA)	0.21	0.20	-0.050	-0.050	0.547	0.523
Mumbai (MU)	0.21	0.24	0.125	0.125	0.454	0.522
Visakhapatnam (VP)	0.26	0.37	0.297	0.305	0.181	0.431
Vijaywada (VD)	0.25	0.45	0.440	0.450	0.083	0.5
Panaji (PJ)	0.27	0.28	0.035	0.071	0.416	0.458
Madgaon (MA)	0.26	0.30	0.133	0.133	0.361	0.446
Gokarna (GK)	0.27	0.29	0.068	0.068	0.395	0.437
Manglore (ML)	0.24	0.15	-0.600	-0.533	0.642	0.452
Banglore (BL)	0.45	0.42	-0.071	-0.071	0.106	0.042
Yesvantpur (YS)	0.48	0.39	-0.230	-0.230	0.152	-0.04
Pondicherry (PC)	0.61	0.42	-0.450	-0.480	0.046	-0.418
Ernakulam (ER)	0.27	0.35	0.228	0.228	0.225	0.425
Thiruvananthapuram (TR)	0.29	0.31	0.064	0.064	0.34	0.382
Kanniyakumari (KR)	0.27	0.33	0.181	0.212	0.266	0.422

H_o, observed average heterozygosity; H_e, expected average heterozygosity; F, population inbreeding coefficient; F_{IS}, inbreeding coefficient due to non random mating; F_{ST}, inbreeding coefficient due to population subdivision; F_{IT}, inbreeding coefficient due to effect of non random mating with subpopulation and the effect of population subdivision.

Table 2. Pairwise estimates of Fst values in Indian natural populations of *D.ananassae*

	JU	DH	KG	DN	HD	MD	GT	LK	GU	RP	CW	DM	SH	PN	AB	IM	GY	UJ	BP	IN	JR	HW	SD	KL	RJ	DW	AD	PA	BN	PU	SI	NA	MU	VP	VD	PJ	MA	GK	ML	BL	YS	PC	ER	TR	KR
JU	0	0.23	0.2	0.2	0.2	0.34	0.24	0.34	0.24	0.18	0.33	0.366	0.42	0.22	0.27	0.16	0.25	0.26	0.2	0.325	0.31	0.264	0.27	0.3	0.37	0.415	0.224	0.29	0.247	0.38	0.38	0.339	0.197	0.144	0.322	0.29	0.31	0.43	0.16	0.181	0.13	0.2	0.28	0.2
DH		0.23	0.2	0.2	0.21	0.34	0.27	0.34	0.23	0.17	0.32	0.363	0.41	0.21	0.26	0.15	0.197	0.25	0.24	0.321	0.31	0.258	0.26	0.3	0.367	0.413	0.218	0.29	0.241	0.38	0.38	0.333	0.19	0.136	0.318	0.29	0.31	0.43	0.15	0.174	0.12	0.2	0.28	0.2
KG			0.2	0.2	0.14	0.36	0.29	0.36	0.26	0.2	0.34	0.38	0.43	0.24	0.29	0.19	0.222	0.27	0.27	0.21	0.34	0.33	0.28	0.29	0.32	0.364	0.428	0.241	0.29	0.263	0.4	0.4	0.352	0.215	0.163	0.336	0.29	0.33	0.44	0.18	0.2	0.15	0.2	0.3	0.3
DN				0.2	0.21	0.35	0.28	0.35	0.24	0.19	0.33	0.369	0.42	0.22	0.27	0.17	0.211	0.26	0.2	0.329	0.31	0.269	0.26	0.31	0.373	0.417	0.23	0.3	0.252	0.39	0.38	0.34	0.204	0.152	0.326	0.3	0.29	0.43	0.16	0.188	0.14	0.2	0.29	0.2
HD					0.17	0.32	0.25	0.32	0.21	0.14	0.3	0.34	0.39	0.19	0.24	0.12	0.174	0.23	0.16	0.298	0.28	0.235	0.24	0.28	0.344	0.39	0.195	0.26	0.218	0.36	0.35	0.309	0.166	0.113	0.295	0.26	0.26	0.4	0.13	0.151	0.1	0.2	0.25	0.2		
MD						0.33	0.26	0.28	0.22	0.17	0.26	0.322	0.39	0.31	0.36	0.15	0.191	0.24	0.18	0.305	0.29	0.247	0.25	0.28	0.347	0.389	0.21	0.27	0.231	0.38	0.36	0.293	0.184	0.135	0.312	0.27	0.29	0.4	0.15	0.17	0.12	0.2	0.26	0.2		
GT							0.41	0.47	0.36	0.33	0.45	0.484	0.53	0.35	0.39	0.33	0.333	0.39	0.33	0.446	0.43	0.391	0.39	0.43	0.489	0.531	0.351	0.41	0.372	0.49	0.51	0.461	0.329	0.273	0.442	0.41	0.43	0.55	0.29	0.311	0.27	0.4	0.3	0.4		
LK								0.41	0.31	0.25	0.39	0.426	0.48	0.28	0.34	0.24	0.264	0.32	0.26	0.386	0.37	0.325	0.33	0.36	0.454	0.363	0.284	0.33	0.306	0.45	0.33	0.4	0.258	0.202	0.382	0.35	0.37	0.49	0.24	0.241	0.19	0.3	0.34	0.3		
GU									0.38	0.33	0.45	0.484	0.53	0.35	0.42	0.33	0.33	0.39	0.32	0.44	0.43	0.391	0.39	0.43	0.489	0.531	0.351	0.41	0.372	0.51	0.51	0.461	0.329	0.273	0.442	0.52	0.43	0.55	0.29	0.311	0.27	0.4	0.4	0.3		
RP										0.32	0.3	0.39	0.4	0.18	0.29	0.19	0.226	0.28	0.22	0.301	0.34	0.289	0.29	0.33	0.4	0.397	0.247	0.32	0.27	0.41	0.41	0.365	0.219	0.162	0.297	0.32	0.34	0.46	0.18	0.202	0.15	0.2	0.25	0.2		
CW											0.3	0.345	0.4	0.18	0.24	0.11	0.17	0.24	0.16	0.301	0.28	0.234	0.24	0.28	0.349	0.397	0.192	0.26	0.21	0.36	0.35	0.31	0.16	0.167	0.297	0.27	0.29	0.41	0.12	0.14	0.08	0.2	0.25	0.2		
DM												0.469	0.51	0.32	0.37	0.3	0.31	0.36	0.3	0.42	0.41	0.36	0.37	0.4	0.46	0.51	0.32	0.39	0.35	0.48	0.26	0.43	0.3	0.25	0.42	0.39	0.41	0.52	0.26	0.29	0.24	0.3	0.38	0.3		
SH													0.54	0.42	0.46	0.35	0.397	0.4	0.34	0.46	0.45	0.408	0.41	0.44	0.505	0.547	0.368	0.43	0.389	0.52	0.52	0.478	0.347	0.291	0.458	0.43	0.45	0.57	0.29	0.329	0.29	0.4	0.42	0.4		
PN														0.42	0.45	0.42	0.397	0.46	0.39	0.51	0.5	0.456	0.46	0.26	0.553	0.595	0.414	0.48	0.436	0.57	0.57	0.527	0.395	0.336	0.505	0.48	0.49	0.62	0.36	0.376	0.33	0.4	0.47	0.4		
AB															0.27	0.17	0.206	0.26	0.2	0.372	0.31	0.267	0.27	0.3	0.37	0.42	0.22	0.29	0.25	0.38	0.38	0.34	0.2	0.146	0.325	0.3	0.31	0.43	0.16	0.183	0.13	0.2	0.28	0.2		
IM																0.24	0.258	0.31	0.25	0.37	0.35	0.31	0.32	0.35	0.41	0.457	0.276	0.34	0.29	0.42	0.41	0.38	0.25	0.2	0.36	0.34	0.35	0.47	0.21	0.23	0.18	0.3	0.32	0.2		
GY																	0.154	0.22	0.14	0.305	0.29	0.228	0.24	0.28	0.361	0.397	0.18	0.28	0.2	0.37	0.36	0.322	0.14	0.054	0.308	0.26	0.28	0.43	0.11	0.12	0.05	0.2	0.27	0.2		
UJ																		0.24	0.19	0.311	0.3	0.252	0.26	0.29	0.35	0.39	0.21	0.27	0.23	0.36	0.36	0.32	0.18	0.13	0.3	0.28	0.29	0.4	0.15	0.17	0.12	0.2	0.26	0.2		
BP																			0.24	0.367	0.35	0.305	0.31	0.34	0.41	0.45	0.26	0.32	0.28	0.42	0.42	0.38	0.23	0.18	0.36	0.33	0.35	0.47	0.19	0.22	0.16	0.3	0.32	0.2		
IN																				0.304	0.29	0.244	0.25	0.28	0.34	0.39	0.2	0.27	0.27	0.35	0.35	0.31	0.17	0.12	0.3	0.27	0.29	0.4	0.14	0.16	0.11	0.2	0.26	0.2		
JR																					0.41	0.355	0.36	0.4	0.456	0.5	0.315	0.39	0.35	0.41	0.48	0.426	0.3	0.25	0.42	0.39	0.4	0.52	0.26	0.29	0.24	0.3	0.37	0.3		
HW																						0.35	0.35	0.39	0.45	0.5	0.31	0.38	0.22	0.47	0.47	0.42	0.29	0.23	0.4	0.38	0.39	0.51	0.25	0.27	0.22	0.3	0.33	0.3		
SD																							0.31	0.34	0.414	0.457	0.279	0.34	0.297	0.43	0.43	0.384	0.252	0.2	0.368	0.34	0.36	0.47	0.21	0.236	0.19	0.3	0.33	0.3		
KL																								0.35	0.41	0.45	0.27	0.34	0.29	0.42	0.42	0.38	0.25	0.2	0.36	0.34	0.35	0.47	0.31	0.23	0.18	0.3	0.32	0.3		
RJ																									0.446	0.489	0.3	0.37	0.32	0.46	0.4	0.27	0.23	0.4	0.37	0.38	0.44	0.24	0.26	0.22	0.31	0.4	0.32	0.4		
DW																										0.55	0.37	0.43	0.39	0.52	0.51	0.48	0.35	0.33	0.46	0.43	0.45	0.46	0.3	0.33	0.28	0.4	0.42	0.4		
AD																											0.41	0.47	0.43	0.57	0.57	0.41	0.39	0.33	0.5	0.47	0.49	0.61	0.35	0.36	0.33	0.4	0.46	0.4		
PA																												0.3	0.25	0.38	0.38	0.34	0.2	0.15	0.32	0.29	0.31	0.42	0.17	0.19	0.14	0.2	0.28	0.3		
BN																													0.319	0.45	0.44	0.4	0.27	0.22	0.38	0.36	0.37	0.44	0.23	0.25	0.21	0.3	0.31	0.3		
PU																														0.4	0.4	0.36	0.23	0.17	0.34	0.31	0.33	0.44	0.19	0.21	0.17	0.3	0.3	0.3		
SI																															0.54	0.5	0.36	0.3	0.477	0.45	0.45	0.6	0.31	0.344	0.29	0.4	0.44	0.3		
NA																																0.5	0.36	0.3	0.47	0.44	0.46	0.6	0.31	0.45	0.29	0.4	0.43	0.4		
MU																																	0.318	0.26	0.43	0.4	0.42	0.54	0.27	0.3	0.25	0.4	0.28	0.4		
VP																																		0.13	0.3	0.27	0.29	0.4	0.14	0.16	0.11	0.2	0.21	0.2		
VD																																			0.25	0.22	0.23	0.34	0.11	0.11	0.06	0.2	0.2	0.2		
PJ																																				0.38	0.4	0.52	0.26	0.28	0.24	0.3	0.35	0.3		
MA																																					0.38	0.49	0.23	0.25	0.21	0.3	0.35	0.3		
GK																																						0.51	0.25	0.28	0.23	0.3	0.36	0.3		
ML																																							0.35	0.38	0.34	0.4	0.48	0.4		
BL																																								0.12	0.07	0.2	0.25	0.2		
YS																																									0.1	0.2	0.2	0.2		
PC																																										0.2	0.2	0.2		
ER																																											0.29	0.3		
TR																																												0.3		
KR																																														

Table 3. Estimates of Nem based on Fst values in Indian natural populations of *D.ananassae*

	JU	DH	KG	DN	HD	MD	GT	LK	GU	RP	CW	DM	SH	PN	AB	IM	GY	UJ	BP	IN	JR	HW	SD	KL	RJ	DW	AD	PA	BN	PU	SI	NA	MU	VP	VD	PJ	MA	GK	ML	BL	YS	PC	ER	TR	KR
JU		0.9	0.83	0.83	1.03	1	0.49	0.65	0.49	0.8	1.15	0.52	0.43	0.35	0.91	0.68	1.22	0.75	0.73	1.03	0.52	0.56	0.7	0.68	0.58	0.43	0.352	0.866	0.606	0.76	0.402	0.41	0.49	1.02	1.486	0.53	0.61	0.55	0.33	1.342	1.13	1.69	0.81	0.64	0.79
DH			0.86	0.92	1.18	0.93	0.48	0.67	0.48	0.84	1.21	0.53	0.44	0.36	0.95	0.7	1.38	1.02	0.75	0.81	0.53	0.57	0.72	0.7	0.59	0.43	0.355	0.896	0.621	0.79	0.407	0.41	0.5	1.07	1.588	0.54	0.62	0.57	0.34	1.427	1.19	1.83	0.84	0.66	0.81
KG				0.8	0.99	1.51	0.44	0.6	0.44	0.73	1	0.49	0.41	0.33	0.81	0.63	1.08	0.88	0.67	0.92	0.49	0.52	0.64	0.63	0.54	0.4	0.334	0.787	0.627	0.7	0.397	0.38	0.46	0.91	1.283	0.49	0.63	0.52	0.32	1.178	1	1.43	1.27	0.59	0.72
DN					1.07	0.96	0.46	0.64	0.46	0.8	1.09	0.51	0.43	0.35	0.87	0.66	1.2	0.93	0.71	0.99	0.51	0.55	0.68	0.72	0.56	0.42	0.349	0.836	0.594	0.74	0.397	0.4	0.49	0.98	1.394	0.52	0.59	0.6	0.33	1.274	1.08	1.57	0.79	0.63	0.76
HD						1.22	0.53	0.97	0.53	0.97	1.49	0.59	0.49	0.39	1.1	0.79	1.78	1.19	0.86	1.27	0.59	0.64	0.81	0.79	0.66	0.48	0.391	1.032	0.696	0.9	0.45	0.46	0.56	1.26	1.962	0.6	0.7	0.71	0.37	1.734	1.41	2.35	0.96	0.74	0.93
MD							0.52	0.72	0.64	0.89	1.26	0.72	0.53	0.39	0.55	0.45	1.42	1.06	0.8	1.12	0.57	0.61	0.76	0.74	0.63	0.47	0.392	0.94	0.665	0.83	0.414	0.45	0.6	1.11	1.601	0.55	0.67	0.61	0.38	1.45	1.22	1.83	0.88	0.7	0.86
GT								0.36	0.28	0.44	0.52	0.31	0.31	0.22	0.46	0.39	0.5	0.5	0.39	0.52	0.31	0.33	0.39	0.39	0.34	0.26	0.222	0.462	0.353	0.42	0.256	0.25	0.29	0.51	0.665	0.32	0.35	0.33	0.2	0.366	0.55	0.69	0.44	0.59	0.43
LK									0.36	0.55	0.77	0.4	0.34	0.27	0.64	0.49	0.78	0.7	0.53	0.73	0.4	0.42	0.52	0.51	0.44	0.33	0.438	0.63	0.509	0.57	0.309	0.52	0.38	0.72	0.987	0.4	0.46	0.43	0.26	0.8	0.79	1.07	0.6	0.49	0.58
GU										0.91	0.52	0.31	0.27	0.22	0.46	0.3	0.51	0.51	0.39	0.53	0.32	0.33	0.39	0.39	0.34	0.26	0.22	0.462	0.353	0.42	0.245	0.25	0.29	0.51	0.665	0.32	0.23	0.33	0.2	0.621	0.55	0.69	0.94	0.38	0.47
RP											0.91	0.46	0.39	0.31	0.79	0.6	1.07	0.86	0.64	0.91	0.46	0.49	0.62	0.6	0.51	0.38	0.309	0.762	0.538	0.68	0.353	0.43	0.89	1.29	0.468	0.54	0.49	0.29	1.17	0.987	1.44	0.99	0.72	0.57	0.79
CW												0.58	0.47	0.38	1.13	0.79	1.94	1.22	0.81	1.31	0.58	0.63	0.82	0.79	0.65	0.47	0.379	1.052	0.726	0.91	0.44	0.45	0.55	1.29	2.068	0.59	0.69	0.63	0.36	1.833	1.46	2.6	0.98	0.74	0.95
DM													0.29	0.24	0.51	0.42	0.57	0.55	0.43	0.57	0.34	0.36	0.43	0.42	0.37	0.28	0.24	0.509	0.386	0.46	0.267	0.27	0.32	0.56	0.742	0.34	0.39	0.36	0.22	0.693	0.61	0.77	0.49	0.4	0.47
SH														0.21	0.43	0.36	0.45	0.46	0.36	0.5	0.29	0.3	0.36	0.36	0.32	0.25	0.207	0.429	0.33	0.39	0.229	0.23	0.27	0.47	0.609	0.3	0.33	0.31	0.19	0.63	0.51	0.66	0.41	0.34	0.4
PN															0.35	0.3	0.35	0.38	0.29	0.39	0.24	0.25	0.3	0.3	0.26	0.2	0.17	0.353	0.273	0.32	0.187	0.19	0.22	0.38	0.494	0.25	0.27	0.26	0.16	0.462	0.41	0.51	0.34	0.28	0.33
AB																0.67	1.26	0.96	0.72	1.02	0.51	0.55	0.69	0.67	0.57	0.42	0.345	0.851	0.597	0.75	0.394	0.4	0.48	1	1.462	0.52	0.6	0.55	0.33	1.322	1.12	1.67	0.8	0.63	0.77
IM																	0.81	0.72	0.56	0.25	0.42	0.45	0.54	0.53	0.46	0.35	0.297	0.655	0.485	0.59	0.334	0.35	0.4	0.74	1	0.43	0.49	0.45	0.28	0.929	0.81	1.08	0.62	0.51	0.6
GY																		1.37	0.91	1.51	0.57	0.63	0.85	0.81	0.65	0.94	0.35	1.138	0.7	0.95	0.414	0.42	0.54	1.49	4.379	0.58	0.7	0.62	0.33	2.04	1.73	4.06	1.04	0.75	1
UJ																			0.77	1.09	0.55	0.59	0.74	0.72	0.61	0.46	0.379	0.912	0.646	0.81	0.433	0.44	0.53	1.08	1.561	0.56	0.65	0.59	0.36	1.416	1.2	1.78	0.86	0.68	0.84
BP																				0.81	0.43	0.46	0.57	0.56	0.48	0.36	0.294	0.696	0.528	0.62	0.334	0.32	0.41	0.8	1.131	0.44	0.5	0.46	0.28	1.032	0.89	1.24	0.66	0.53	0.64
IN																					0.57	0.62	0.77	0.25	0.64	0.47	0.389	0.963	0.672	0.85	0.446	0.45	0.55	1.15	1.687	0.58	0.67	0.61	0.37	1.523	1.27	1.56	0.9	0.71	0.88
JR																						0.36	0.43	0.42	0.36	0.28	0.24	0.509	0.386	0.46	0.346	0.27	0.32	0.56	0.742	0.34	0.39	0.36	0.22	0.693	0.61	0.77	0.49	0.4	0.47
HW																							0.45	0.45	0.39	0.3	0.25	0.543	0.407	0.85	0.28	0.28	0.34	0.61	0.809	0.36	0.41	0.38	0.23	0.25	0.66	0.8	0.52	0.43	0.5
SD																								0.54	0.47	0.36	0.298	0.672	0.494	0.6	0.336	0.34	0.4	0.76	1.045	0.43	0.49	0.46	0.28	0.963	0.84	1.13	0.64	0.52	0.62
KL																									0.46	0.35	0.297	0.646	0.985	0.59	0.334	0.34	0.4	0.74	1	0.43	0.49	0.45	0.28	0.533	0.81	1.08	0.62	0.51	0.6
RJ																										0.31	0.261	0.561	0.472	0.51	0.292	0.29	0.36	0.65	0.832	0.38	0.42	0.39	0.31	0.774	0.68	0.88	0.53	0.49	0.52
DW																											0.202	0.422	0.323	0.39	0.227	0.23	0.27	0.46	0.494	0.29	0.32	0.3	0.29	0.561	0.5	0.62	0.4	0.34	0.39
AD																												0.353	0.273	0.32	0.187	0.19	0.35	0.38	0.494	0.25	0.27	0.26	0.16	0.462	0.43	0.5	0.34	0.28	0.33
PA																													0.591	0.73	0.401	0.4	0.49	0.95	1.342	0.52	0.59	0.59	0.34	1.22	1.05	1.49	0.77	0.62	0.75
BN																														0.53	0.305	0.31	0.37	0.66	0.881	0.39	0.44	0.41	0.31	0.818	0.72	0.93	0.56	0.46	0.54
PU																															0.365	0.37	0.44	0.84	1.154	0.47	0.53	0.49	0.31	1.058	0.91	1.26	0.69	0.56	0.67
SI																																0.21	0.25	0.44	0.572	0.27	0.31	0.29	0.17	0.536	0.48	0.59	0.38	0.32	0.61
NA																																	0.25	0.44	0.533	0.28	0.31	0.29	0.16	0.546	0.3	0.6	0.39	0.32	0.37
MU																																		0.54	0.711	0.33	0.37	0.34	0.21	0.662	0.58	0.74	0.46	0.63	0.45
VP																																			1.673	0.57	0.66	0.6	0.37	1.51	1.26	1.94	0.89	0.7	0.87
VP																																				0.75	0.88	0.8	0.48	1.942	1.87	3.6	1.24	0.94	1.2
PJ																																					0.39	0.37	0.23	0.7	0.62	0.79	0.49	0.44	0.48
MA																																						0.41	0.26	0.818	0.72	0.93	0.56	0.46	0.54
GK																																							0.24	0.742	0.64	0.84	0.52	0.43	0.5
ML																																								0.446	0.4	0.48	0.32	0.27	0.31
BL																																									1.69	3	1.14	0.87	1.11
YS																																										2.23	0.98	0.76	0.95
PC																																											1.36	1	1.32
ER																																												0.59	0.71
TR																																													0.57
KR																																													

Table 4. Genetic distance (D) estimated from the gene arrangement frequencies among the natural populations of *D. ananassae*

	JU	DH	KG	DN	HD	MD	GT	LK	GU	RP	CW	DM	SH	PN	AB	IM	GY	UJ	BP	IN	JR	HW	SD	KL	RJ	DW	AD	PA	BN	PU	SI	NA	MU	VP	VD	PJ	MA	GK	ML	BL	YS	PC	ER	TR	KR
JU		0	0	0.13	0.04	0.03	0.12	0.32	0.09	0.16	0.01	0.07	0.09	0.08	0	0.04	0.06	0.32	0.01	0.03	0.05	0.03	0.08	0.04	0.03	0.06	0.07	0.03	0.07	0.05	0.04	0.32	0.04	0.01	0.07	0.09	0.06	0.14	0.14	0.07	0.07	0.11	0.11	0.11	0.19
DH			0	0.03	0.03	0.03	0.18	0.07	0.18	0.06	0.04	0.12	0.15	0.15	0.02	0.07	0.14	0.32	0.01	0.02	0.08	0.03	0.13	0.16	0.07	0.1	0.12	0.07	0.11	0.09	0.05	0.33	0.09	0.04	0.1	0.04	0.07	0.14	0.08	0.07	0.04	0.1	0.1	0.1	0.18
KG				0.01	0.01	0.07	0.19	0.1	0.13	0.08	0.06	0.13	0.16	0.17	0.04	0.08	0.15	0.12	0.12	0.12	0.09	0.04	0.12	0.06	0.08	0.07	0.13	0.07	0.05	0.09	0.07	0.34	0.11	0.04	0.08	0.08	0.06	0.1	0.08	0.06	0.11	0.1	0.07	0.07	0.13
DN					0.01	0	0.15	0.06	0.05	0.06	0.06	0.01	0.05	0.11	0.03	0.06	0.11	0	0	0.07	0.06	0.06	0.04	0.03	0.07	0.04	0.11	0.02	0.05	0.05	0.03	0.35	0.03	0.01	0.05	0.06	0.04	0.08	0.08	0.03	0	0.03	0.03	0.1	0.15
HD						0.02	0.2	0.07	0.19	0.06	0.03	0.13	0	0.17	0.07	0.08	0.15	0.33	0.02	0.02	0.15	0.04	0.07	0.09	0.08	0.11	0.16	0.06	0.09	0.09	0.08	0.35	0.1	0.03	0.07	0.1	0.06	0.13	0.1	0.05	0.02	0.05	0.39	0.09	0.16
MD							0.12	0.35	0.06	0.04	0.06	0.08	0.11	0.12	0.02	0.04	0.1	0.32	0.02	0	0.1	0.02	0.02	0.03	0.03	0.05	0.07	0.01	0.03	0.04	0.06	0.34	0.05	0.01	0.03	0.04	0.03	0.06	0.06	0.02	0.02	0.02	0.34	0.02	0.09
GT								0.06	0.05	0.12	0.14	0.05	0.02	0.04	0.09	0.04	0.05	0.39	0.12	0.13	0.03	0.09	0.05	0.05	0.03	0.02	0.02	0.04	0.07	0.03	0.07	0.37	0.03	0.07	0.08	0.07	0.05	0.12	0.06	0.14	0.23	0.16	0.13	0.05	0.2
LK									0.05	0.01	0.02	0.05	0.02	0.04	0.02	0.04	0.05	0.34	0.03	0.05	0.1	0.03	0.03	0.04	0.03	0.03	0.04	0.02	0.07	0.04	0.03	0.32	0.01	0.02	0.09	0.1	0.06	0.44	0.02	0.09	0.08	0.11	0.15	0.15	0.23
GU										0.11	0.13	0.01	0.01	0.02	0.09	0.04	0.01	0.39	0.12	0.13	0.04	0.1	0.08	0.05	0.53	0.03	0.03	0.03	0.08	0.03	0.08	0.37	0.02	0.07	0.09	0.09	0.06	0.04	0.07	0.14	0.23	0.22	0.14	0.14	0.11
RP											0.01	0.1	0.12	0.09	0.03	0.08	0.1	0.34	0.02	0.05	0.12	0.03	0.04	0.06	0.06	0.05	0.06	0.04	0.09	0.06	0.05	0.22	0.03	0.02	0.1	0.12	0.06	0.18	0.03	0.09	0.07	0.1	0.16	0.17	0.02
CW												0.12	0.14	0.12	0.02	0.08	0.13	0.35	0.03	0.05	0.15	0.15	0.07	0.08	0.07	0.09	0.1	0.06	0.01	0.08	0.07	0.35	0.08	0.03	0.12	0.15	0.42	0.2	0.07	0.09	0.07	0.1	0.43	0.17	0.17
DM													0.03	0.01	0.06	0.01	0	0.35	0.08	0.08	0.01	0.06	0.04	0.02	0.01	0.01	0	0.01	0.03	0.03	0.04	0.34	0.01	0.04	0.06	0.04	0.02	0.08	0.04	0	0.16	0.17	0	0.08	0.16
SH														0.01	0.09	0.01	0	0.38	0.11	0.11	0.01	0.04	0.02	0	0.02	0.02	0.02	0.03	0.02	0.02	0.05	0.35	0.03	0.08	0.07	0.04	0.03	0.08	0.05	0.11	0.2	0.2	0.1	0.09	0.17
PN															0.12	0.05	0.01	0.39	0.09	0.13	0.02	0.07	0.08	0.05	0.07	0.01	0.01	0.03	0.06	0.03	0.04	0.34	0.01	0.06	0.11	0.07	0.05	0.13	0.04	0.15	0.23	0.11	0.14	0.13	0.23
AB																0.03	0.06	0.01	0.02	0.02	0.05	0.03	0.09	0.03	0.03	0.06	0.08	0.02	0.07	0.05	0.04	0.32	0.04	0.01	0.06	0.09	0.05	0.13	0.05	0.15	0.07	0	0.1	0.1	0.18
IM																	0.02	0.32	0.05	0.04	0.01	0.03	0.05	0	0	0.02	0.03	0.01	0.02	0.04	0.03	0.32	0.03	0.02	0.04	0.02	0.01	0.06	0.03	0.04	0.1	0.11	0.36	0.05	0.1
GY																		0.37	0.09	0.1	0.01	0.06	0.04	0.03	0.02	0.01	0.01	0.02	0.05	0.02	0.04	0.34	0.02	0.05	0.08	0.05	0.01	0.09	0.13	0.12	0.2	0.21	0.11	0.09	0.18
UJ																			0.01	0	0.09	0.01	0.01	0.02	0.02	0.04	0.05	0.01	0.02	0.02	0.03	0.32	0.04	0	0.03	0.03	0.02	0.15	0.04	0.02	0.01	0.27	0.04	0.01	0.1
BP																				0.01	0.09	0	0.02	0.03	0.02	0.08	0.05	0.01	0.02	0.04	0.02	0	0.03	0.01	0.07	0.06	0.03	0.1	0.03	0.05	0.03	0.07	0.09	0.09	0.16
IN																					0.09	0.01	0.02	0.02	0.03	0.04	0.06	0.01	0.03	0.03	0.06	0.34	0.05	0.01	0.03	0.04	0.02	0.06	0.05	0.02	0.01	0.02	0.36	0.05	0.1
JR																						0.08	0.05	0.06	0.06	0.07	0.06	0.06	0.07	0.07	0.01	0.31	0.07	0.08	0.12	0.08	0.07	0.12	0.08	0.11	0.12	0.14	0.16	0.05	0.13
HW																							0.01	0.02	0.02	0.02	0.03	0.02	0.02	0.03	0.26	0.32	0.03	0.01	0.06	0.03	0.02	0.06	0.02	0.04	0.03	0.07	0.07	0.05	0.14
SD																								0	0	0.01	0.01	0	0.01	0.01	0.03	0.33	0.01	0.01	0.04	0.02	0.02	0.06	0.02	0.03	0.04	0.06	0.1	0.1	0.16
KL																									0	0.01	0.02	0.01	0.01	0	0.03	0.32	0.02	0.02	0.04	0.02	0	0.04	0.03	0.04	0.06	0.06	0.04	0.04	0.11
RJ																										0	0.01	0.01	0.01	0.01	0.02	0.31	0.01	0.02	0.06	0.03	0.01	0.06	0.01	0.05	0.06	0.06	0.06	0.06	0.13
DW																											0	0.01	0.02	0.01	0.02	0.31	0.01	0.03	0.08	0.03	0.01	0	0.01	0.07	0.08	0.1	0.1	0.08	0.17
AD																												0.02	0.05	0.02	0.02	0.31	0.01	0.04	0.09	0.04	0.02	0.09	0.01	0.08	0.1	0.12	0.11	0	0.19
PA																													0.02	0	0.03	0.32	0.01	0.01	0.03	0.02	0.02	0.06	0.02	0.04	0.05	0.05	0.07	0.07	0.13
BN																														0.01	0.14	0.32	0.04	0.02	0.04	0.01	0	0.02	0.04	0.03	0.06	0.01	0.03	0.02	0.02
PU																															0.02	0.32	0.02	0.02	0.04	0.03	0.03	0.06	0.03	0.04	0.14	0.06	0.36	0.08	0.12
SI																																0.31	0.01	0.03	0.09	0.05	0.03	0.1	0.01	0.11	0.13	0.19	0.39	0.08	0.18
NA																																	0.01	0.01	0.09	0.04	0.02	0.1	0.02	0.09	0.1	0.17	0.39	0.08	0.18
MU																																		0.07	0.08	0.06	0.03	0.12	0.11	0.08	0.09	0.11	0.1	0.12	0.2
VP																																			0.04	0.05	0.03	0.11	0.03	0.03	0.03	0.04	0.37	0.04	0.06
VP																																				0	0.04	0.05	0.11	0.02	0.03	0.01	0.33	0.05	0.06
PJ																																					0.01	0.01	0.05	0.07	0.15	0.07	0.33	0.07	0.07
MA																																						0.03	0.04	0.04	0.05	0.06	0.34	0.02	0.09
GK																																							0.09	0.05	0.07	0.06	0.32	0	0.04
ML																																								0.12	0	0.16	0.13	0.1	0.22
BL																																									0.02	0.02	0.35	0.04	0.15
YS																																										0.02	0.38	0.08	0.13
PC																																											0.1	0.11	0.15
ER																																												0.01	0.02
TR																																													0.03
KR																																													

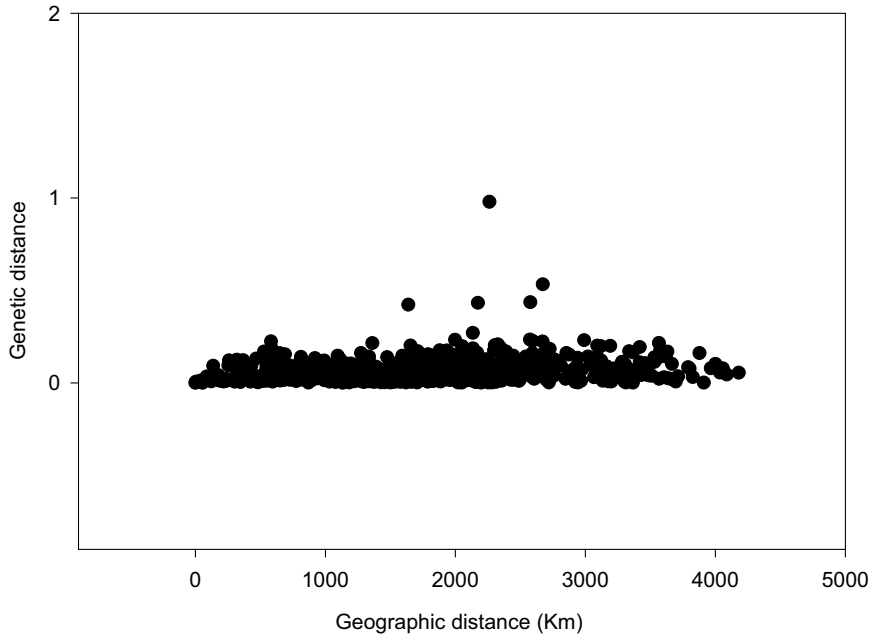

Figure 1. Correlation between geographic distance (Km) and genetic distance in natural population of *Drosophila ananassae*

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Does an Actomyosin System Supplement the Axoneme in Mammalian Sperm

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Abstract

Boar sperm fixed in paraformaldehyde, pemeabilized in cold acetone and incubated in the presence of DAPI and Rhodamine-conjugated phalloidin or with fluorescein-conjugated cytochalasin D show brilliant blue staining of the nucleus and brilliant red or green fluorescence respectively in the middle piece and tail regions of the sperm. The results suggest the presence of F-actin in the boar sperm middle piece. Immunocytochemical studies also show the presence of tropomyosin in the middle piece region of boar sperm.

Western Blot studies, using lysates of isolated boar sperm middle pieces and tails, indicate prominent sperm-actin bands with similar electrophoretic patterns of rat cardiac and striated muscle. The results strongly suggest that a 41 kDa actin, similar to cardiac or striated muscle actin, is present in sperm middle pieces and corroborates the phalloidin and cytochalasin D localization by fluorescence microscopy.

We suggest that the simple sliding filament mechanism of the axoneme may not, by itself, impose the propulsive forces needed to propel large spermatozoa through the viscous prefertilization milieu. We further propose that an actomyosin system associated with the microtubule/dynein complex might play a role in sustained motility of mammalian spermatozoa.

Keywords: Actomyosin, Axoneme, Flagella, Boar sperm

1. Introduction

Analysis of the motion of flagella and cilia would suggest a mechanism based on the sliding of microtubules (1-2). They may be sliding in relation to each other, and the sliding out of a tubule preferentially on one side of the cilium could produce a bending in that direction (3). Thus the dynein cross bridges or motors that form connections with microtubules are implicated in this movement. Presumably, a dynein complex attached to the A tubule of a doublet is in a position to interact with the B tubule of an adjacent complex. Studies with isolated *Tetrahymena* cilia suggest that free dynein arms are polarized and tilt uniformly away from the direction of active sliding towards the base of the cilium (2). More recent studies confirm dynein-tubulin interactions for microtubule-based motility (4). In all cases ATP hydrolysis appears to be involved in motion. Thus the sliding-filament/microtubule hypothesis of ciliary motility, generated by ATP, is considered acceptable dogma for single cells, including simple sperm, and ciliated epithelia. In these cells, microtubular structures are arranged linearly, and thus impose a vectoral displacement of matter in an aqueous medium. The velocity of sliding is directly related to the propulsive force generated in an aqueous environment and is proportionately related to the mass of the organism.

What happens when a massive unicellular organism, equipped with a single cilium or flagellum, must move in a viscous medium? Structurally, filaments and fibers that are accessory to the microtubule/dynein complexes are present in some of these large cells. A case in point is sperm of most mammals that show the presence of accessory coarse fibers and a plethora of axonemal filaments and fibers (5). Biochemically, isolated coarse fibers appear not to contain contractile proteins and might just represent stiffening rods in the middle pieces and tails of large sperm. Might these coarse fibers represent attachment sites for contractile proteins also located in the axoneme? Conceivably, an ATP-regulated contractile system accessory to the microtubule/dynein system may be essential to generate the motive and propulsive forces needed to propel large cumbersome sperm cells in a viscous milieu.

We suggest that the simple sliding filament mechanism of the axoneme may not, by itself, impose the propulsive forces needed to propel large spermatozoa through the viscous prefertilization milieu. We further propose to establish that an actomyosin system associated with the microtubule/dynein complex might play a role in sustained motility of mammalian spermatozoa.

2. Materials and Methods

Fresh boar semen was purchased commercially from the Zierke Company. Sperm were checked for motility and viability and maintained at 26°C, prior to further use. Sperm maintained viability up to 7 days under these conditions.

2.1 Sperm isolation and purification

To remove the seminal fluid from sperm, semen was diluted in two changes of PBS-PMSF and centrifuged at 3000g for 20 min in a refrigerated swinging bucket rotor. The pellet was resuspended and layered over a 35% and 70% Percoll/PBS gradient in a 15ml conical centrifuge tube and centrifuged at 2000 x g for 5 min followed by centrifugation at 4000 x g for 10 min. The fluffy mass of sperm near the middle of the 70% Percoll layer was aspirated using a pipette and transferred to a centrifuge tube. The collected sperm were resuspended in PBS, washed, and pelleted by centrifugation.

2.2 Sperm tail isolation

Spermatozoa suspended in PBS-PSMF were homogenized at 30 second intervals until the head was separated. Complete separation of sperm head and tail was checked by microscopy. The decapitated spermatozoa were washed in PBS-PSMF and centrifuged for 10 min. The pellet was resuspended in 20% (w/v) sucrose in PBS-PMSF before layering over a 20%, 40% and 60% sucrose gradient and centrifuged at 3000 x g for 90 min in a refrigerated swinging bucket rotor. Sperm tail fractions were collected from the 40-60% sucrose interface and purity was assessed by microscopy. The sperm tail fraction was re-suspended in 20% (w/v) sucrose in PBS-PMSF and centrifuged again at 3000 x g through a 20%, 40% and 60% sucrose step gradient for 60 min. Sperm tails were collected from 40-60% interface, which contained <1% sperm head contamination. The sperm tails were subsequently diluted with PBS-PMSF and pelleted by centrifugation at 15000 x g for 10 min.

2.3 Western Blot Analysis for Actin

For detection of actin, purified sperm tails, rat striated and cardiac muscle were lysed in 3 different lysis buffers (a) ice-cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, and 50 mM NaF), (b) 0.1M Tris –HCL, 1M EDTA, 8M urea and 0.05M DDT and (c) 100mM Tris-HCL, 100mM KCl, 200UM EDTA, and 1.5mM MgCl2.The resulting lysates were centrifuged at 14000 x g for 10 min to remove cell debris. The samples were mixed with SDS sample buffer and boiled for 5 min. 20 µl of the prepared lysates, as well as purified actin (Abcam, Cambridge, Ma) were resolved on 12% SDS-polyacrylamide gels and electroblotted to PVDF membrane (Hybond, Amersham). Membranes were blocked for 1 hr at room temperature with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with antibodies specific to actin (0.5 µg/mL, Molecular probes, Eugene Oregon, USA) for 24 hr in the same buffer. After washing, the blots were incubated for 1 hr with a 1:4000 dilution of horseradish peroxidase (HRP)–conjugated secondary antibody and visualized using an enhanced chemiluminescence detection system (Amersham).

2.4 Localization of Actin by Phalloidin and Cytochalasin

Sperm were resuspended with PBS-PMSF and washed three times before pelleting by centrifugation and fixed for 30 min with a 4% paraformaldehyde buffered with PBS or sodium cacodylate. The sperm samples were rinsed overnight with several changes of PBS and then permeabilized with cold acetone (-20°C) for 5-10 min. Cells were washed and incubated for 1 hr in a 1:1000 dilution of Texas Red, Alexa, or Fluorescein phalloidin (Invitrogen, Molecular Probes) in PBS. The cells were washed three times, counterstained with DAPI for 3 min and washed several times with PBS before mounting in Slow Fade. Some sperm were fixed, washed and permeabilized as above and incubated in fluorescein-conjugated cytochalasin D (Invitrogen, Molecular Probes). The samples were viewed under a Zeiss fluorescence microscope using a Rhodamine filter (wavelength 550 nm), or a fluorescent triple excitation filter for UV, Rhodamine and Fluorescein.

2.5 Immunocytochemical Procedres

Sperm, washed of seminal fluid, were fixed for 30 min in 4% buffered paraformaldehyde. The fixed samples were washed thoroughly with PBS and permeabilized with cold acetone (-20°C) for 5 min. Sperm were washed with PBS then blocked with 1% Bovine serum albumin for 30 min, washed and incubated in a 1:500 dilution of primary antibody to tropomyosin (Abcam, Cambridge, MA.) for 1 hour except the control which was incubated with PBS. The samples were washed three times with PBS then incubated with FITC or Rhodamine conjugated secondary antibody (1:500 dilution) for 30 min. The samples were washed three times with PBS and counterstained with diluted DAPI, washed and mounted in Slow Fade and visualized under a Zeiss fluorescence microscope.

3. Results

Sperm fixed in paraformaldehyde, pemeabilized in cold acetone and incubated in the presence of DAPI and Rhodamine conjugated phalloidin show brilliant blue staining of the nucleus and brilliant red middle pieces (Fig. 1). The phalloidin reaction started just below the neck region of the sperm, passed through the middle piece region and extended into the sperm tail (Fig. 2). To verify the presence of actin, sperm that were fixed in paraformaldehyde, permeabilized with acetone and incubated with fluorescein-conjugated cytochalasin D, showed similar green fluorescence in the middle piece and tail regions. The results confirm the presence of F-actin in the boar sperm middle piece. When reacted with fluorescein-conjugated mitotracker and phalloidin, faint staining of the actin was observed. The presence of the surrounding mitochondria of the middle piece obscures actin staining in the axoneme.

To confirm the presence of actin in boar sperm middle pieces, sperm tails were lysed with several buffers specific for mammalian cell actin isolation and electrophoresed to immunodetect actin. Sperm tail lysates were electrophoresed alongside rat cardiac and striated muscle lysates and reacted to reveal actin. Western blot results revealed actin in sperm, striated muscle and cardiac muscle (Fig. 5). These actin bands had identical molecular weights around 41KDa.

Sperm fixed in paraformaldehyde, permeabilized with acetone, washed and incubated with anti-tropomyosin antibodies reveal a strong red fluorescence reaction after treatment with a Fluorescein-conjugated secondary antibody to antitropomyosin (Fig. 6). The results suggest a possible actin-tropomyosin binding site in the middle pieces and tails of the boar sperm.

4. Discussion

The phallotoxins are reliable probes for labeling, identifying and quantitating F-actin in formaldehyde-fixed and permeabilized tissues and cells. Conjugated to green or red fluorescent dyes, fluorescein (FITC) or rhodamine (RITC) these probes have been used to accurately visualize actin filament assemblies, cytoskeletal reorganizations, and actin-related movement of intracellular organelles, and cell movements (6-13) Studies with cytochalasins B and D conclusively indicate that this cell-permeant fungal toxin binds to the barbed end of actin filaments preventing association of dissociation of subunits. Green or orange-fluorescent cytochalasin D (BODIPY FL; BODIPY TMR) are accurate indicators of F-actin in formaldehyde and permeabilized tissues and cells (Invitrogen, Molecular Probes). The literature is replete with examples showing the consistency of fluorescent-phalloidin and cytochalasin D probes for actin in a broad variety of cells (14-18). Our results consistently show the presence of actin from the neck to tail regions of formaldehyde-fixed and permeabilized boar spermatozoa. Previous studies have used electron microscopy to localize filaments, presumably actin, in cytosols of invertebrate spermatozoa and it has been speculated that thin axonemal filaments associated with microtubules may have actomyosin properties.

Western Blot studies, using lysates of isolated boar sperm middle pieces and tails, indicate prominent sperm-actin bands with similar electrophoretic patterns of rat cardiac and striated muscle. The results strongly suggest that a 41 kDa actin, similar to cardiac or striated muscle actin, is present in sperm middle pieces and corroborates the phalloidin and cytochalasin D localization by fluorescence microscopy.

How much and what role axonemal actin plays in sperm motility is not yet determined, as is their localization with respect to the axonemal microtubules and coarse fibers, and spermatozoan motility. Isolated coarse fibers show no actin reactivity. It might be construed that the coarse fibers, that seem keratin-like in nature, may represent binding sites for an active actmyosin system. The perimicrotubular filaments in close proximity to the axonemal microtubules and coarse fibers might represent an accessory actomyosin complex which together with the microtubular system generates the motive forces for spermatozoan propulsion, especially in a viscous internal fertilization environment.

By binding specifically to actin, tropomyosin plays a central role in the regulation of actonmyosin contractile systems (19-20). Immunocytochemical studies show the presence of tropomyosin in the middle piece region of boar sperm. Conceivably, actin-tropomyosin conjugation might represent a functional actomyosin complex needed for motility.

Papers by Fawcett (5) and others clearly reveal the presence of accessory filaments and fibers adjacent to the axonemal micotubular elements of the sperm tail. The filamentous configurations are especially prominent in sperm of the 13-lined ground squirrel and other species (5)). Less dramatic filamentous assemblies occupy the axonemal region of the middle piece of other mammalian sperm and are not prominent in sperm shed in an aqueous, non-viscous environment. Indeed, filamentous assemblies are not prominent in boar sperm, but if we are to believe the images after phalloidin and cytochalasin treatment the conclusion can be made to support the presence of actin at these sites. Incubation of sperm in DAB/OsO4 solutions reveals the existence of a matrix between the coarse fibers and surrounding the axonemal microtubules (Fig. 7). The Western blot data confirm actin in the middle piece and sperm tails. Similarly, tropomyosin immunofluorescence suggests the presence of a functional actomysoin system in the middle piece region of the boar sperm. More work is needed to determine the exact structural and functional relationship between the microtubular and actomyosin filaments and their contributions to sperm motility.

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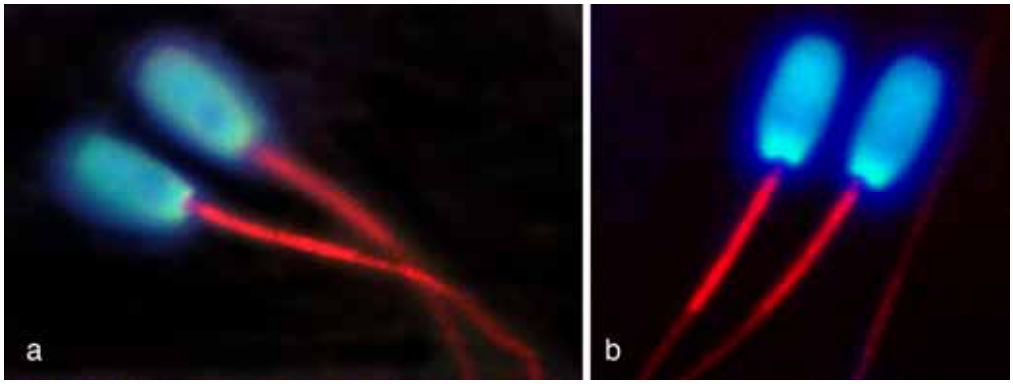
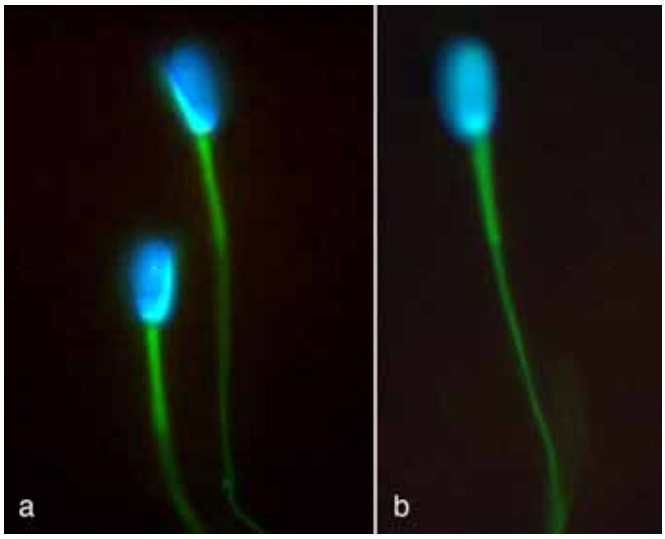


Figures 1 and 2. Texas Red-phalloidin actin is present in the middle piece and tails of paraformaldehyde-fixed and acetone permeabilized boar sperm. DAPI-reacted nuclei are also revealed. X400



Figures 3 and 4. Fluorescein-conjugated cytochalasin D reacts with F-actin in the middle piece and sperm tails. DAPI-reacted nuclei are stained blue. X200

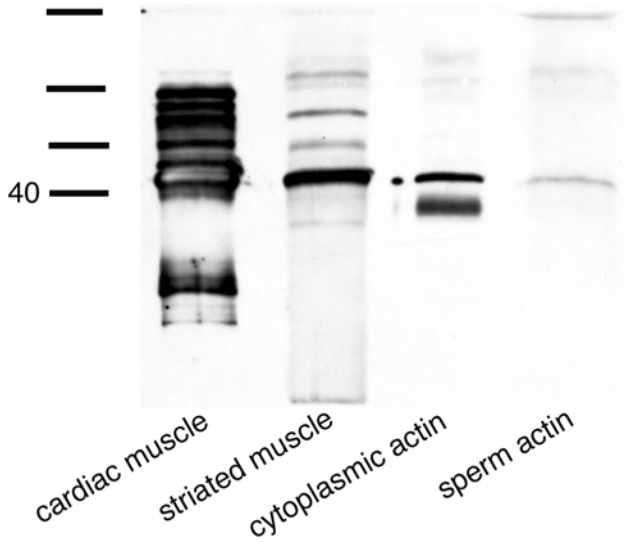


Figure 5. Western blot results revealed 41KDa actin bands cardiac (Lane 2), striated muscle (Lane 3), and in sperm lysates (Lane 5). Band 1 shows the standard and Band 4 shows pure actin (Abcam, Cambridge, MA).

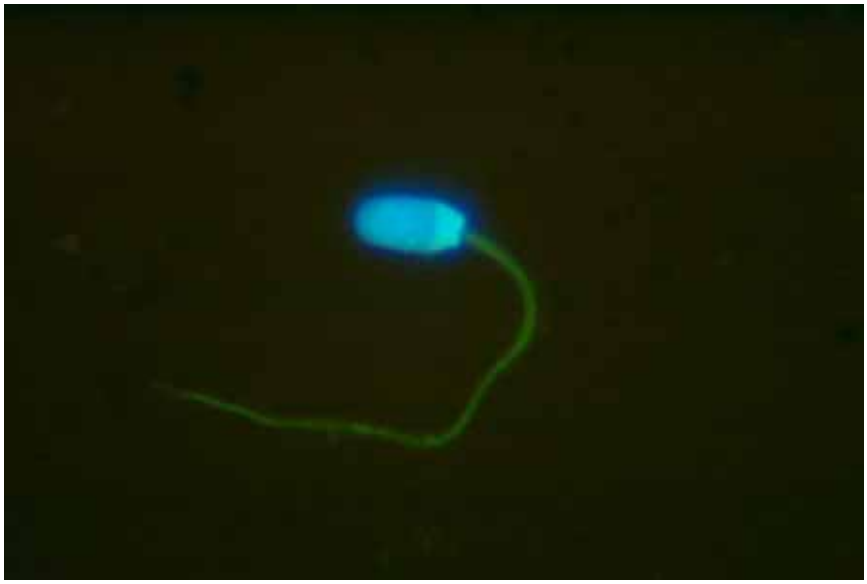


Figure 6. Immunocytochemical image reveals the presence of tropomyosin in the middle piece and tails of boar sperm. X200

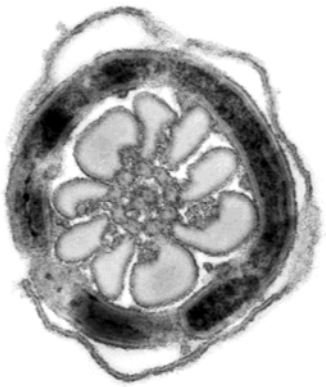


Figure 7. Sperm incubated in Diaminobenzidine and reacted with OsO4 reveal a matrix material between coarse fibers and surrounding the axonemal microtubules. X20,000

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Environmental Estrogens Stimulate Gene Transcription in the Prolactin Promoter

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Abstract

The ability of environmental estrogens to regulate gene transcription on model and physiologically complex promoter was examined in GH_3 cells, a pituitary cell line. In transient transfection studies, the pGL3 model promoter and the physiologically complex prolactin promoter were both responsive to the xenoestrogen bisphenol A and the phytoestrogen daidzein in a dose related manner. These transcriptional responses were mediated by estrogen receptors, as responses were ameliorated in the presence of ICI 180, 782, a pure antiestrogen. Cotransfection of Pit-1 significantly enhanced the transcriptional response of the prolactin promoter to stimulation by environmental estrogens. The nature and magnitude of transcriptional responses to estradiol sensitive genes following challenge by environmental estrogens is likely dependent on regulatory elements found in the promoter and their ability to recruit transcription factors.

Keywords: Pituitary, Bisphenol A, Daidzein, Transcription, Activation, Promoter, Genistein

1. Introduction

The steroid hormone 17 -estradiol (E_2) is the primary estrogen generated in the ovaries (Gruber et al., 2002) and regulates gene expression in a number of target tissues. This gives way to physiological effects which include cell differentiation, cell proliferation, and protection from pathological insult (Gruber et al., 2002, Heldring et al., 2007). Arguably, the most significant reproductive neuroendocrine event mediated by E_2 is the initiation of the luteinizing hormone (LH) surge in mammals during the estrous or menstrual cycle. This LH surge is driven by positive feedback

effects of estrogen on the pituitary and hypothalamus and these events subsequently trigger ovulation (de Ziegler et al., 2007).

Estrogen receptors alpha and estrogen receptor beta (ER , ER) mediate the action of E_2 in a target cell (Kuiper et al. 1996; Shughrue et al., 2007). Like other nuclear receptors, ERs can be divided into several functional domains that govern ligand dependent and independent gene activation and DNA binding (Dahlman-Wright et al., 2006). Cell and/or promoter specific ligand independent gene activation (as is the case of stimulation of the estrogen receptor by growth factors) is mediated at the amino terminus of the ER via activation function-1 (AF-1). The central receptor domain is characterized by the presence of two zinc fingers mediating DNA binding. The carboxyl terminus of the ER, containing the AF-2 domain, is required for ligand binding and ligand dependent transcription. ER and ER differ primarily in the amino acid sequence identity between them, particularly in the AF-1 domain (Tora et al., 1989; Zhao et al., 2008) and the respective distribution of the proteins in target tissues. In the rat pituitary, ER . and ER can be detected but ER. is the predominantly expressed in lactotrophs and gonadotrophs (Gonzalez et al., 2008).

The reproductive effects of E_2 are largely initiated via a genomic mechanism of action (Greeley et al., 1975). Ligand binding induces the estrogen receptor (ER) dimerization and dissociation from stabilizing heat shock proteins (Dahlman-Wright et al., 2006; Heldring et al., 2007). The receptor complex translocates to the nucleus to bind an estrogen response element (ERE), in the promoter region of a responsive gene (Gruber et al., 2004; Dahlman-Wright et al., 2006; Heldring et al., 2007). The displacement of corepressor proteins and the recruitment of coactivators and integrator proteins to the receptor results in chromatin remodeling and the establishment of a preinitiation complex (Moggs & Orphanides, 2001). With the subsequent recruitment of RNA polymerase I, transcription the target gene commences.

The actions of endogenous E_2 may be mimicked by environmental estrogens (EEs) which can bind ERs and alter gene transcription. Phytoestrogens are non-steroidal plant derived compounds with estrogenic activity and include lignans, coumestans, and isoflavones. Of particular interest to this study, the abundant isoflavones tend to be introduced into the body as a dietary component or generated as bioactive products during metabolic processing (Atkinson et al., 2005). Xenoestrogens are non-steroidal, synthetic estrogen mimics that may enter the environment via a number of routes as components of plastics, industrial pollutants or pesticides (Safe, 2004). Xenoestrogens are endocrine disruptors—their negative effects on physiology, particularly reproduction, are well documented in a number of non-mammalian species (Pickford & Morris 1999; Olmstead & LeBlanc, 2000; Cheshenko et al., 2008).

Collectively, phytoestrogens and xenoestrogens act as selective estrogen receptor modulators—their specific actions (as agonists or antagonists) on a given gene may depend on number of factors: dose, length of exposure, cell type, and the ability to recruit various regulatory promoter elements in the targeted gene (Nowakowski et al., 1994; Gould et al., 1998; Lascombe et al., 2000). Thus, the prevalence of EE and our frequent exposure to them may have significant transcriptional effects on E_2 regulated genes. In that E_2 regulates the coordinated release of pituitary hormones, it is critical to characterize the consequences of environmental estrogen exposure on transcriptional regulation of gene expression in anterior pituitary gland cell types. We examined the ability of EEs to regulate the transcriptional activity in a relatively simple model promoter and a pituitary specific, complex, estrogen regulated promoter.

2. Materials and Methods

2.1 Cells

The mammosomatotroph GH_3 cell line (ATCC® CCL-82.1) secretes prolactin (PRL) and growth hormone and was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 5% charcoal-stripped newborn calf serum (NCS) (HyClone, Logan, UT) at 37C in 95% O_2/5% CO_2.

2.2 Plasmids

The model ERE reporter plasmid (250 ng/well in all experiments) used in transfection experiments was pGL3 containing 2 consensus EREs fused to the SV40 viral promoter (gift of Dr. Margaret Shupnik). Plasmid constructs 2.5 kb of the 5' flanking region of the rat PRL gene (PRLluc, gift of Dr. Margaret Shupnik) was also used. Rat Pit-1 was inserted into the pCDNA 3.1 expression vector (Invitrogen, San Diego, CA). Total plasmid DNA concentrations used in transfections were normalized with pcDNA 3.1.

2.3 Transient transfection

GH_3 cells were seeded to $1x10^6$ 6 well plates (BD Falcon, San Jose, CA) with phenol red-free DMEM (Mediatech, Manassas, VA) supplemented with 5% charcoal-stripped newborn calf serum and 100U/ml penicillin, and 100 ug/ml streptomycin to a final concentration of 1 x 10^6 cells/well. 250 ng PRL-luc or pGL3 per well was transiently transfected into cells via GeneJuice® transfection reagent (Novagen, Gibbstown, NJ) according to manufacturer's instructions. To assess the effect of Pit-1 on EE induced transcriptional activity, 100 ng/well of Pit-1 expression vector was cotransfected with PRL-luc. Following a 16 hour transfection, media was changed and cells were treated with vehicle or

10nM E_2. In addition, some cells will be treated with a range of doses of bisphenol A (BPA, a xenoestrogen), kepone (K, a xenoestrogen), genistein (G, a phytoestrogen), or daidzein (D, a phytoestrogen) for 24 hours. All treatments were purchased from Sigma (St. Louis, MO) with the exception of BPA (Oakwood Products, Columbia SC) and K (Chem Service, West Chester, PA). Cells were washed twice with PBS (pH 7.4) and collected in 200ul 1X lysis buffer (Promega, Madison, WI). 50 l lysate was assayed for luciferase activity. Luciferase assays of lysate samples were performed with a Turner TD-20E luminometer and protein content determined by total lysate protein using protein dye (Promega, Madison, WI). Promoter activity is expressed as ALU/ g protein.

2.4 Data analysis

Values are expressed as mean ± SEM. Transfections were performed in triplicate; experiments were performed at least 4 times. To determine if there are differences in promoter activity as a function of treatment, a one way analysis of variance (ANOVA) was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). The Tukey's post hoc test was used to determine significant differences between the means. Statistical significance was achieved at $p<0.05$.

3. Results

3.1 EEs stimulate E2 sensitive promoters in a dose related fashion

We first examined the ability of two EEs, BPA, a xenoestrogen and D, phytoestrogen to stimulate pGL3, a model ERE-containing promoter (Figure 1). Untransfected cells exhibited negligible basal transcriptional activity, which increased only slightly upon transfection of reporter (Figure 1, Panel 1). The pGL3 promoter exhibited a 3.43 fold increase in transcriptional activity following challenge with 10nM E_2. As cells were treated with increasing concentrations of BPA (10^{-15}M-10^{-6}M), promoter activity was observed to increase in a dose related fashion, though response to highest dose of BPA was not significantly different that of E_2 alone (3.23 fold increase in activity as compared to vehicle treated control). In subsequent experiments, 1 M BPA was used as a stimulatory dose. Daidzein was more effective at stimulating promoter activity than BPA (4.6 fold at maximum dose as compared to control) and stimulated a slightly more robust response increase in pGL3 activity than E_2.

The E_2 regulated PRL promoter was also stimulated by EE (Figure 1, Panel B) with the exclusion of the lowest dose tested (10^{-15}M). BPA and D significantly increased PRL promoter activity as compared to vehicle treated controls and E_2 treated cells (3.6 and 4.2 fold respectively at the highest doses), however, BPA and D were equally as effective at stimulating promoter activity.

3.2 EEs regulate promoter activity via interactions with ERs

To demonstrate that EE indeed interact with ERs to bring about a transcriptional response, transfected cells were co treated with stimulatory doses of estrogens (including genistein (G), a phytoestrogen) and kepone (K), a xenoestrogen) in the absence and presence of ICI 182, 780 a pure ER antagonist. ICI 182,780 reduced basal pGL3 activity and prevented BPA- or D-induced increases in transcriptional activity (Figure 2, Panel A). Similar results were observed in the PRL promoter. While, ICI had no effect on basal activity of the PRL (Figure 2, Panel B) promoter, estrogen-induced activity was soundly prevented in cells co treated with ICI and E, BPA, or D. Thus, the activation of promoter activity by EE is mediated by ER and/or ER.

3.3 Stimulation of PRL promoter by EEs is enhanced in the presence of the transcription factor Pit-1

Having observed some basic aspects of EE activity on model promoters, we also examined their effects in physiologically complex promoters. The PRL promoter contains an imperfect ERE in the distal promoter region. GH_3 cells were transfected with 250 ng PRL promoter fused to and ERE and treated for 24 hours with E_2, BPA, and D. Promoter activity was significantly increased by all estrogenic compounds, with D being especially potent (4.2 fold). Cotransfection of the pituitary transcription factor Pit-1 significantly enhanced the stimulatory effects that were observed. BPA-induced transcription in the PRL promoter was increased by 40%, while D-induced transcription was increased by 20%.

4. Discussion

The goal of this study was to assess transcriptional activity in a model gene promoter and physiologically complex gene promoter following exposure to EEs. A wealth of *in vitro* and *in vivo* studies clearly demonstrates that xenoestrogens and phytoestrogens can exert significant effects on E_2 regulated target tissues. For example, isoflavones (phytoestrogens) bind and activate ER and ER in uterine and breast cancer cell lines with varying degrees of potency depending on cell and promoter context (Willard & Frawley, 1998, Massaad & Barouki, 1999; Bowers et al., 2000), but preferentially bind ER (Kuiper et al., 1994; Mueller et al., 2004; Harris et al., 2005). Regarding pituitary effects in whole animal studies, ingestion of BPA (via drinking water) in rats reduces serum LH levels, suggesting that BPA may act on the hypothalamus or pituitary gland (Rubin et al., 2001). Subcutaneous injection of BPA increases PRL levels in rats (Goloubkova et al., 2000).

In primary, transfected pituitary cultures, BPA treatment stimulates an ERE and PRL promoter and increases PRL secretion in cell culture and whole animal studies (Steinmetz et al., 1997). Oral administration of G to rats for 21 days or the D metabolite equol for 2-3 months significantly increased serum levels of PRL (Santell et al., 1997; Rachon et al., 2007). Whole animal studies are less abundant, but ingestion of the daidzein itself increases serum PRL levels while decreasing serum concentrations of LH (Rachon et al., 2007). We focus here more closely on the physiology of the PRL promoter as a complex, E_2 sensitive pituitary promoter and challenged it with both the xenoestrogen BPA and the phytoestrogen D in the presence and absence of Pit-1, a pituitary specific transcription factor, and assessed transcriptional activity via reporter assay.

Our results are consistent with the results of Steinmetz et al. (1997). In our system, transcriptional activity in both promoter constructs were stimulated by EEs in a dose related fashion. These stimulatory effects are mediated via AF-2 interactions between the EEs and the ER, since ICI (which competitively inhibits the binding of estrogen to the ER) is observed to abolish these effects. Relative to the potency E_2, BPA, G and D proved to be more effective at stimulating transcription in the PRL promoter. Hormonal regulation of the PRL gene is mediated by transcription factors that bind in the proximal promoter and distal enhancer; these upstream regions then act synergistically to facilitate gene expression (Crenshaw et al., 1989). ERs, acting as ligand inducible transcription factors, are tethered to an imperfect ERE in the distal portion of the PRL promoter to stimulate gene transcription. The ability of the PRL promoter to be stimulated by estrogen requires the binding the transcription factor Pit-1 in the proximal and distal regions of promoter (Nelson et al., 1988) with the Pit-1 binding site adjacent to the imperfect ERE being of particular importance (Schaufele, 1999).

In our experiments, the cotransfection of Pit-1 resulted in a moderate increase in EE-induced transcription. Similar results have been seen in the stimulation of the PRL promoter using E_2 (Day et al., 1990) and confirmed here. Thus, occupancy of the ER by EEs may elicit responses in the PRL promoter that require Pit-1 for full transcriptional activity. Collectively, these data indicate that EEs indeed behave similarly as E_2 in the stimulation of the prolactin promoter with regard to mechanism but not magnitude. These data in these studies are significant because they demonstrate the effects of EEs on a pituitary specific gene promoter that is not regulated by a palindromic ERE, but multiple promoter elements.

How can differences in promoter response and magnitude of stimulation be explained? It is likely that sensitivity of the two promoters to EEs appears to be due to cell/promoter context. Routledge et al. (2000) demonstrated in transfection studies and GST pull down assays an increased recruitment of ER coactivators SRC-1 and TIF-2 by ER (as compared to ER) in the presence of BPA and G. While E_2 and EE utilize the same receptor and their respective abilities to stimulate transcription in sensitive genes is bolstered by a common transcription factor, Pit-1, it is possible the ligands may initiate discrepant interactions between other proteins within the nucleus that drive changes in gene transcription; EEs may initiate the recruitment of a different amounts or different/additional transcription factors to the nucleus upon binding ERs.

PRL is of physiological significance for its ability to initiate and maintain lactation in mammals, but also regulates the reproductive cycle, general body growth, and maternal behavior (Ben-Jonathan et al. 2008). The prolactin gene, however, is not the only E_2 sensitive gene expressed in the pituitary. Indeed, E_2 regulation gene expression and hormone release in the anterior pituitary is evidenced by the ubiquitous expression of ERs in the APG (Shupnik, 2002). Thus, the ability of xenoestrogens and phytoestrogens to stimulate complex promoter activity in pituitary cells may present some particular implications for overall health, but reproductive health in particular (Adeyoa-Osiguwa et al., 2003). With detailed information on specific nuclear mechanisms of EE action, we may be better able to assess risk of incidental or deliberate exposure to such compounds.

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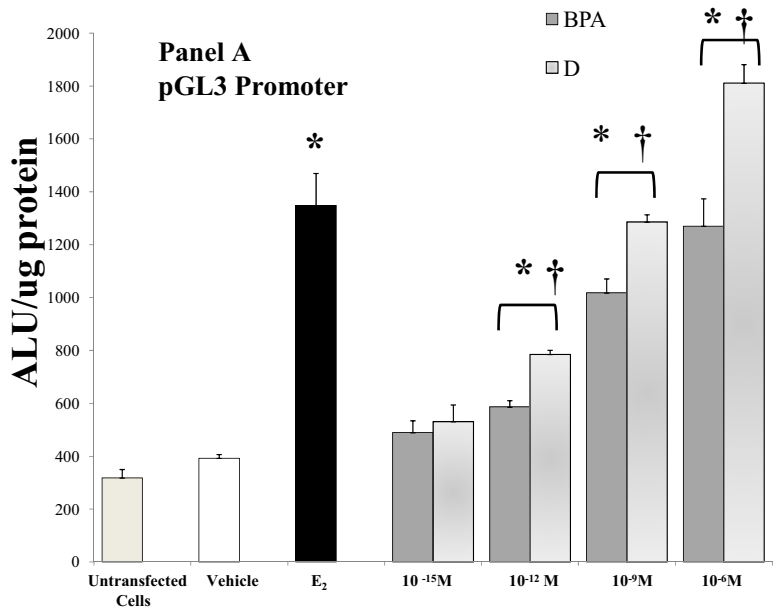
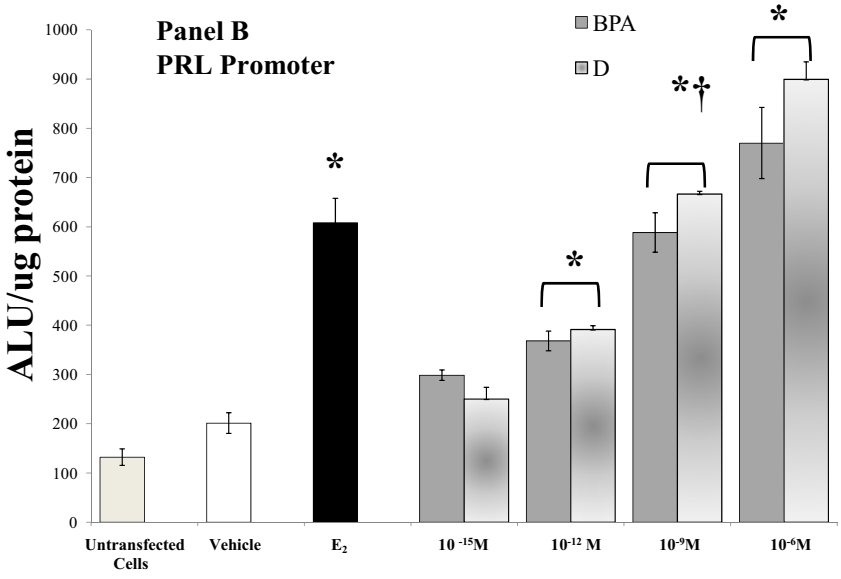
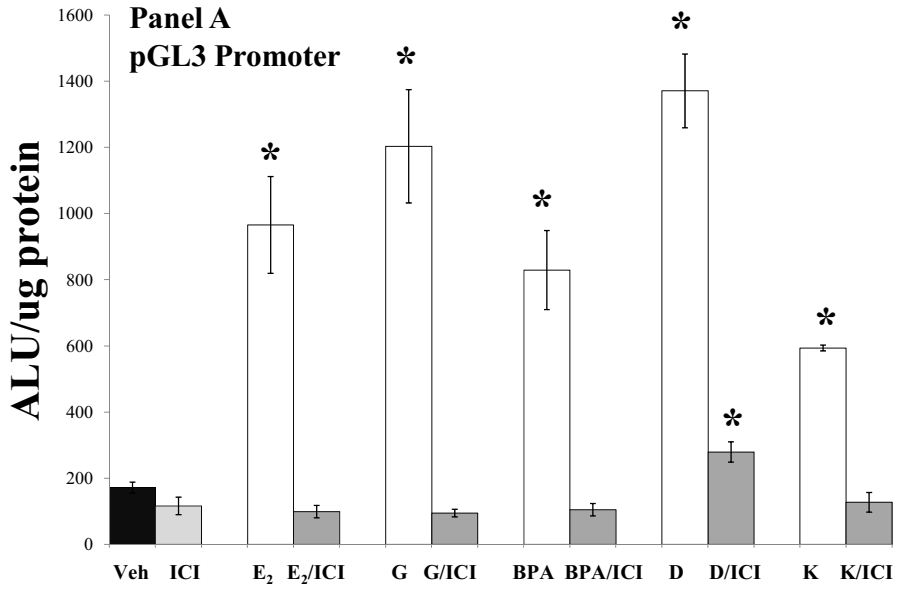


Panel A

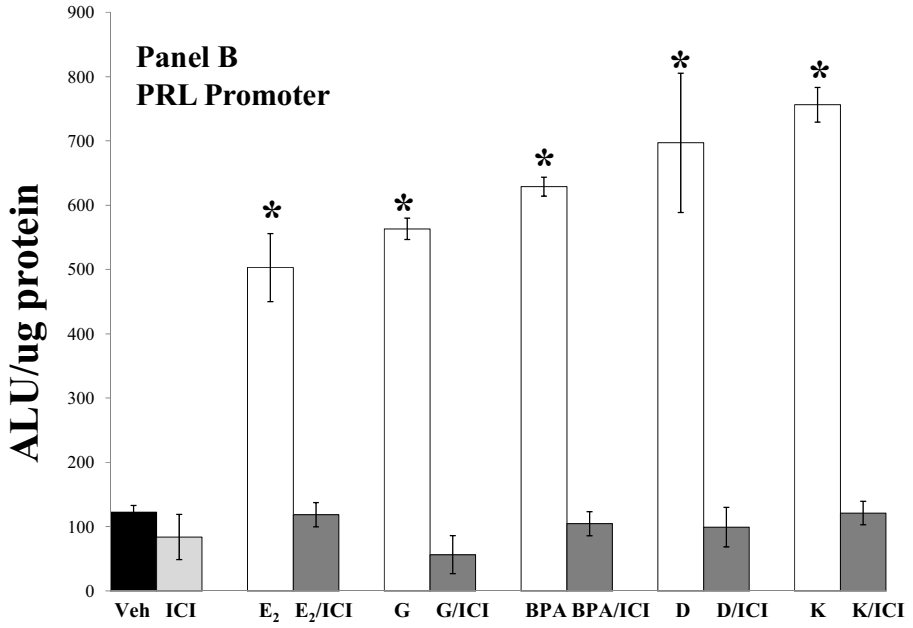


Panel B

Figure 1. Environmental estrogens stimulate transcription in a dose related manner. GH_3 cells were transfected with the 250 ng pGL3 model promoter (Panel A) or 250 ng of the physiologically complex PRL promoter (Panel B). Cells were treated for 24 hours with BPA (solid grey bars) or D (shadowed bars) at the doses indicated in the figure. Cells were collected, lysed and subjected to luciferase assay. Data are expressed as arbitrary light units per microgram of protein. Bars represent mean ± SEM for 4 experiments. For purposes of comparison, response of transfected cells to E_2 is also shown (black bar).*, significantly different from vehicle treated control, $P<0.05$. †, significant difference in respective responses of EEs, $P<0.05$.



Panel A



Panel B

Figure 2. ERs mediate EE-induced transcription of a model and complex promoter. GH_3 cells were transfected with the 250 ng pGL3 model promoter (Panel A) or 250 ng of the physiologically complex PRL promoter (Panel B). Cells were treated for 24 hours with stimulatory doses of E_2, G, BPA, D, and K in the absence (white bars) or presence (gray bars) of ICI 182, 780, a pure estrogen antagonist. Cells were collected, lysed, and subjected to luciferase assay. Data are expressed as arbitrary light units per microgram of protein. Bars represent mean ± SEM for 4experiments. *, significantly different from vehicle treated control (black bar), $P<0.05$.

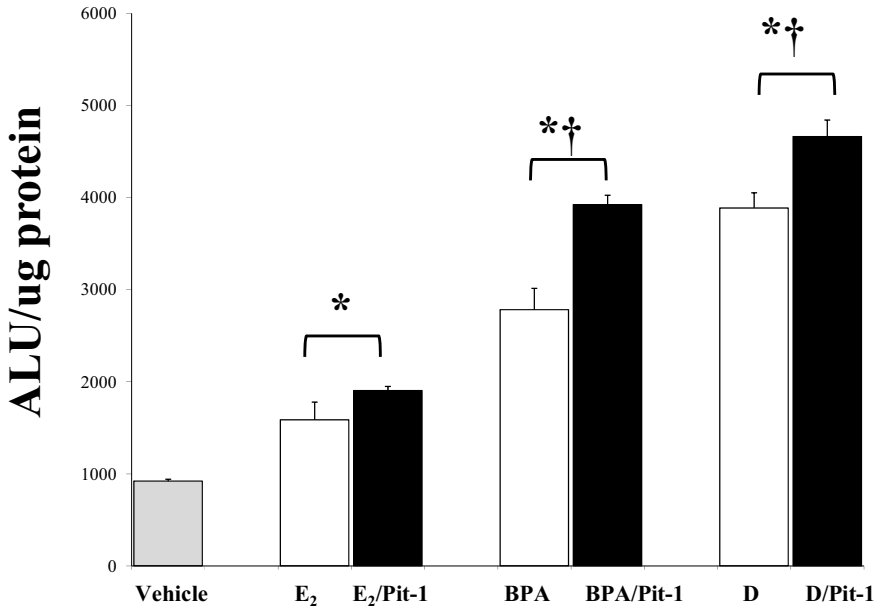


Figure 3. The nuclear transcription factor Pit-1 enhances EE-induced transcriptional responses in the PRL promoter. GH_3 cells were transfected with the 250 ng of the PRL promoter (white bars). Additionally, some cells were cotransfected with Pit-1 (black bars). Cells were treated for 24 hours with stimulatory doses of E_2, BPA or D. Cells were collected, lysed and subjected to luciferase assay. Data are expressed as arbitrary light units per microgram of protein. Bars represent mean ± SEM for 4 experiments. For purposes of comparison, response of transfected cells to E_2 is also shown (black bar). Bars represent mean ± SEM for 4 experiments.*, significantly different from vehicle treated control, $P<0.05$. †, significant difference in transcriptional responses in presence of Pit-1, $P<0.05$

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Comparative Growth Performance of Mixed-Sex and Monosex Nile Tilapia Population in Freshwater Cage Culture System under Indian Perspective

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Abstract

Cage culture is one of the important methods for intensive culture of tilapia in large water impoundments. But, information related to growth performance of androgen-treated monosex tilapia population during cage culture under the ecological conditions of India is limited. The aim of this study was to compare the growth potential of control, mixed-sex and androgen-treated, monosex tilapia in confined environment of cages. Control and hormone treated fish were stocked separately in mesh cages at a density of 50 fry / m^3 and it was found that the androgen treated monosex fish grew significantly larger than their control mixed-sex counterparts. The monosex population showed a significantly higher weight, length, depth, specific growth rate, daily weight gain, protein efficiency ratio and body protein content than the mixed-sex tilapia population. Thus, culture of hormone treated monosex tilapia in cages can be considered ideal for augmented production of the fish under Indian context.

Keywords: Cage culture, Nile Tilapia, Androgen treatment, Mixed-sex, Monosex, Growth potential

1. Introduction

The Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) is a widely cultured species because it grows and reproduces in a wide range of environmental conditions and tolerates stress induced by handling (Tsadik and Bart 2007). The fish is currently ranked second only to carps in global production (Ridha 2006). Previously, tilapia was consumed mainly in Africa and Asia but nowadays it has been touted as the "new white fish" replacing the depleted ocean stocks of cod and heck, leading to a worldwide demand for the fish (Yue and Zhou 2008). The efficiency of reproduction in tilapia has paradoxical consequences. This aptitude allows easy and rapid propagation of the fish in various environmental conditions, but can as well be a source of problem. Within a limited environment, uncontrolled multiplication of the fish not only reduces the faunal diversity of the system but also produces dwarf fish population of poor market value (Coleman 2001; Hepher and Pruginin 1981). Monosex culture of male tilapia is postulated to solve this problem and dietary supplementation of synthetic androgens is a potent method for production of all-male tilapia population (Guerrero 1982; Macintosh et al 1985; Gale et al 1999; Beardmore et al 2001; Smith and Phelps 2001). In a previous study, monosex population of male tilapia was produced by treating fry with a synthetic male hormone 17α-methyltestosterone (17αMT) at a treatment regime of 10 mg/kg food for 30 days (Chakraborty et al 2007). The predominant advantage of monosex culture can be achieved in such aquaculture situations where one sex displays marked growth superiority, as in tilapia (Beardmore et al 2001). Thus, culture of such hormone treated monosex tilapia in confined system might prove effective to induce a positive approach towards tilapia culture in India.

Tilapia has been widely introduced in the shallow and seasonal ponds of eastern region of India (Sugunan 1995). It is rapidly gaining popularity among the fish farmers as a readily available source of animal protein in the diets of rural and urban dwellers belonging to the lower socioeconomic strata. However, such indiscriminate introduction of the exotic fish into the open waters has generated a lot of debate in recent years. The ecologists and aquaculturists are concerned with the adverse effect of this exotic group on the indigenous fish population (Bartley and Martin 2004; De Silva et al 2004). Cage culture can provide an effective measure to this problem. Tilapia can be cultured at high densities in mesh cages that maintain free circulation of water. It ensures flexibility in management practices with easy and low cost of harvesting. Such culture method is found suitable for tilapia as it provides means for not only more intensive production, but also controlling wild spawning and overpopulation. It can be applied in existing bodies of water that cannot be drained or seined and would otherwise not be suitable for aquaculture. But, this culture method is yet to be commercialized on a wide scale basis and is still in its pilot stage in India. Moreover, only a few published data on the growout performance of androgen treated *Oreochromis niloticus* in the Indian context is available (Pandian and Varadaraj 1988). Hence, the propagation potentiality of sex-reversed tilapia population under cage culture system must be clearly documented. Considering these aspects, the present study aims to evaluate the comparative growth performance of the control mixed-sex and androgen treated monosex tilapia in cage culture system under the climatic and ecological conditions prevailing in the Gangetic plains of West Bengal, India.

2. Methods

Three days old mixed sex juveniles of Nile tilapia (mean weight 0.025 ± 0.009 g; mean length 1.25 ± 0.012 cm) were collected from the Fish Hatchery at Naihati, West Bengal. The fish were initially reared in glass aquaria at laboratory for one month and thereafter they were transferred to mesh cages for further five months of culture. In the laboratory, the fish were divided into two equal groups (n=50). During the first 30 days, one of the groups was given control diet at a rate of 20% body weight/day while the other was fed with 17α methyltestosterone (17αMT) treated diet with a dose of 10 mg/kg at the same rate. This optimal hormone treatment regime for production of almost 100% male tilapia population was deduced through our previous study (Chakraborty et al 2007) and hormone treated diets were prepared by the alcohol evaporation technique (Shelton et al 1978). In brief, during the previous study, the fish were fed with differential hormone doses (5 mg/kg, 10 mg/kg, 20 mg/kg and 30 mg/kg food) for differential time period (15 days, 30 days, 45 days and 60 days) (Chakraborty et al 2007). The differential amount of 17αMT was dissolved in 95% ethanol and then mixed with the pellets (1 litre ethanol/kg pellets). The 17αMT containing wet pellets were dried overnight in the open air. Control pellets were prepared similarly without the addition of 17αMT. All the hormone treatment regimes yielded significantly higher (P-value < 0.05) percentage of males compared to the control. Interestingly, the treatment regime of 10 mg/kg for 30 days yielded almost 100% male population that was significantly higher (P-value < 0.05) than other lower dose or duration treatment categories but with no significant difference (P-value > 0.05) from other higher dose or duration treatment categories (Chakraborty et al 2007). Thus, the treatment regime of 10 mg/kg for 30 days was taken as the optimum treatment regime for the present study.

After one month, the control and androgen treated tilapia were transferred to two separate 1 m^3 standing surface cages made of bamboo frames and nylon wires with a mesh size of 1.3 cm at a stocking density of 50 fry / m^3. The cages were equipped with covers to prevent fish losses from jumping or bird predation, placed in a large natural pond selecting an ideal location to allow sufficient water current and easy access for management practices and were separated from each other as well as the bottom substrate by enough distance to optimize water quality. During this period, both the fish groups were given control food (crude protein content 30% and total digestible energy 3000 ± 400 kcal/Kg food) made from a mixture of fine fish meal and rice bran, sieved to a size of less than 1 mm, at a rate of 10% body weight per day for the first two months and 5% per day for the rest three months. The total daily feed ration was divided into four equal portions. The experiment was conducted in three replicating units for statistical validation. Throughout the entire culture period different water quality parameters like temperature, DO_2, free CO_2, pH, total alkalinity and turbidity were regularly monitored using the standard procedures of American Public Health Association (APHA 1998) and maintained within ideal value limits for tilapia growth (data not shown).

Fish from each cage were measured individually for weight, length and depth every 4 weeks and at the end of the trial. Besides, growth parameters like specific growth rate (SGR), daily weight gain (DWG), food conversion ratio (FCR), protein efficiency ratio (PER) and apparent net protein utilization (ANPU) were measured according to standard formulation (Pechsiri and Yakupitiyage 2005) at the end of the culture period. Equal amount of wet muscle tissue from 10 fish from each experimental set were taken to determine proximate body composition using standard methods (AOAC 1984). Moisture content was measured by drying a sample at 105^0C in an oven for 24 hours and ash content was estimated by burning the sample at 550^0C overnight in a muffle furnace. Crude protein and crude lipid were determined using the Kjeltec system 1026 distilling unit and Soxtec system HT 1043 (Tecator, Hognas, Sweden) respectively. To determine these body composites by wet mass, the proportion of dry tissue composed of protein, fat and ash was multiplied by percent solids from the original sample. The percent solid was calculated as the ratio of dry mass to wet mass. The results were reported as the mean ± standard error (SE). When appropriate, Duncan's multiple

test (at 5%) (Duncan 1955) was applied to evaluate the differences among means. The statistically homogenous means were denoted by similar alphabets.

3. Results

The androgen treatment regime applied in the study could yield almost 100% male tilapia population (Chakraborty et al 2007). The survival percentage of the fish for both the mixed-sex and monosex culture was around 90%. The growth patterns (Figure 1) in the mixed-sex and monosex groups depicted that after the first month of culture the MT treated monosex group grew more (~2 fold) than the control mixed-sex. The final growth at the end of 6-month culture duration of the monosex population was ~3 fold higher than that of the mixed-sex (P-value < 0.01) (Figure 1A). Among the mixed-sex population, the control males grew significantly more than the females (P-value < 0.05) (Table 1). Moreover, the control females showed a significantly lower value of length compared to the males (P-value < 0.05) but, for depth, there was no significant difference between the sexes in the growth pattern (Table 1). The monosex tilapia grew significantly more than the mixed-sex fish in these two growth aspects also (Figures 1B, 1C). DWG of the mixed-sex tilapia population was significantly less than that of the monosex group (Table 2). SGR of the monosex fish was significantly high than its mixed-sex counterpart (Table 2). But, FCR for the mixed-sex group was significantly more compared to the monosex group (Table 2). The mixed-sex control group had significantly less PER value compared to the hormone treated monosex category (Table 2). Control males had the highest water content and the lowest protein content of all the groups while the treated males possessed the highest amount of protein and the lowest water content (Table 3). Control females had the highest fat content while control males had the lowest (Table 3). In addition, ash content was found highest in hormone treated group and lowest in control male category (Table 3). ANPU decreased significantly for the monosex category compared to the mixed-sex group (Table 2).

4. Discussion

Increase in freshwater fish production can result from the development and adoption of new technologies and improvement in the economic efficiency of farming operations. Tilapia has good potential for the enhancement of production in the fishery sector of India but considerable research is required to adopt different techniques of tilapia culture that are practiced in other countries.

The high survival percentage of fish for both the mixed-sex and monosex categories may be attributed to the protected environment of cages (Dan and Little 2000) and indicates that hormone treatment has no adverse effect on general fish health. The mesh size and arrangement of cages provide adequate open space for good water circulation through the cage to renew the oxygen supply and remove waste. The growth pattern of mixed-sex and monosex Nile tilapia population in the present study compared well with other studies. The higher growth rates of control males compared to control females (Table 1) might be due to increased energy being channeled towards metabolic maintenance and somatic growth in males while the females use considerable energy for spawning (Tran-Duy et al 2008). The growth curves of mixed-sex and monosex fish in cages were more or less linear (Figure 1A) indicating that the critical standing crop in cages was not exceeded (Diana et al 1994) possibly due to the addition of supplemented food. A few studies have demonstrated the enhanced yield of monosex male Nile tilapia populations under experimental conditions (Mair et al 1995). In *Oreochromis mossambicus* (Peters, 1852) also, 17αMT treated fish is reported to show higher growth compared to the untreated fish reared under similar conditions (Macintosh et al 1985). Dan and Little (2000) observed that on average, monosex tilapia grew more than 10% faster than mixed-sex fish in cages. Several studies are in agreement that testosterone produces muscle hypertrophy by increasing muscle protein synthesis (Bhasin et al 2001). The increased growth performance and greater protein content of the androgen treated fish can surely be analyzed considering this knowledge. In many fish including tilapia, it has been reported that the protein requirement of the fish decreased with increasing size (El-Saidy and Gaber 2005). Our study has also indicated the same as observed from the decreased ANPU value with increased size in monosex population than that in the smaller mixed-sex fish. There was a general decrease in FCR and increase in PER for monosex fish than the mixed-sex ones. Such observation may be related to the fact that FCR decreases while PER increases with increased feeding rate (Pechsiri and Yakupitiyage 2005). Moreover, hormone treatment can increase the SGR and DWG of the fish. Similar values of individual growth for monosex tilapia, as in our study, were also observed by Diana et al (1991, 1994). It has been observed that for a given food composition, the body protein percentage on a wet weight basis is mainly affected by the body weight in salmonids (Shearer 1994). Similar observations have been noticed in Nile tilapia also where body protein content increases with wet weight (Pouomogne and Mbongblang 1993; Abdelghany and Mohammed 2002). This explains the higher protein content of the treated males than corresponding controls. Changes in body composition, particularly in body fat content, might influence feed intake in fish (Tran-Duy et al 2008). In the present study, the highest body fat content but lowest weight of control females might indicate a negative correlation between the two factors. However, the hypothesis was not explicitly expressed considering the comparative weight and body fat content of control and hormone treated males.

In view of the relatively wide regional distribution of tilapia in India and its acceptability by the consumers, high priority is placed on the modification and improvements of the techniques for its culture. Culture of tilapia as a cash crop has two basic options: mixed-sex culture and the culture of monosex male populations. Regular culture without sex separation has often failed in the past because of the "wild spawning" of the tilapia that produces a large number of fry which stunt the entire population. Culture of monosex male tilapia resolves this problem. The management flexibility of intensive close culture systems can be achieved in open water through cage culture method. In spite of a relatively short history, cage culture of tilapia has spread rapidly throughout the world (Coche 1982). It requires a low capital investment compared to other intensive culture methods but allows for the benefits of such close culture systems. Another important benefit of cage culture is the ease and low cost of harvesting. Besides, fish in cages can obtain a small amount of natural food resources along with the necessary supplementary diets. Thus, the major conclusions that can be articulated from the present study are: 1. The hormone treated males show higher growth rates than their control counterparts, 2. Additional advantages of larger fish and higher yields are gained through culture of such sex reversed fish, 3. Intensive culture of tilapia in the confined environment of cages can reduce the adverse ecological impact, if any, of tilapia culture in open water impoundments and 4. Cage culture of androgen treated monosex tilapia population can be postulated as the ideal method of choice for an eco-socio-economically sustainable tilapia culture in India.

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Table 1. Comparative growth performances of control male and female tilapia under cage culture system.

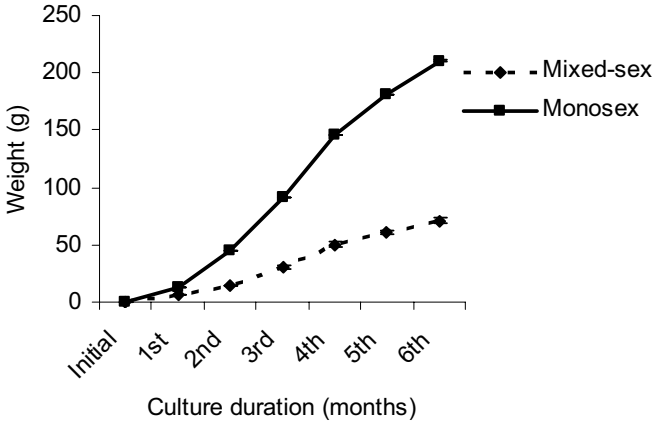
Category	Growth parameters		
	Weight (g)	Length (cm)	Depth (cm)
Control male	77.1 ± 0.07	18.76 ± 0.07	6.4 ± 0.05
Control female	65.4 ± 0.08	13.46 ± 0.05	6.2 ± 0.02

Table 2. Comparative account of different growth parameters for mixed-sex and monosex tilapia under cage culture system. Similar alphabets denote homogenous means.

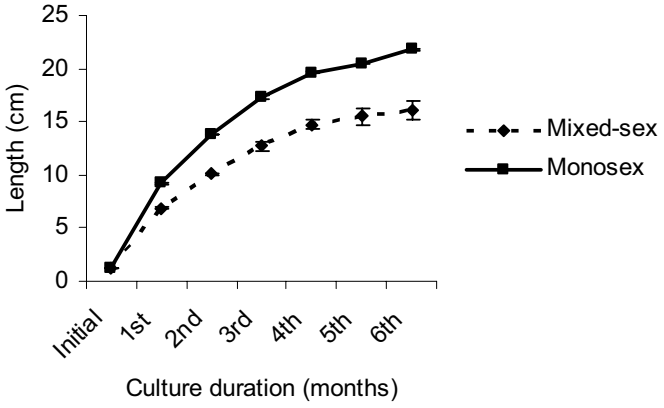
Category	Growth parameters				
	DWG (g/day)	SGR (%)	FCR	PER	ANPU (%)
Mixed-sex	0.39^{b} ± 0.003	4.38^{b} ± 0.05	3.80^{a} ± 0.003	0.85^{b} ± 0.01	12.0^{a} ± 0.4
Monosex	1.17^{a} ± 0.006	4.98^{a} ± 0.05	3.78^{b} ± 0.0006	0.90^{a} ± 0.01	7.0^{b} ± 0.3

Table 3. Body composition analysis of control male, control female and androgen treated male under cage culture system. Similar alphabets denote homogenous means.

Treatment category	Body composition (% wet weight basis)			
	Water	Protein	Fat	Ash
Cotrol male	85.15^{a} ± 0.06	11.23^{c} ± 0.09	1.4^{c} ± 0.07	2.08^{c} ± 0.05
Control female	81.48^{b} ± 0.2	12.25^{b} ± 0.1	2.93^{a} ± 0.09	2.68^{b} ± 0.09
Treated male	78.78^{c} ± 0.1	15.45^{a} ± 0.1	1.65^{b} ± 0.06	3.73^{a} ± 0.09



B.



C.

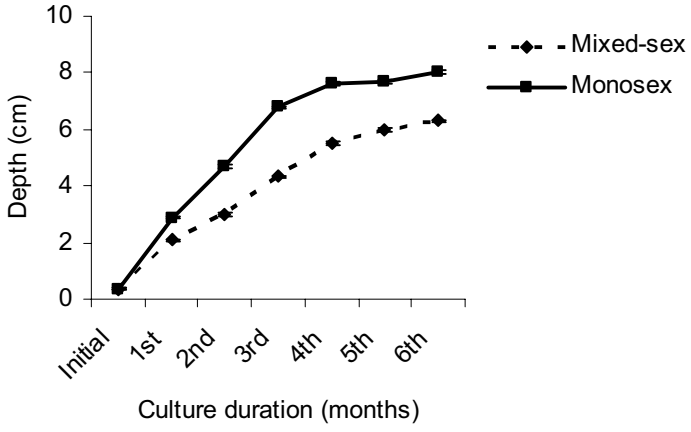


Figure 1. Growth patterns (A: Weight; B: Length; C: Depth) of mixed-sex and monosex fish under cage culture system

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Breeding Ground Profile of Food Fish Species in Damodar River System

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Abstract

Today unwise anthropogenic activity has disturbed the natural ecosystem globally. Civilization on its way to goal has destroyed nature as well as natural diversity. Aquatic ecosystem is also facing the same adverse effect as they are used as waste releasing source. However, anthropogenic activity has drastically damaged the natural habitat of all the living being. Though river water is used for agriculture, fisheries, residential and industrial developments, mining activity, navigation, power generation and variety of other activities including sand digging and disposal of industrial and domestic wastes, but still, some natural breeding do exists in the nature. Identification of those natural breeding ground and to bring them under proper conservation is a most effective way of natural breed conservation. The river Damodar is the most important watershed in the eastern part of India and is one of the main tributaries of the Ganga. The river housed a healthy fish faunal diversity in the recent past but today in spite of drastic deterioration of natural ecosystem, astonishingly no significant difference occurred in faunal composition of the river system.

Keywords: Breeding ground, Conservation, Hydrology

1. Introduction

The river Damodar is the most important watershed in the eastern part of India and is one of the main tributaries of the Ganga. This river is used for agriculture, fisheries, residential and industrial developments, mining activity, navigation, power generation and variety of other activities including sand digging and disposal of industrial and domestic wastes. It had a notable fishery status and fulfilled the demand of the fish eating people of West Bengal in the recent past (Banerjee, 1996).

The river Damodar originates from the hills of Chotonagpur in Jharkhand and drains into a fan shaped catchment area of about 25,820 sq km. The entire Damodar valley can be divided into the upper, middle and lower valleys according to the gradient (Hora, 1947; Job and Motwani, 1952). The undulating upper and the middle valleys are wider than the flat lower valley. The river has a total length of 540 km, out of which 380 km is in Jharkhand and the next 160 km is in West Bengal. The river slope is 1.86 m per km for 241 km, 57 m per km in the next 167 km and 16 m per km in the last reach. In final 145 km the Damodar takes a southward course before joining the river Hooghly. The main tributaries of the river are the Barakar and the Konar. The Damodar was east flowing up to the year 1757 when it was out-falling into the extended bay, well north of present Kolkata. Around the year 1762, it took a sharp turn southwards somewhere in Burdwan and presently enters the Bay of Bengal far south of Kolkata. The upper and middle catchment area, constituting over $4/5^{th}$ of the total catchment area is a hilly terrain with a steep slope while the lower valley is strikingly narrow and flat. Thus, in the event of heavy rain in the upper valley, there is a natural tendency for water to overflow in the lower alluvial plain where most of the farm lands and human habitats are located. To cope up with the flood situation five dams have been constructed over the entire river system. The Tilayia dam was filled during 1953, the Konar during 1955, the Maithon in 1957, the Panchet during 1959 and the Durgapur in 1966. These dams as in case of other dams of the globe have affected the hydrological system of the river and altered the natural habitats of the river system. These changes, in turn, affected the fish population in the river stretch.

The Damodar is seasonal and flood prone mainly on account of reasons, which are physiographic and meteorological in nature. Frequent floods ravage the lower valley area, which is not only very fertile owing to its alluvial plain suitable for irrigation and agriculture but also used for various industrial activities.

2. Literature review

Survey of international literature on river fish diversity, hydrology and ecology of rivers provides extensive data concerning the Ichthyofauna of river systems both in India and abroad (Matthews, *et al*.,1986, 1987, 1988a, 1994; Matthews, 1986b, 1987a, 1998; Sakar and Yadav, 1996; Poff, N.L., 1996, 1997; Chen *et al.,* 2002; Steffy, 2003; Ahmed, 2004; Ahmed and Alfasana, 2004a; Anning, 2004; Ashfaque and Alfasana, 2004b; Garcia *et al*., 2004; Hauer and Habersack, 2005; Piazza *et al*., 2005).

Fish fauna of the Damodar along the stretch of 160 km from below Rajarappa upto Anderson Weir was reviewed by Job and Motwani (1952) before the construction of dams to understand the extent of the future adverse effects on the fish fauna after the construction of dams. Hora (1947) discussed the effects on fish and fisheries due to the flow regulation and recommended some measures to minimize adverse effects for dams. Hora (1955) also enlightened about the problems regarding the river valley projects in India with special reference to the Damodar basin. Several authors (Job and Motwani, 1952; Hora, 1955; Gopalkrishnan *et al*., 1966; Pantulu *et al*., 1966; Natarajan, 1968, 1975; Jhingran and Natarajan, 1969; Ramkrishnaiah and Banerjee, 1979; Mukherjee *et al*., 1986; Sarkar, 1999; Sarkar and Banerjee, 2000, 2002) studied the composition, stocking, breeding ground, seasonal effects and flood effects on fish species of Tilayia, Maithon, Konar and Panchet reservoirs and the Damodar main stream fish fauna. Pollution of the Damodar river has also been studied by several workers like Mukherjee *et al*. (1986), Karmakar *et al.* (1994), as well as by the Central Pollution Control Board (1975, 1987, 2000, 2001), Singh *et al*. (2003).

3. Materials and methods

The present study was conducted for consecutive four years (1995, 1996, 1997, 1998) during premonsoon (March to June), monsoon (July to October), and post monsoon (November to February) periods through out the entire Damodar river system. A total of 13 stations were selected for this study. Among them eight stations are related with breeding ground.

4. Collection of Ichthyofauna

4.1 Fishing equipments

Fishing gear includes gill nets, cast nets, drift nets, hand nets, hooks of all kinds, poles and lines, traps, fish fences, cutlasses and bare hands. Typical gill nets have a mesh size of 7.5 mm. When set parallel to the shore, these nets have no harmful effect on the fishery. If set across the river, they can block upstream breeding movement and migration and capture large numbers of juvenile fishes. Cast nets have a small mesh size of 1.5 – 2 mm that can damage fish stocks through harvesting of premature juvenile fishes. Drift nets usually target breeding adults as they migrate upstream to spawn. Traps probably cause the highest mortality of these species. Fish fences catch all the species of fishes during the juvenile's migration from the streams to the main river during November and January.

4.2 Collections of fish samples

Site selection was made according to the gradient of the river bed in the upper valley, middle valley, lower valley and tributaries. The collection sites were selected according to the presence and absence of industries and also considering the presence of fish ladder. Each site was sampled throughout the day. Fishing was performed between 6 AM and 3 PM engaging fishermen. While sampling with gill net, it was fixed for 72 hour at the same places like upstream and downstream of Panchet, Durgapur, Ramgarh, Tori, shifted in the morning and fish removed within 24 hours (Cowx, 1983; Spindler, 1995; Allen *et al*., 1999; Paukert, 2004). This method was applied mainly in those areas where water depth is about 7.6 cm and above and also in reservoir during postmonsoon and monsoon periods. But in premonsoon when water depth is below 7.6 cm use of cast net, Chaba and other hand netting devices were employed.

This is to mention here that fish juveniles and oozing females caught during the collections were counted, identified in the field and released in the habitat. To avoid biasness, seining was done sufficiently in all the collection sites so that such collection could be compared with validity across the years. Repeatability of the collection is exemplified by collecting fishes in consecutive four years during three seasons from 13 stations.

4.3 Preservation

All the fishes captured, were counted, measured (length and weight) and grouped under two age class categories, namely, juvenile and adult following Matthews (1998). Thereafter, these were immediately segregated according to the family.

The sampled specimens were preserved in 4% Formaldehyde in the field and brought to the laboratory of the Zoological Survey of India, Spirit Building, Kolkata for confirming species identification. The fish species was identified following standard taxonomic procedure.

5. Hydrological parameters of water samples

Water samples were collected from 8 study sites from where fish samples were procured. These water samples were collected during three seasons in four years (1995, 1996, 1997 and 1998) for estimation of routine hydrological parameters following standards procedures (APHA, 1995).

6. Results of Hydrological study

Hydrological parameters of the river water of the Damodar, namely, pH, dissolved oxygen, free carbon-di-oxide; methyl orange alkalinity, water and air temperature and transparency were examined from thirteen stations during 1995-1998 in premonsoon, monsoon and post monsoon.

6.1 Tori

pH in river water ranged from 6.22 to 7.5 in premonsoon 5.54 to 8.72 in monsoon and 5.3 to 9.22 in post monsoon seasons.

Dissolve oxygen in river water varied from 1.6 mg/l to 2.83 mg/l in premonsoon seasons. Its value varied from 1.5 mg/l to 4.4 mg/l in monsoon seasons and during post monsoon seasons it varied from 4.45 mg/l to 4.85 mg/l.

Free Carbon-di-oxide in river water oscillated from 31.56 mg/l to 61.68 mg/l in premonsoon seasons, its value ranged from 10.9 mg/l to 31 mg/l in monsoon seasons and during post monsoon seasons it varied from 18.21 mg/l to 43.46 mg/l.

Alkalinity in river water ranged from 235.5 mg/l to 354 mg/l in premonsoon seasons; its value varied from 90 mg/l to 112.85 mg/l in monsoon seasons and during post monsoon seasons it varied from 90 mg/l to 182 mg/l.

Transparency in river water varied from 24.12 cm to 33.2 cm in premonsoon; its value ranged from 12.38 cm to 13.16 cm in monsoon and during post monsoon it varied from 16 cm to 55.65 cm.

Temperature in air is characteristically higher than that of the river water. In river water it ranged from 25.18°C to 40.2°C in premonsoon seasons, 16.14 °C to 22.24°C in monsoon seasons, 9.88°C to 26.8 °C in post monsoon seasons, whereas in air temperature ranged from 29°C to 42°C in premonsoon seasons,19 °C to 26 °C in monsoon seasons, 12 °C to 29 °C in post monsoon seasons.

6.2 McCluskiegunj

pH in river water ranged from 5.22 to 7 in premonsoon seasons, from 7.66 to 8.72 in monsoon seasons and 5.2 to 6.8 in post monsoon seasons.

Dissolve oxygen in river water ranged from 1.85 mg/l to 2.83 mg/l in premonsoon seasons; its value varied from 1.5 mg/l to 2.86 mg/l in monsoon seasons and during post monsoon seasons it varied from 3.65 mg/l to 6.1 mg/l.

Free Carbon dioxide in river water oscillated from 9.65 mg/l to 21.5 mg/l in premonsoon seasons, its value varied from 5.21 mg/l to 30.2 mg/l in monsoon seasons and during post monsoon seasons it varied from 5.75 mg/l to 37.5 mg/l.

Alkalinity in river water varied from 234.5 mg/l to 255 mg/l in premonsoon seasons; its value varied from 74 mg/l to 135mg/l in monsoon seasons and during post monsoon seasons it varied from 125 mg/l to 182 mg/l.

Transparency in river water varied from 28.97 cm to 43.4 cm in premonsoon, its value varied from 10.8 cm to 21.8 cm in monsoon and during post monsoon it varied from 32 cm to 47.7cm.

Temperature in air is higher than river water. In water it ranged from 33.18°C to 40.2 °C in premonsoon seasons, 18 °C to 22.6°C in monsoon seasons, 12°C to 25 °C in post monsoon seasons, whereas in air it ranged from 40°C to 42°C premonsoon seasons, 21°C to 26.2°C in monsoon seasons, 15°C to 28.5 °C in post monsoon seasons.

6.3 Ramgarh

pH in river water varied from 6.3 to 6.8 in premonsoon seasons, its value ranged from 5.7 to 98.8 in monsoon seasons and during post monsoon seasons it varied from 6.3 to 8.8.

Dissolve oxygen in river water displayed a range from 5 mg/l to 5.8 mg/l in premonsoon seasons, from 4.9 mg/l to 5 mg/l in monsoon seasons and from 6 mg/l to 7.8 mg/l in post monsoon season.

Free Carbon dioxide in river water oscillated from 6 mg/l to 19.6 mg/l in premonsoon seasons; its value ranged from 7.9 mg/l to 17.2 mg/l in monsoon seasons and during post monsoon seasons it varied from 17 mg/l to 27 mg/l.

Alkalinity in river water ranged from 110 mg/l to 454 mg/l in premonsoon seasons, its value ranged from 7.15 mg/l to 159 mg/l in monsoon seasons and during post monsoon seasons it varied from 143 mg/l to 310 mg/l.

Transparency in river water during the years 1995 to 1998 varied from 5 cm to 36 cm in premonsoon, its value ranged from 10 cm to 26 cm in monsoon and during post monsoon it varied from 42 cm to 86 cm

Temperature in air is higher than river water. In water it ranged from 33°C to 37°C in premonsoon seasons, 22 °C to 30 °C in monsoon seasons, 13°C to 25 °C in post monsoon seasons. Whereas in air it ranged from 33 °C to 37°C in premonsoon seasons, 23 °C to 29 °C in monsoon seasons and 14 °C to 26°C in post monsoon seasons.

6.4 Rajarappa

pH in river water varied from 6.5 to 6.98 in premonsoon seasons, its value ranged from 6.55 to 9.36 in monsoon seasons and during post monsoon seasons it varied from 7.35 to 8.46.

Dissolve oxygen in river water displayed a range from 2.12 mg/l to 4.7 mg/l in premonsoon seasons, from 2.3 mg/l to 3.46 mg/l in monsoon seasons, from 2.05 mg/l to 6.13 mg/l, in post monsoon seasons.

Free Carbon dioxide in river water oscillated from 12.1 mg/l to 39.14 mg/l in premonsoon seasons, its value ranged from 9.02 mg/l to 34 mg/l in monsoon seasons and during post monsoon seasons it varied from 2.32 mg/l to 32.38 mg/l.

Alkalinity in river water varied from 19 mg/l to 41.5 mg/l in premonsoon seasons, its value ranged from 7.15 mg/l to 27 mg/l in monsoon seasons and during post monsoon seasons it varied from 8.24 mg/l to 30.75 mg/l.

Transparency in river water varied from 1.12 cm to 5.94cm in premonsoon, its value ranged from 1.42 cm to 5.9 cm in monsoon and during post monsoon it ranged from 1.58 cm to 5.77cm.

Temperature in air is characteristically higher than that in river water. It ranged from 35.14°C to 43.56 °C in premonsoon seasons, 26.08 °C to 32.6 °C in monsoon seasons, and 13.16°C to 32.48 °C in post monsoon seasons in air. Whereas in water it ranged between 33.26 °C to 42°C in premonsoon seasons, 25 °C to 31.06°C in monsoon seasons, 12.6 °C to 29.7°C in post monsoon seasons.

6.5 Chandrapura

pH in river water ranged from 4.3 to 7.35 in premonsoon seasons, from 5.65 to 7.45 in monsoon seasons and 6.02 to 7.15 in post monsoon seasons.

Dissolve oxygen in river water varied from 3.15 mg/l to 3.78 mg/l in premonsoon seasons, its value ranged from 2.45 mg/l to 3.98 mg/l in monsoon seasons and during post monsoon seasons it varied from 3.8 mg/l to 6.55 mg/l.

Free Carbon dioxide in river water oscillated from 12.06 mg/l to 30.1 mg/l in premonsoon seasons; its value varied from 12.1 mg/l to 22 mg/l in monsoon seasons and during post monsoon seasons it varied from 7.2 mg/l to 32.4 mg/l.

Alkalinity in river water varied from 28 mg/l to 49.49 mg/l in premonsoon seasons, its value varied from 7.15 mg/l to 27 mg/l in monsoon seasons and during post monsoon seasons it varied from 8.24 mg/l to 30.75 mg/l.

Transparency in river water varied from 1.62 cm to 9.05 cm in premonsoon, its value ranged from 16.6 cm to 36.1 cm in monsoon and during post monsoon it varied from 13.8 cm to 76.62 cm.

Temperature in air is higher than that in river water except in premonsoon of 1997, 1998. It ranged from 37.28°C to 38.16 °C in premonsoon seasons, 28.4 °C to 32.12 °C in monsoon seasons and 26.6°C to 30.14 °C in post monsoon seasons in air. Whereas in water it ranged between 34.14 °C to 39.84°C in premonsoon seasons, 25.36 °C to 30.14 °C in monsoon seasons and 22.6 °C to 29.7°C in post monsoon seasons.

6.6 Panchet

pH in river water varied from 4.65 to 6.85 in premonsoon seasons, its value ranged from 3.23 to 6.67 in monsoon seasons and during post monsoon seasons it varied from 4.9 to 7.15.

Dissolve oxygen in river water displayed a range from 5.44 mg/l to 6.75 mg/l in premonsoon seasons, from 4.95 mg/l to 5.18 mg/l in monsoon seasons, from 5.65 mg/l to 9.63 mg/l in post monsoon seasons.

Free Carbon-di-oxide in river water oscillated from 21.18 mg/l to 47.64 mg/l in premonsoon seasons; its value varied from 17.5 mg/l to 24.5 mg/l in monsoon seasons and during post monsoon seasons it varied from 6.24 mg/l to 37.6 mg/l.

Alkalinity in river water varied from 230 mg/l to 632.12 mg/l in premonsoon seasons, its value varied from 120.5 mg/l to 205 mg/l in monsoon seasons and during post monsoon seasons it varied from 114 mg/l to 687 mg/l.

Transparency in river water during the years 1995 to 1998 varied from 61.2 cm to 150.8 cm in premonsoon, its value ranged from 47.2 cm to 135.5 cm in monsoon and during post monsoon it varied from 72.15 cm to 115.83 cm.

Temperature in air is higher than that in river water. It varied from 26.1°C to 29 in premonsoon seasons, 22°C to 30°C in monsoon seasons, 27 °C to 29°C post monsoon seasons in air during the years 1995 to 1998. Whereas in water it ranged between 23°C to 24.1°C in premonsoon seasons, 18.3°C to 25°C in monsoon seasons, 23°C to 26°C in post monsoon seasons.

6.7 Durgapur

pH in river water varied from 4.9 to 5.77 in premonsoon seasons, from 3.4 to 9.48 in monsoon seasons, 4.9 to 6.9 in post monsoon seasons.

Dissolve oxygen in river water ranged from 5.73 mg/l to 7.17 mg/l in premonsoon seasons, its value varied from 5.8 mg/l to 6.82 mg/l in monsoon seasons and during post monsoon seasons it varied from 7.46 mg/l to 9.6 mg/l.

Free Carbon-di-oxide in river water oscillated from 31.56 mg/l to 61.68 mg/l in premonsoon seasons; its value varied from 10.9 mg/l to 31 mg/l in monsoon seasons and during post monsoon seasons it varied from 18.21 mg/l to 43.46 mg/l.

Alkalinity in river water varied from 123 mg/l to 533 mg/l in premonsoon seasons, its value ranged from 83 mg/l to 211mg/l in monsoon seasons and during post monsoon seasons it varied from 123 mg/l to 270 mg/l.

Transparency in river water varied from 38.85 cm to 134.1 cm in premonsoon, its value varied from 29.05 cm to 90.1 cm in monsoon and during post monsoon it varied from 33.4 cm to 90.1 cm.

Temperature in air is higher than that in river water. It ranged from 35°C to 41°C in premonsoon seasons, 20 °C to 30.1 °C in monsoon seasons and 20°C to 30 °C in post monsoon seasons in water, whereas in air it ranged from 30.3 °C to 42.3°C in premonsoon seasons 22.3 °C to 33 °C in monsoon seasons and 22.9 °C to 32.3°C in post monsoon seasons.

6.8 Tarakeshwar

pH in river water displayed a range from 4.2 to 7.95 in premonsoon seasons, from 4.02 to 7 in monsoon seasons and 4.95 to 7.78 in post monsoon seasons.

Dissolve oxygen in river water varied from 3 mg/l to 6.08 mg/l in premonsoon seasons, its value ranged from 2.64 mg/l to 6.35 mg/l in monsoon seasons and during post monsoon seasons it varied from 3.05 mg/l to 6.5 mg/l.

Free Carbon-di-oxide in river water oscillated from 18.25 mg/l to 32 mg/l in premonsoon seasons, its value ranged from 11.6 mg/l to 30.05 mg/l in monsoon seasons and during post monsoon seasons it varied from 16.5 mg/l to 42.25 mg/l.

Alkalinity in river water varied from 21.75 mg/l to 47.71 mg/l in premonsoon seasons, its value ranged from 11.6 mg/l to 42.34 mg/l in monsoon seasons and during post monsoon seasons it varied from 12.27 mg/l to 42.25 mg/l.

Transparency in river water varied from 13.74 cm to 18.27 cm in premonsoon, its value ranged from 1.2 cm to 1.62 cm in monsoon and during post monsoon it varied from 13.1 cm to 30 cm.

Temperature in air is higher than that in river water. It ranged from 33 °C to 36.4°C in premonsoon seasons, 17.5 °C to 27.5 °C in monsoon seasons and 18.2°C to 21.14°C in post monsoon seasons in water whereas in air it ranged from 33.2°C to 38.04 °C in premonsoon seasons, 21 °C to 29.18 °C in monsoon seasons, 24.5°C to 32.4 °C in post monsoon seasons.

7. Result of fish fauna

The Damodar River is considerably diverse in fish fauna in number of species. This is quite evident from the fact that Job *et al*. (1952) recorded the occurrence of 89 species of fishes under 48 genera and 20 families. During the present study also 79 species of fishes belonging to 51 genera and 22 families have been recorded. The important breeding grounds of the upper valley, middle valley and lower valley are discussed with respect to the occurrence of the brood fish, juveniles, and fingerlings.

Tori (Stn.1) is the special breeding ground of brood fishes. The fishes are found in juvenile forms in these riffles and pools in almost dry river bed during the pre monsoon. The oozing females were caught by gillnet from this site during monsoon in all the study years. Not only the brood fishes, the juveniles (fingerlings) of *Labeo dero, Labeo boggut, Cirrhinus mrigala, Nemacheilus* sp*., Esomus* sp. and *Lepidocephalicthys guntia* were found confined within refill or pools with water depth of 1.5 m during summer. In post monsoon inside the weeds, spawn and fingerlings of *Esomus danricus*, *Barilius bendilisis* are found. But *Cirrhinus reba, Brachydanio rario* is found in all seasons in all the stages throughout the study period. *Parluciosoma daniconius* is found only in adult form. Large oozing females and matured males of *Crossocheilus latius latius* are found in the postmonsoon seasons but spawns and fingerlings are found in McCluskieganj. Three species of *Nemacheilus* have been recorded at this station. The oozing female of *Nemacheilus* are found in the refills during post monsoon (1996, 1997, 1998) indicates that this rocky loach increases its population in these refills. *Rhinomugil corsula* male and female with matured ovary was observed during premonsoon and spawns are found in early monsoon but fingerlings were not observed. *Macrognathus pancalus, Mastacembelus armatus* male and female are found during postmonsoon.

At McCluskieganj (Stn.2) 17 species of fishes were recorded during the study period. Out of these *Lepidocephalicthys guntia* was found to be most abundant species and of all stages of lifecycle. Here, *Macrognathus pancalus, Cirrhinus reba,* were found during the summer, in shallow water flow in thick population but very rare during monsoon and post monsoon period. At McCluskieganj adult forms of the fingerlings and the spawns of almost all the fishes found in this particular stretch were observed in late postmonsoon and early premonsoon season. During heavy monsoon flow only adult forms are recorded except *Cirrhinus reba*, *Mastacembelus armatus*, *Amblypharyngodon mola*, *Barilius bendilisis* they are found in all seasons in all forms. Fingerlings of *Brachydanio rario, Danio acquipinnatus Parluciosoma*

daniconius are recorded once during monsoon but due to flooded condition, collection was difficult during 1996 and 1998.

At Ramgarh (Stn. 3) the river bed is sandy and rocky. The sewage outlet of Central Coalfield Limited directly falls in the river near over bridge. About 5 km upstream of Ramgarh bridge, there is a fish ladder and above the ladder there is a pool like area with about 6 m depth. The oozing females and spawns and juveniles of almost all those fishes that were collected from this river stretch were found in that pool during early monsoon and postmonsoon seasons throughout the study period. It is a good breeding ground in the upper valley (Sarkar and Banerjee, 2000). This region was found to house 36 fish species and the most abundant was *Garra annandeli*. *Chagunius chagunio* and *Garra* sp. breeds and completes its lifecycle at this site only because starting from oozing female the seeds as well as fingerlings are only found in this region. Ramgarh has high turbidity of water and the *Mystus* species with oozing female and fingerlings are observed. Four species of *Puntius*, namely, *P. chola, P. sophore, P. sarana* and *P. ticto* were observed at Ramgarh along downstream with oozing females. Out of these, the last two species were considerably abundant in this site.

At Rajarappa (Stn.4) only 19 species of fishes were found and most abundant species was *Cirrhinus reba*. Large fishes like *Notopterus notopterus* (Total Length 58 cm) and *Labeo rohita* (TL 68cm) were found in all the three seasons but no fingerlings of these species were observed. This may be due to the reason that the fingerlings are more susceptible to pollution and the site shows very low oxygen content (2.05-6.25 mg/l) and high turbid water. The situation indicates that these fishes do not breed in this site.

At Chandrapura (Stn.5) in Bhandaridaha the river bank lagoons were over flooded during high flood in monsoon and fish migration occurred from river to flood plains. Juveniles of *Amblyphayngodon mola, Aspidoparia morar* and *Crossocheilus latius latius* were observed in the rock lagoons at Bhandaridaha. At Chandrapura the river bed is about 1 km wide. Water flows in summer, along the left bank with a depth of 125-140 cm and 350 cm width, and along the right bank water flows with 280 cm depth and width of river is 465 cm. Stagnant pools exist in the middle and near the edge of the river where large fishes and fingerlings were observed. 15 species of fishes were recorded from Chandrapura during the present study. The most abundant species was *Cirrhinus reba*. *Glossogobius giuris giuris* is found in pre-adult stages, 4 km downstream of Chandrapura Thermal Power Station (the survey site) at Bhandaridaha in the shallow and deep pools temporarily formed on the rocky bank, where river water floods in the monsoon and remains up to early premonsoon. *Cirrhinus mrigala*, *Cirrhinus reba*, *Osteobrama cotio cotio*, *Puntius chola*, *puntius conchonius, Aspidoparia morar* are found to complete their lifecycle in the slow flowing shallow water at Chandrapura. But the *Chela labuca, Salmostoma phulo* and *Salmostoma bacaila* are only found in adult forms. Oozing females and juveniles of *Garra lamta* (sucker fish) were found throughout the year during the entire study period, which indicates that this fish could tolerate high water temperature ranging from 34-39°C during the premonsoon season and can also complete their life cycle.

Panchet (Stn.6) is a large mainstream reservoir, impounded in the year 1959. The river bed below the reservoir is sandy and rocky. A hydral power station is situated below the dam. At this site species diversity of fishes were found to be 53 and most abundant species was *Gudusia chapra*. From this reservoir, spawn of *Labeo rohita, Aorichthys aor, Cirrhinus mrigala, Cirrhinus reba* and *Labeo calbasu*, *Labeo bata* were obtained in early post monsoon season. Oozing females of *Mystus seenghala, Mystus cavasius, Mystus tengara, Ompok bimaculatus* were recorded from all the permanent and temporary fish landing stations near the lockgates of the Panchet reservoir. Juveniles of these fishes were recorded from the head water of the reservoir as well as downstream of the reservoir.Juveniles of *Labeo rohita, Aorichthys aor, Cirrhinus mrigala, Cirrhinus reba* and *Labeo calbasu* were observed in the area where the river enters the reservoir and bordering Purulia district. This area is undisturbed and covered with forest. The juveniles of minnows like *Colisa fasciatus, Colisa lalia, Aspidoparia morar*, *Securicula gora*, were observed in the over flooded marshy lowland near the Panchet hill during the early postmonsoon. The oozing females and spawns and juveniles of almost all those fishes that were collected from this reservoir are found here.*Channa orientalis, Channa punctatus, Channa striatus, Macrognathus pancalus, Mastacembelus armatus* these fishes were found in large population in the water pockets along the eastern bank of the reservoir near Bachara gram after heavy monsoon every year. People collect juveniles of these fishes from these area and introduce in the village pond.

Reservoir of Durgapur barrage (Stn.7) houses a good number of fish species. The maximum species diversity of fishes was observed in this reservoir (56 species). The most abundant species was *Gudusia chapra*. The river bed below the dam a sandy and water remains only in deep and shallow ditches during postmonsoon season sheltering large carps. Small minnows were found in the shallow ditches. Whereas in deep pools large oozing female fishes like *Labeo rohita, Mystus aor, Cirrhinus mrigala, Ompok bimaculatus* and *Barilius bendilisis bendilisis* were trapped. Mostly these fishes could not survive because people are always engaged in catching those fishes with bamboo sticks by hitting them. Here thick populations of minnows and fish seeds are also destroyed by local people. They use mosquito nets to collect those fish seed and sell in local market. Seeds of *Catla catla Labeo rohita, Aorichthys aor, Cirrhinus mrigala, Cirrhinus reba, Mystus seenghala, Mystus cavasius,Mystus tengara, Ompok bimaculatus* and *Labeo bata* are introduced regurly in the

reservoir from the Government rearing pond beside the reservoir. Here the siltation rate is very high and as a result several small and large islands locally called Manachar where many brood fishes and spawns and fingerlings are found at the semi stagnant water of the reservoir, those fishes are *Glossogobius giuris giuris, Labeo rohita, Mystus aor, Ompok bimaculatus*, *Channa orientalis, Channa punctatus, Channa striatus, Macrognathus pancalus, Mastacembelus armatus.* The oozing females and spawns and juveniles of almost all those fishes that were collected from this river stretch.People living in those islands rear *Monopterus cuchia* and *Heteropneustes fossilis* in cage and sell in local market. Small marshy lands inside the reservoir is the ideal breeding place of all the *Puntius* sp. collected from this reservoir. They are found in thick population there.

Tarakeshwar (Stn.8) is an agricultural area. Water depth is very low in this site. Here the river was found to house only 27 fish species. The most abundant species was *Aoricthes aor*. Oozing females spawns and fingerlings of *Aoricthes aor, Xenentodon cancila* are found in this region. At Tarakeshwar fingerlings of *Glossogobius giuris giuris, Notopterus notopterus, Osteobrama cotio cotio, Puntius phutunio, Chela cachius, Gonialosa manmina* were found during the postmonsoon seasons throughout the study period and it is also a breeding ground in the lower valley.

8. Discussion

In the Damodar valley drought is worse during premonsoon period. Anthropogenic modification of stream channels may act synergistically to make drought worse for fish. Surface flow is important and substantial discharge is necessary to maintain the surface flow on which most fishes depend. Because during the dry season, immigration and emigration cannot occur, reproduction is negligible and the numerical abundances of species can only decrease (Matthews, 1998). During summer, the rain fed Damodar runs through severe droughts, which decimate fish populations in stream.

Specially in the headwater area at Tori and Piparwar fishes in high concentration were able to survive for a limited period of time under the most unfavorable condition not due to the anthropogenic activity but natural drought. In the headwater area only a discrete pool remain during summer, because of seasonal drying but not due to sudden drying where brood fish takes shelter.

At Tori and McCluskieganj adult forms of the fingerlings of almost all the fishes found in this particular stretch were observed in late post monsoon and early premonsoon seasons. Moore and Gregory (1988a, 1988b) observed that fingerlings show a specific character in the lotic system they are found in the stream edge where bushes and water weeds are thick.

Though Tori is the special breeding ground of brood fish. But the number of fingerlings is less in Tori than McCluksigaunge.

The larger oozing females of *Labeo dero, Labeo boggut, Cirrhinus reba,* shows upward migration from McCluksigaunge breeding team and are caught by gillnet in this site during monsoon. The fish fry which can exist after the summer drought migrate to the down stream during first monsoon. Large densities of small fishes residing in shallow habitats inside the bushes are observed at McCluksigaunge. A specific character of the fingerlings that they are found in the stream edge where bushes and water weeds are thick. This phenomenon is distinct in the pre and post monsoon at McCluksigaunge and Tori. They also show a tendency to avoid the swiftest water current (Moore and Gregory 1988a, 1988b). At Rajarappa large fishes like *Notopterus notopterus* (Total Length 58 cm) and *Labeo rohita* (TL 68cm) were found in all the three seasons but no fingerlings of these species were observed. This may be due to the reason that the fingerlings are more susceptible to pollution and the site shows very low oxygen content (2.05-6.25 mg/l) and high turbid water. The situation indicates that these fishes do not breed in this site.

This phenomenon is also observed in pre monsoon season at Chandapura. The river bed about 2km wide and water flow along the left bank with 3.5-4 ft 35-40 cm depth and width on the right bank stagnant pools and shallow flow (3 – 4 cm) exist where numerous fish fingerlings are observed.

The present observation reveals that fingerlings of *Rasbora* sp.*, Amblypharyngodon* sp.*, Macrognathus* sp. and *Nemacheilus* sp., show a tendency to avoid the swiftest water current and take shelter in the bushes and water weeds along the river bed. The present study shows that the adjacent sites like Tori and McCluskieganj have more or less same species composition. However, number of fingerlings is less in Tori than in McCluskieganj.

At Ramgarh and Rajarappa large brood fishes were found. At Rajarappa only large fishes were found but not a single fingerlings was observed. But in Ramgarh the deep pools is the upstream of fish ladder houses fish fry as well as brood fishes. The large fish tries to complete their life cycle in the deep pools (Scholler 1995) said the same.

The good breeding rate of *Puntius* species at Ramgarh indicates that they need somewhat lentic ecosystem to breed and prefer muddy, Rocky River bed which is congenial to them at this site.

Oozing females of *Puntius conchonius* is found in Chandrapura, Tarakeshwar and Panchet in the mainstream. Oozing females of *Puntius sophore* is found in Durgapur, Panchet and Rajarappa. Fingerlings of *Puntius sarana* is found in the upper valley in Ramgarh and Rajarappa.

In lotic system extensive netting may also produce some gap in the observed and actual data but the deviation is least in lentic systems. In the long term sampling where species disappears, there is often one or more anthropogenic effects that can be linked to the declines (Burr, 1991).

The seasonal variation of fishes indicates a rapid increase in availability in the monsoon and post monsoon seasons. This is because of the over flooded condition of river bed and migration of the species from the river to flood plain inside the crop fields and shallow low lands on the bank. This increases the aggregation of fish spawns by number and diversity in partial stagnant water.

However, during drought period most of the fishes take shelter in the small pools which ultimately become disconnected from the main stream. Each major disconnected pool seems to retain more or less its population of fish plus the riffle species. These fishes are crowded for weeks or months, if excessive decomposition does not develop (which lowers pH) or if the pool does not dry up(lowers transparency). The fries and spawns become confined in the refills. The pools water becomes acidic at Tori during premonsoon 1998(value-6.16), Dissolve Oxygen (1.6 mg/l), Free Carbondioxide (9.58 mg/l) and high alkalinity (245.7 mg/l). It needs mention here that several workers like Starrett (1951), Canton *et al*. (1984), Moore and Georgy, (1988a,1988b) and Angelo *et al*.(1995), reviewed the effects of late summer reduction in stream flow on fishes emphasizing their crowding and fingerlings crowd in shallow stagnant pools. The present observation in the Damodar River, as stated above, corroborate their findings. The upper level shows 4 mg/l or 5-6 mg/l dissolved oxygen (DO) in average while the lower water showing 0-1 mg/l (DO) (results of hydrology). Difference of temperature to the extent of 5 °C from upper to lower levels of water in these pools is also observed.

Mastacembelus sp. was sharply reduced in number during drought condition. During the premonsoon Tori and McClukskieganj suffer from scorching heat and the Damodar water flow almost dries up. Small scattered pools remain in the river bed where water remains very low and submerged vegetation could only be seen. In those pools spawn of *Cirrhinus reba, Perluciosoma daniconius* and *Barilius* sp. take shelter under damp slime of the drying pools in almost unswimable condition for weeks. Therefore huge number of those fishes with oozing females, fingerlings, crowd into refuge pools where hydrology of water is not within the tolerance limit (results of hydrology) and fishes has to wait until next rain. But, as the river bed dries up gradually within mid summer and water remains only in shallow water bodies, the spawns may die. The same scenario was observed in McCluskieganj. The composition of premonsoon fishes includes only young ones and fry.

Though anthropogenic changes has disturbed the natura ecosystem of this river a lot. However, astonishingly no significant difference occurred in faunal composition in these two regions during low water flow in premonsoon. In the headwater area only discrete pools remain during summer, because of seasonal drying but not due to sudden drying. The middle and the lower valley also shows no drastic effect on occurrence of fishes. The Natural breeding grounds do exists in nature.

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Table 1. Description of study sites related with breeding ground.

Valley	Selected Sites (Stn.1-8)	Brief description
Upper valley (Tori - Parjori)	Tori(1) McCluskieganj (2) ,Ramgarh(3) Rajarappa (4).	The upper valley length 241.5 km.it is rocky and with deep gorge at Rajarappa, Bhandaridaha and adjacent areas.
Middlevalley (Parjori-Ranigunj)	Chandrapur (5) Panchet (6) Durgapur (7)	A stretch of about 105 km is regarded as the middle valley. The river bed is wide being about 1.61 km near Panchet dam, almost covered by fine sand.
Lower Valley Ranigunj to the confluence of Hooghly	Tarakeshwar (8)	This part of the valley is also called the trans-Damodar region. Here the river has a number of distributaries of take off channels on the left bank.

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Characterization of Gene Encoding Type-I Gonadotropin Releasing Hormone Receptor in Goat (*Capra hircus*)

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Abstract

In the present study, complete ORF of GnRH receptor transcript from goat pituitary was amplified, cloned in prokaryotic system and sequenced. The goat type-I GnRH receptor sequence shows high homology with sheep, cow, buffalo, equine and human. Like in other mammalian species in goat too, the GnRH receptor lacks a C terminal tail. Amino acids which are critical for structure and function of the receptor were found conserved. Few amino acid residues conserved amongst ruminants were found to be replaced in other mammalian species suggesting divergent evolutionary path of this gene in these groups.

Keywords: Gonadotropin releasing hormone receptor, *Capra hircus*, Pituitary, Accession number EF150356 and EU596505

1. Introduction

Gonadotropin Releasing Hormone (GnRH) is a key neuroendocrine regulator of the hypothalamic-pituitary-gonadal axis and plays an important role in mammalian reproduction by regulating synthesis and release of gonadotropins. GnRH is synthesized in hypothalamus and binds to a specific receptor called gonadotropin releasing hormone receptor (GnRH-R) located on plasma membrane of gonadotroph cells of anterior pituitary. In non mammalian vertebrates several subtypes of GnRH and it's receptor are known. In mammals two subtypes of this hormone called GnRH-I and GnRH-II are found, but the only functional subtype of GnRH receptor found in mammals is type-I GnRH-R as in no species a type-II GnRH-R gene lacking a frame shift mutation is reported so far (Millar et al., 2004). Type-I GnRH-R is a member of seven transmembrane domain G protein coupled receptor super-family and is hypothesized to couple to

multiple G proteins (Conn and Croeley 1994). Unlike other members of this super-family, type-I GnRH-receptor lacks the carboxy terminal tail (Stojilkovic et al., 1994; Sealfon et al., 1997) which participates in several downstream reactions through several receptor-associated proteins (Bockaert et al 2003; Ferguson et al., 2001). GnRH receptor transcripts have also been detected in some extrapituitary tissues, including ovary (Choi et al., 2001; Kang et al., 2000 and 2001) and placenta (Siler-Khodr et al., 2001; Khodr et al., 1980; Chou et al., 2004) and there are increasing evidences that GnRH may act as an autocrine or paracrine factor in regulating local cellular functions in these organs. In the ovary GnRH modulates both basal and gonadotropin-stimulated steroidogenesis (Olofsson et al., 1995) and induces transcription of several genes involved in the follicular maturation process and ovulation. High-affinity binding sites for GnRH-I have been reported in human corpus luteum, luteinized granulose cells, and a number of ovarian cancer cell lines (Bramley et al., 1987; Emons et al., 1989 and 1993; Brus et al., 1997). Though goat is an economically important animal for Asian countries, particularly India, no information is available on GnRH receptor of goat. In the present study, transcript of type-I GnRH receptor has been characterized in goat pituitary. The goat type-I GnRH receptor sequence shows high homology with sheep, cow, buffalo, equine and human. Like in other mammalian species in goat too, the GnRH receptor lacks a C terminal tail. Amino acids which are critical for structure and function of receptor were found conserved. Few amino acid residues conserved amongst ruminants were found to be replaced in other mammalian species suggesting divergent evolutionary path of this gene in these groups.

2. Materials and methods

2.1 Total RNA isolation

For total RNA isolation, pituitaries were collected from local abattoir, immediately after slaughter in RNAlater solution (Qiagen, Germany). Total RNA isolation was done using RNeasy Mini kit (Qiagen, Germany) according to manufacturer's protocol. Total RNA was quantified and its integrity was assessed using 1% formaldehyde agarose gel electrophoresis.

2.2 cDNA synthesis

For cDNA synthesis 5µg of total RNA was reverse transcribed into first strand cDNA, using oligodeoxythymidine primers (MBI-Fermantas). Each reaction included 1 X RT buffer 50 mM Tris-HCl (pH 8.3), 50mM KCl, 4mM $MgCl_2$, 5mM DTT (MBI-Fermentas), 2.5 mM of each of the four deoxy-NTPs, 0.5µl oligo-dT primers, 20 U Ribonuclease inhibitor, and 200 U Moloney murine leukemia virus reverse transcriptase (MBI-Fermentas) and was carried out at a volume of 25 µl for 1 h at 37°C. Reaction was stopped by heating at 70°C. A parallel control reaction was carried out without adding reverse transcriptase. After the end of the reaction, the volume was increased to 100µl with ultrapure water, and the mixture was stored at -20°C.

2.3 PCR amplification

The primers were designed to amplify two PCR products a 408 bp product and a 1026 bp product on the basis of the available bovine and ovine GnRH receptor sequences. Primers used to amplify 1026 bp product were, forward 5'ATAAAGGATGGCAAACAGTGAC3', reverse 5'GCCTTTCTTTGACTTTCTATGC3' and primers for 408 bp product were forward 5'CCT CCC CAC CCT GAC CCT ATC T-3' and reverse 5'GCCAAGCCAATCATGAACTGTCC3'. Standard protocol according to Sambrook and Russel (2001) was used for PCR amplification. Reaction was carried out in 25 µl reaction volume containing 70 ng of cDNA, 10 pM of each primer, 400µM of dNTP mix, 2.5 unit of Taq DNA polymerase and 1.5mm of $MgCl_2$ using T personal thermocycler. The PCR programme used to amplify 408 bp product involved initial denaturation at 95°C for 3 min followed by 32 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 1 min ending with a final extension at 72°C for 10 min. Same reaction volumes, conditions of temperature and time were used to amplify 1026 bp product, except that annealing temperature was 48°C. The amplified PCR products were electrophorized in with 100 bp DNA ladder (MBI Fermentas) on ethidium bromide stained 1% agarose gel and were visualized and photographed under UV.

2.4 Cloning and sequencing of PCR product

Both 1026 and 408 bp PCR products were cloned in prokaryotic system and sequenced. For this PCR products were purified using gel extraction kit (Qiagen) following manufacturers protocol. The purified PCR product was ligated to pTZ57R/T (MBI Fermentas) cloning vector using 50 ng purified PCR products 50ng of vector, and 5U of T_4 DNA Ligase. The reaction mixture was incubated at 22°C overnight in water bath. The transformation of DH5α E.Coli cells with ligated product was done using Insta cloning kit (MBI Fermentas) following manufactures procedure. The transformed cells were spread over ampicillin agar plate coated with 40 µl of 0.1 m IPTG and 40 µl of 20mg/ml X Gal. The white colonies were propagated in 3 ml LB broth containing ampicillin by overnight incubation in a shaker incubator at 37°C. Presence of insert was confirmed by plasmid isolation using alkaline lyses method followed by restriction digestion with *EcoR*I and *Sal*I enzymes. For sequencing cloned PCR products, DNA sequencing facility at University of Delhi South Campus (UDSC), New Delhi was availed.

3. Results

3.1 RNA isolation and RT-PCR

The RNA yield using RNAeasy mini kit was found 1.5 μg/mg of tissue and 260/280 of the RNA sample obtained was 1.98 indicating the high purity of the RNA sample. (Fig. 1) Primers used for the amplification were designed on the basis of published sequences of bovine and sheep GnRH-R gene. PCR with these primers gave products of expected size i.e. 1026 bp (Fig. 2) and 408 bp products (Fig. 3) from pituitary.

3.2 Cloning and Sequencing of PCR products

About 110 colonies were observed on the LB plates. Colonies were screened by blue and white selection method. Most colonies were white and few blue. The white colonies were propagated for the plasmid isolation and further confirmation. Alkaline lysis method was found to be an easy and fast method for plasmid isolation. Isolated plasmid was free from genomic DNA and RNA contamination. The yield of plasmid was 484 ng/μl and has shown a 260/280 ratio of 1.8 which was sufficient for carrying out subsequent screening steps. InsT/A cloning vector has restriction sites for *EcoR*I and *Sal*I, which were absent in the insert. Therefore for screening of recombinant clones, plasmids isolated from different white colonies were subjected to double digestion with restriction enzymes, *EcoR*I and *Sal*I. Insert release of about 1026 bp and 408 bp were confirmed by agarose gel electrophoresis. Clones confirmed for the presence of desired fragments were sequenced at UDSE New Delhi. The obtained sequences were analyzed using DNASTAR software. The sequence homology was confirmed using Basic Local Alignement Search Tool (BLAST) programme of National Centre For Biotechnology Information Gene Bank (www.ncbi.nlm.nih.gov). Sequences were submitted to Gene Bank NCBI and Accession number EF 150356 and EU 516505 were obtained.

4. Discussion

The amplified 1026 bp product spans complete ORF, which is 987 bp long, position 8 to 994 (Fig. 5) The 408 bp product corresponds to position 81 to 488 of the ORF. The 408 bp product resides completely within the Exon-I region and thus can be used as a probe for type-I GnRH-R gene identification in southern dot blot analysis with RT-PCR product as well as with genomic DNA. The ORF of goat type I GnRH was aligned with that of other mammalian species using MegAlign programme of DNASTAR software. The goat type-I GnRH ORF shows more than 85% homology with different animal species. The sequence shows high homology with GnRH receptor sequences of sheep (99%), cow (98%), buffalo (98.4%), equine (91.5%) and human (88.4%). The phylogenetic tree of type-I GnRH-R gene of different mammalian species show different evolutionary pattern as GnRH-R gene of goat and sheep fall in one group which in turn share close evolutionary resemblance with primate and distinct relation with rodents (Fig. 4). The amino acid sequence of goat type-I GnRH-R bears all the characteristic features for transmembrane receptor. High numbers of hydrophobic amino acid residues (141 out of 328) in deduced amino acid sequence were found, which is a characteristic feature of transmembrane protein. The length of the protein suggests that like other mammalian species in goat too, the GnRH-R lacks a C terminal tail. The deduced amino acid sequence was aligned with that of other species using MegAlign programme (Fig. 6). Upon alignment of the amino acid sequence of type-I GnRH receptor of several mammalian species it was found to be more than 90% conserved. Goat type I GnRH-R share close similarity with sheep (98.5%), buffalo and cow (97.9%) and equine (92.7%), lower similarity with human and macaca (89.7% and 89.9% respectively) and lowest similarity with mouse and rat (86.0% and 84.8%). These results are in accordance with the nucleotide sequence analysis. The putative transmembrane domains were found almost identical with in these mammalian species. Amino acid residues important for receptor function for instance, Ala (261) in the third intracellular loop which is crucial for G protein coupling and receptor internalization (Myburgh et al., 1998), Asp (98), Trp (101) Asn (102), Lys (121), Asn (212) and asp (302) which are important for ligand binding (Hoffmann et al., 2000) were found conserved in all species. The GnRH receptor was found conserved amongst mammalian species but species wise difference were found at several positions, for example, Ser (27) is well conserved amongst ruminants (cow, buffalo, sheep and goat), but is replaced by Lys in rodents (mouse and rat) and by Asn in human and macaca monkey, residue Ile (51) which is conserved amongst ruminants is replaced by Ala in rodents and Thr in human and macacca. Several such species wise variations were observed in amino acid sequences of Type-I GnRH-Receptor in different mammalian species. These results suggest the divergent evolutionary path of Type-I GnRH receptor in different animal groups. Type-I GnRH-R appears to have undergone a relatively recent period of rapidly accelerated molecular evolution (Fig 4) that may have been important for the development of mammalian reproductive strategies. The present study provide the necessary information about Type-I GnRH-R in goat and paves the way for further molecular work related to this receptor in goat, like PCR based confirmation of expression of this receptor in different tissues and quantitative PCR (real time PCR) to study different expression levels of this receptor.

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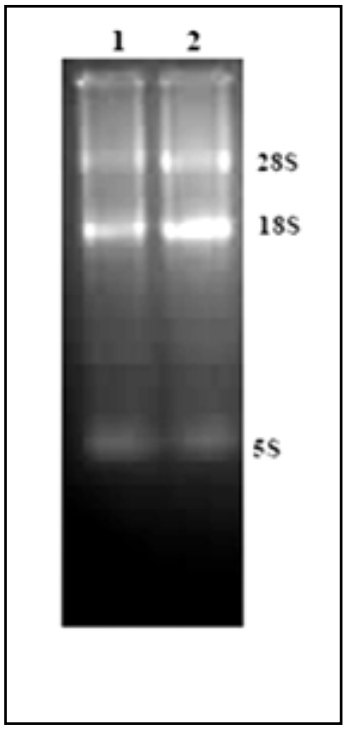


Figure 1. Formaldehyde Agarose Gel electrophoresis of Total cellular RNA from Goat Pituitary

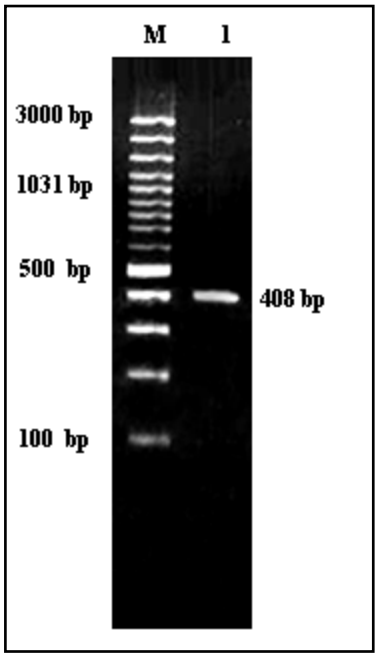


Figure 2. Agarose Gel Electrophoresis of 408 bp purified PCR Product from goat pituitary cDNA. Lane M Molecular size marker, Lane 1 purified 408 bp product

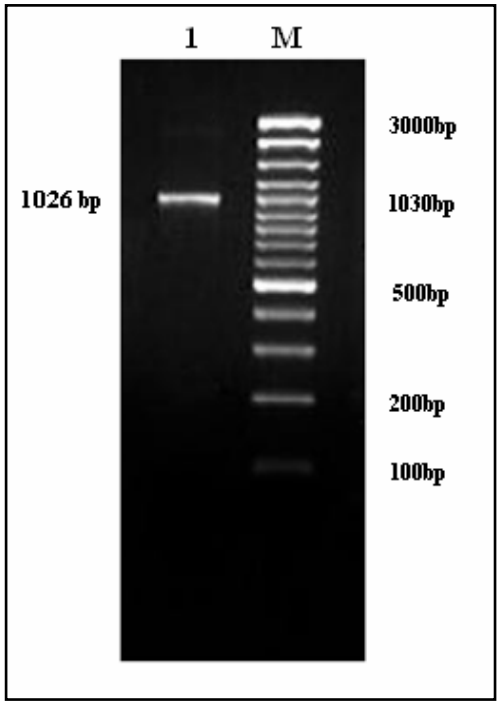


Figure 3. Agarose gel electrophoresis of purified 1026bp PCR product Lane M Molecular size marker, Lane 1 purified 1026 bp purified product

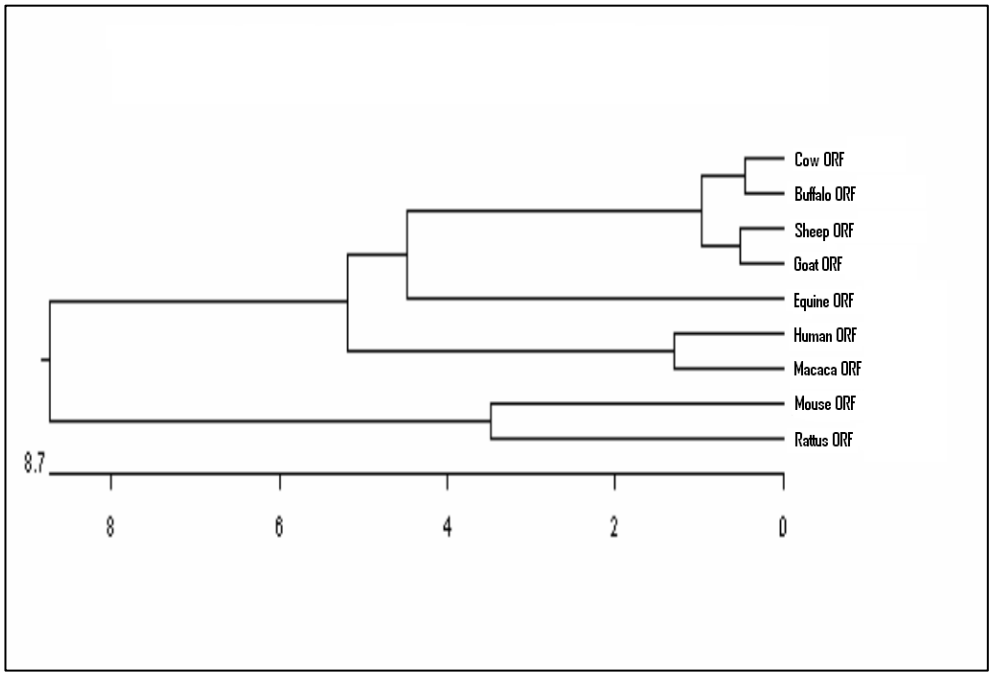


Figure 4. Phylogenetic tree of Type-I GnRH Receptor gene based on the sequences of different animal species

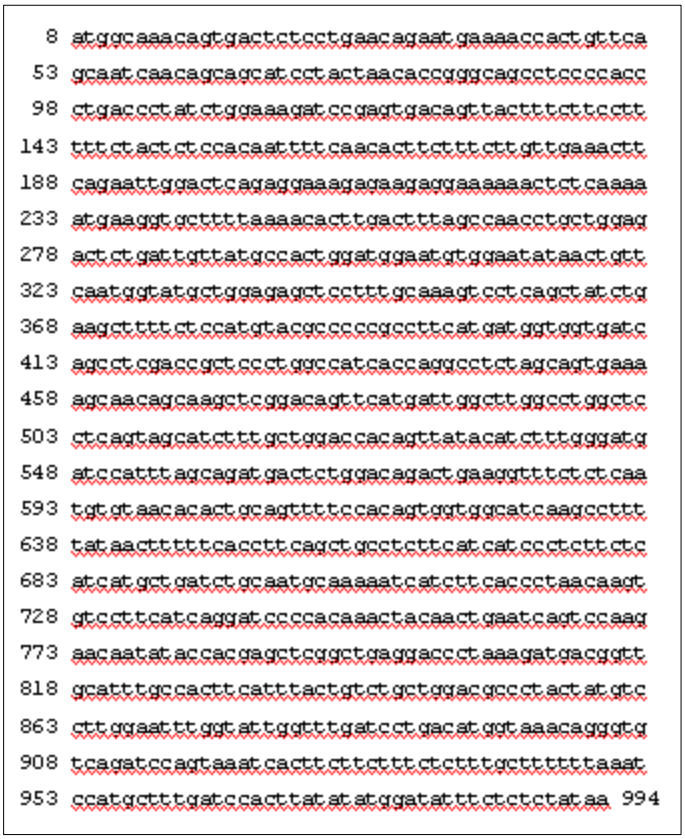
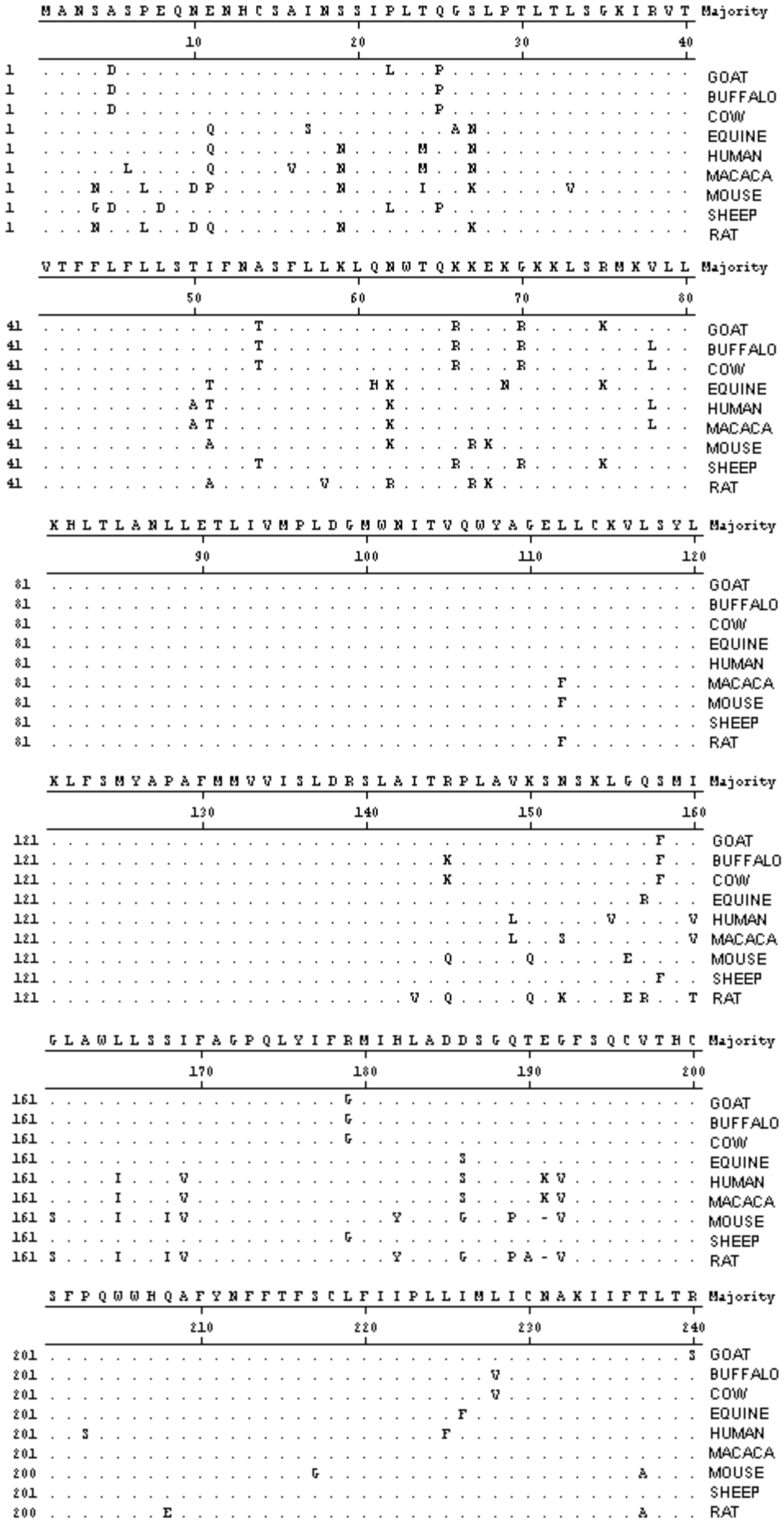


Figure 5. ORF of goat type-I GnRH-R gene


M A N S A S P E Q N E N H C S A I N S S I P L T Q G S L P T L T L S G K I R V T Majority
10 20 30 40
1 . . . . D . . . . . L . . P . . . . . GOAT
1 . . . . D . . . . . P . . . . . BUFFALO
1 . . . . D . . . . . P . . . . . COW
1 . . . . . Q . . . . . S . . . . . A N . . . . . EQUINE
1 . . . . . Q . . . . . N . . . . M . . N . . . . . HUMAN
1 . . . . . L . . . . Q . . . . V . . N . . . . M . . N . . . . . MACACA
1 . . . N . . L . . D P . . . . . N . . . . I . . K . . . . . V . . . . . MOUSE
1 . . . G D . . D . . . . . L . . P . . . . . SHEEP
1 . . . N . . L . . D Q . . . . . N . . . . . K . . . . . RAT
V T F F L F L L S T I F N A S F L L K L Q N W T Q K K E K G K K L S R M K V L L Majority
50 60 70 80
41 . . . . . T . . . . . R . . . R . . . . K . . . . . GOAT
41 . . . . . T . . . . . R . . . R . . . . . L . . BUFFALO
41 . . . . . T . . . . . R . . . R . . . . . L . . COW
41 . . . . . T . . . . . H K . . . . . N . . . . . K . . . . EQUINE
41 . . . . . A T . . . . . K . . . . . L . . HUMAN
41 . . . . . A T . . . . . K . . . . . L . . MACACA
41 . . . . . A . . . . . K . . . . R K . . . . . MOUSE
41 . . . . . T . . . . . R . . . R . . . . K . . . . . SHEEP
41 . . . . . A . . . . . V . . . R . . . . R K . . . . . RAT
K H L T L A N L L E T L I V M P L D G M W N I T V Q W Y A G E L L C K V L S Y L Majority
90 100 110 120
81 . . . . . GOAT
81 . . . . . BUFFALO
81 . . . . . COW
81 . . . . . EQUINE
81 . . . . . HUMAN
81 . . . . . F . . . . . MACACA
81 . . . . . F . . . . . MOUSE
81 . . . . . SHEEP
81 . . . . . F . . . . . RAT
K L F S M Y A P A F M M V V I S L D R S L A I T R P L A V K S M S K L G Q S M I Majority
130 140 150 160
121 . . . . . F . . GOAT
121 . . . . . K . . . . . F . . BUFFALO
121 . . . . . K . . . . . F . . COW
121 . . . . . R . . . EQUINE
121 . . . . . L . . . . . V . . . . V HUMAN
121 . . . . . L . . S . . . . . V MACACA
121 . . . . . Q . . . . Q . . . . . E . . . . MOUSE
121 . . . . . F . . SHEEP
121 . . . . . V . Q . . . . Q . K . . . E R . . T RAT
G L A W L L S S I F A G P Q L Y I F R M I H L A D D S G Q T E G F S Q C V T H C Majority
170 180 190 200
161 . . . . . G . . . . . GOAT
161 . . . . . G . . . . . BUFFALO
161 . . . . . G . . . . . COW
161 . . . . . S . . . . . EQUINE
161 . . . . I . . . V . . . . . S . . . . K V . . . . . HUMAN
161 . . . . I . . . V . . . . . S . . . . K V . . . . . MACACA
161 S . . . I . . I V . . . . . Y . . . G . . P . - V . . . . . MOUSE
161 . . . . . G . . . . . SHEEP
161 S . . . I . . I V . . . . . Y . . . G . . P A - V . . . . . RAT
S F P Q W W H Q A F Y N F F T F S C L F I I P L L I M L I C N A K I I F T L T R Majority
210 220 230 240
201 . . . . . S GOAT
201 . . . . . V . . . . . BUFFALO
201 . . . . . V . . . . . COW
201 . . . . . F . . . . . EQUINE
201 . . S . . . . . F . . . . . HUMAN
201 . . . . . MACACA
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201 . . . . . SHEEP
200 . . . . . E . . . . . A . . . RAT

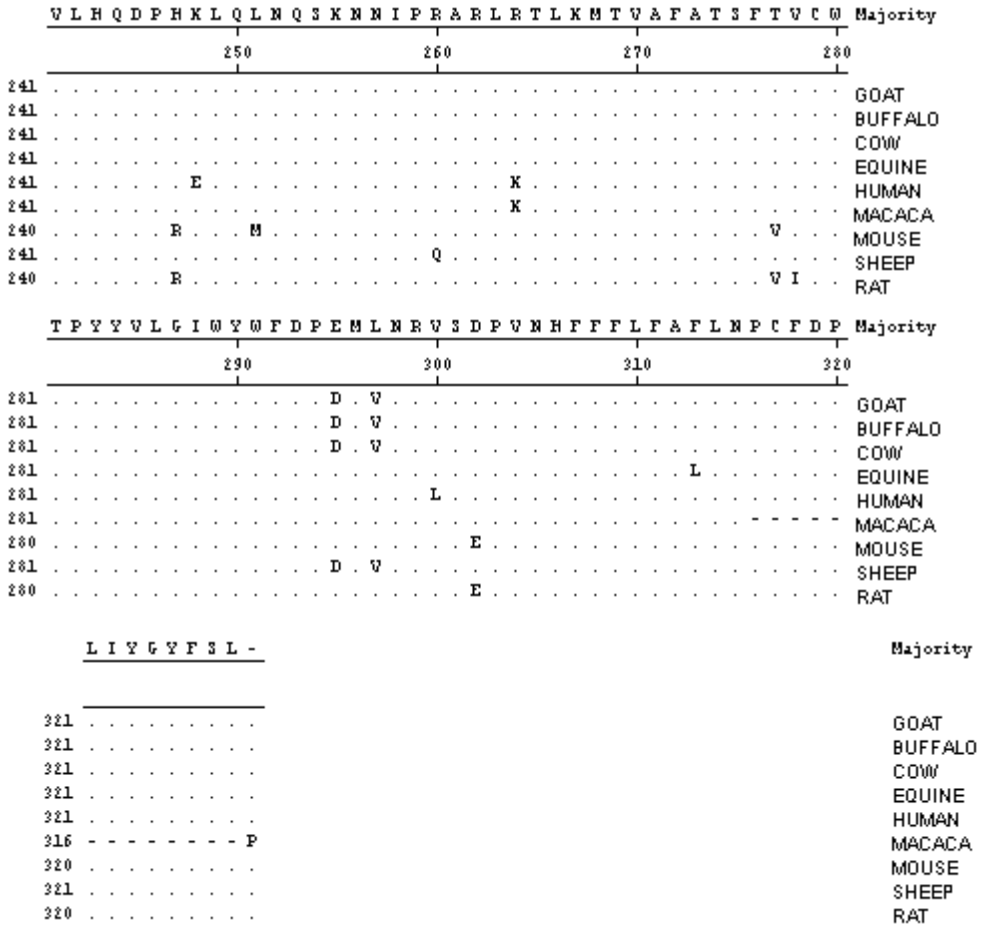

Figure 6. Alignment of goat type-I GnRH-R deducted amino acid sequence with other animals

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Characterization of Human Adipose Tissue Derived Hematopoietic Stem Cell, Mesenchymal Stem Cell and Side Population Cells

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Abstract

Adipose tissue represents an abundant and accessible source of multipotent adult stem cells, which appear to possess a yet-undetermined degree of plasticity. Subcutaneous adipose tissue is much studied in recent years than omentum fat tissue. Hence the objective of this work is to characterize the freshly isolated human adipose tissue derived stromal vascular fraction cell relative to passage 0 from subcutaneous fat and the omentum fat. The stromal vascular fraction of both subcutaneous and omentum fat contains hematopoietic and mesenchymal stem cell population where as in P0 the mesenchymal stem cell alone was retained. The side population ABCG2 is present in subcutaneous fat, whereas no ABCG2 expression is found in omentum fat. This study showed that the omentum fat exhibit higher percentage of hematopoietic cells compared to subcutaneous fat in both SVF and P0. It is concluded from this study that not only subcutaneous fat, but also omentum fat derived stem cells serve as a therapeutic potential in Regenerative medicine.

Keywords: Stromal vascular fraction, Adipose derived stem cells, Subcutaneous fat, Omentum fat, Hematopoietic stem cells, Mesenchymal stem cells, Side population stem cells

1. Introduction

Adult stem cells hold great promise for use in tissue repair and regeneration (Fuchs et al 2000, Kondo M et al 2003). In recent years, research on subcutaneous adipose tissue is of great interest (Tholpady Sunil S et al 2009, Kotaro Yoshimura et al 2009). Subcutaneous adipose tissues are the fat depots which are present beneath the cutaneous layer and are easily accessible and abundant. The Stromal vascular fraction (SVF) is a heterogenous cell population derived from the adipose tissue. Several recent findings demonstrates that mesenchymal stem cells with in the stromal vascular fraction (SVF) of subcutaneous adipose tissue display multilineage developmental potential in vitro and in vivo (Giuseppe Astori et al 2007, Adam J Katz 2005, Aust L et al 2004). However there is another type of fat called the omentum fat obtained from the omentum which is present beneath the abdomen and around the intestine. Singh Ashok and his team in the year 2008 isolated, cultured and characterized the omentum fat obtained from rat and concluded that these cells exhibit stem cell properties enabling to be used for repair and possibly for the regeneration of damaged tissues (Singh Ashok et al 2008). However there has been a scarcity of information on human omentum fat tissues and its implications in therapeutics. The paucity is due to lack of easy accessibility and its influence on the manifestation of pathogenecity in the bowel as omentum fat surrounds and protects the bowel beneath the intestine. Hence, as a maiden attempt, human omentum fat tissue samples have been investigated for their cytotherapeutic potentiality in contrast with subcutaneous adipose tissue by isolation and characterization of hematopoietic stem cells, mesenchymal stem cells and side population cells in stromal vascular fraction relative to passage 0 (P0). By doing so this study identifies efficient and alternative stem cell population for future therapeutics.

2. Materials and methods

2.1 Reagents

The following antibodies conjugated with corresponding flurochromes (CD34-PE; Cat No: 348057, CD117-PE-Cy7; Cat No: 339195, CD-90-PER-CP-Cy5; Cat No: 555597, Cell viability dye 7-AAD; Cat No: 555816) were purchased from BD Biosciences, (http://www.bd.com/). CD105-APC; Cat No: 17-1057, ABCG2-PE; Cat No: 12-8888 and CD49d-PE; Cat No: 12-0499 were purchased from eBioscience, (www.ebioscience.com). Aldefluor; Cat No: 01700 and Ficoll Paque Plus; Cat No: 07917 were purchased from stem cell technologies, (www.stemcell.com). DMEM; Cat No: AL007, Fetal Bovine Serum; Cat No: RM1112, Antibiotic antimycotic solution (Anti-anti); Cat No: A002A, Collagenase Type I; Cat No: RM2075, Trypsin-EDTA; Cat No: TCL007, Phosphate Buffer Saline (PBS); Cat No: TL1032 were purchased from Himedia.

2.2 Adipose tissue collection

Subcutaneous adipose tissue and omentum fat tissue were obtained from the obese patients undergoing bariatric surgeries to reduce their weight and who underwent abdominal surgeries respectively. The tissues were collected after the consent of the patients. The tissues were collected in a sterilized container.

2.3 Cell isolation procedure

Cells were isolated from adipose tissue using a procedure modified from Zuk et al 2001. The collected tissues were washed two to three times with phosphate buffer saline (PBS). The tissues were taken in a sterile petridish and minced in to pieces using forceps and surgical blade with 1-3 mm in diameter. The minced tissues were then treated with 0.075% collagenase type I prewarmed to 37°C with continuous agitation for 1-2 hours and centrifuged at 600g for 10 minutes. The supernatant containing the mature adipocytes was discarded. The pellet was identified as the SVF cells. Ficoll density gradient centrifugation technique was performed on the isolated stromal vascular fraction cells. This is a modified step without filtration. The usage of Ficoll technique without filtering the sample in a 100 μ cell strainer and 40μ cell strainer is to avoid all the debris and using this protocol, high yield of stem cells were obtained than filtration method.

2.3.1 Ficoll method

The washed pellet termed the Stromal Vascular Fraction (SVF) isolated from Adipose tissue was layered on to Ficoll density gradient medium slowly at the sides of the tube at 45° angle and centrifuged. After centrifugation at 400 g for 30 min at room temperature in a swinging bucket rotor, the SVF was collected from the adipocyte -Ficoll interface. Further, cells were washed twice with phosphate buffer saline (PBS) at 450 g for 10 minutes at room temperature to remove residual Ficoll and other contaminants. The cells were resuspended with RBC lysis buffer solution to remove any left over erythrocytes (RBC's) for 10 minutes and immediately treated with 0.9% cold NaCl to stop the lysis reaction and centrifuged at 300 g for 5 minutes at 4º C. Cell viability was determined using the Trypan blue dye exclusion method using hemocytometry. The Stromal vascular fraction cells were characterized for various hematopoietic, mesenchymal and side population cell surface markers using flowcytometry.

2.4 Cell culture of adherent cells of adipose tissue

The cells were suspended with DMEM, 10% Fetal Bovine Serum and 10 % antibiotic- antimycotic solution (anti-anti) and plated immediately in 110 mm culture dish (Cat No: PWO46; Himedia). The cells were incubated at 37º C, 5% CO2, in a humid air. This initial passage of the primary cell culture was referred to as passage 0 (P0). The medium was replaced every 3 days. The adherent cells at P0 were removed by 0.25% Trypsin-EDTA and characterized for various surface marker analysis using flowcytometry.

2.5 Characterization of SVF cells using flowcytometry

$1X10^6$ cells from stromal vascular fraction and P0 cells were taken and characterized for various hematopoietic, mesenchymal and side population markers using BD FACS Aria. The CD34+ hematopoietic population, the CD34-CD117-CD90+CD105+CD49d+ mesenchymal markers and the CD117+ABCG2+ALDH+ side population cells were used for characterization in flowcytometry.

2.5.1 Flowcytometric protocol for characterisation

Flow cytometry was performed on a Becton, Dickinson FACS Aria (http://www.bd.com/) using a 488-nm argon-ion LASER and 632nm red LASER for excitation; fluorescence emission was collected using its corresponding detectors. $1X10^6$ cells were stained with appropriate amount of conjugated antibodies in each of 12X75 mm falcon polystyrene FACS tube, BD Bioscience; Cat No: 352054. The quantity of each antibody conjugated with fluorochromes added to the cells in each tube were 20µl of CD34-PE, 5µl of CD90- PER CP CY5, 20µl of CD 105-APC, 5µl of CD117-PE CY7, 20µl of ABCG2-PE,20µl of 7-AAD (BD Via probe), 20µl of CD49d-PE respectively. All tubes were incubated for 20 minutes in dark. After incubation, cells were washed in phosphate buffer saline to remove the unbound antibodies. The pellet was further resuspended to 500µl. Data analysis and acquisition was then performed using DIVA Software, Becton Dickinson. Flow cytometer instruments were set using unstained cells. Cells were gated by forward versus side scatter to eliminate debris. The number of cells staining positive for a given marker was determined by the percentage of cells present within a gate established. A minimum of 10 000 events was characterized and recorded.

3. Results

3.1 Flowcytometric analysis of subcutaneous fat

Flowcytometric analysis of both SVF and P0 of subcutaneous fat showed that hematopoietic stem cell populations were present in both SVF and P0. Cells expressing CD90+CD105+CD49d+ mesenchymal stem cells population increases in P0 compared to SVF in subcutaneous fat (Figure 1, Line diagram1).

When side population cells are considered, it is clear that SVF cell expresses higher percentage of CD117+, ALDH+ cells in SVF compared to P0 whereas ABCG2+ cells shows no significant changes in both SVF and P0 (figure 2 and bar diagram 1).

3.2 Flowcytometric analysis of omentum fat

Flowcytometric analysis of both SVF and P0 of omentum fat showed that hematopoietic stem cell population was found to be higher in SVF. Cells expressing CD90+CD105+CD49d+ mesenchymal stem cell population is found to be higher in P0 compared to SVF (Figure3 and Line diagram 2).

When side population cells are considered, it is clear that stromal vascular fraction expresses more percentage of CD117+, ALDH+ cells compared to P0, whereas no expression of ABCG2+ cells were found in both SVF and P0 (figure 4 and Bar diagram 2).

3.3 Comparitive flowcytometric results of both subcutaneous and omentum fat

Flowcytometric analysis of SVF and P0 of both subcutaneous and omentum fat showed that the omentum fat contains higher percentage of hematopoietic stem cells compared to subcutaneous fat in both SVF and P0 (Line Diagram 3).

4. Discussion

The stromal vascular fraction derived from the adipose tissue is not a homogenous cell population and usage of exact markers for characterization for therapeutic purpose remains unknown (Giuseppe Astori et al 2007). Several researchers have proved that adipose tissue derived stem cells is of great use in tissue repair, regeneration and organ transplantation (Kotaro Yoshimura et al 2009, Planat-Benard V et al 2004). Though extensive research on hematopoietic stem cells and mesenchymal stem cell exists (Gimble et al 2003, Zuk PA et al 2002), very few studies pertaining to side population characterization exists (Zhou S et al 2001, Kim M et al 2002). Moreover there is paucity in human omentum fat derived stem cell research because of the niche where the omentum fat lies and the difficulty in complications of the bowel after isolation. However we feel that the human omentum fat has multiple differentiation potential and plasticity after the findings of Singh et al and his team who found that rat omentum fat tissue serves as a potential source of tissue repair and regeneration (Singh Ashok et al 2008). Thus we chose to pursue the study on characterization of human omentum fat derived stem cell population in contrast to subcutaneous fat tissue with certain definitive markers in flowcytometry. From the results obtained, we found that side population stem cell ABCG2 is present in SVF and P0 of subcutaneous fat. Whereas research from Katz et al suggests that the SVF of subcutaneous fat lacks sidepopulation ABCG2 (Adam J Katz et al 2005). Additionally, we found that omentum fat contains high percentage of hematopietic stem cells compared to subcutaneous fat tissue in both SVF and adherent P0 population. The advantage of omentum fat derived stem cell isolation is that the SVF of this omentum fat contain maximum of homogenous hematopoietic stem cell population and lack heterogeneity. Whereas the disadvantage of SVF of subcutaneous fat tissue is that it suffers from heterogeneity (Giuseppe Astori et al 2007). Thus from this study we found that subcutaneous and omentum fat tissues are qualitatively and quantitatively different. Hence, it is concluded from the results that not only subcutaneous fat, but also human omentum fat can be used as a high therapeutic potential in regenerative medicine.

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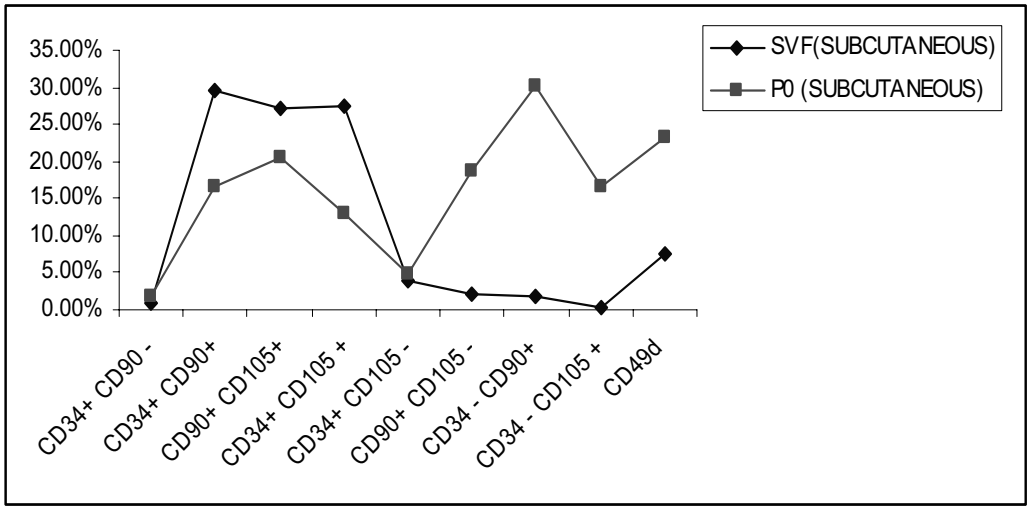
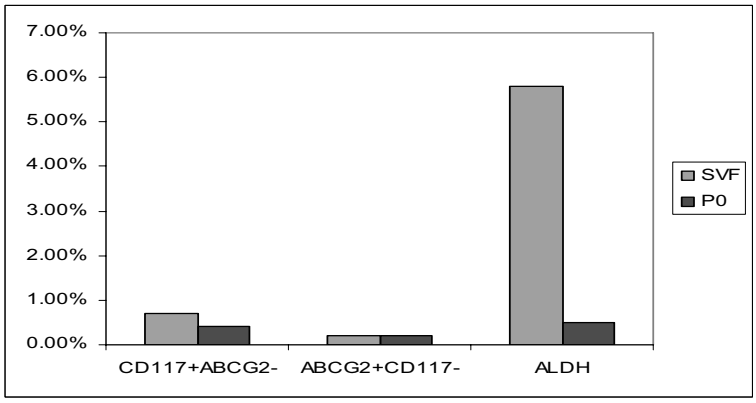
Note: The first and second author contributed equally to this work.

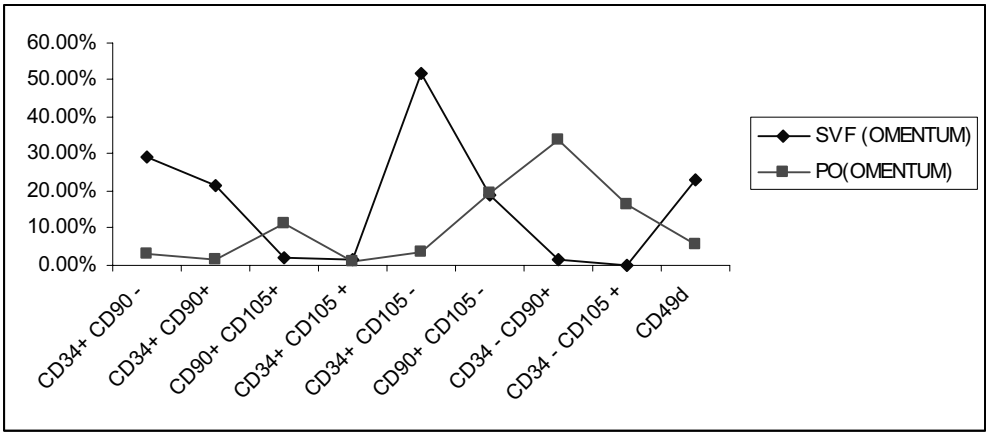
Line diagram 1. Comparison of HSC and MSC of SVF and P0 of subcutaneous fat



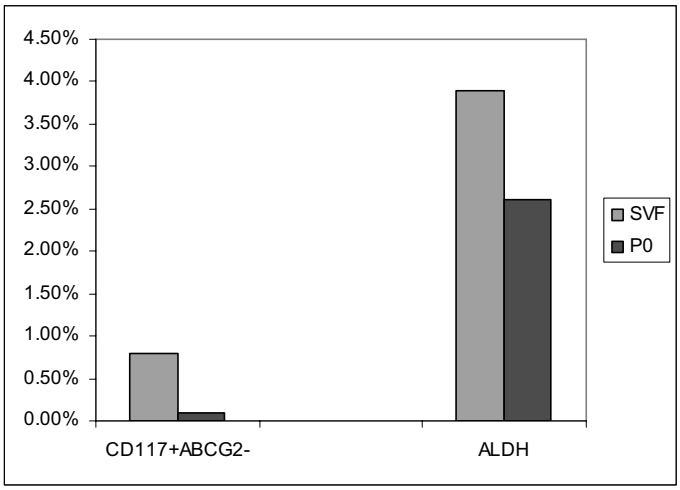
Bar diagram 1. Comparison of side population cells of SVF and P0 of subcutaneous fat



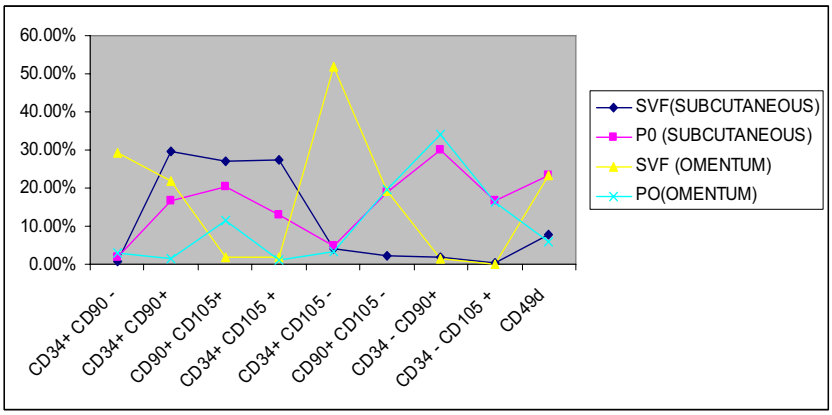
Line diagram 2. Comparison of side population cells of SVF and P0 of omentum fat



Bar diagram 2. Comparison of side population cells of SVF and P0 of subcutaneous fat



Line diagram 3. Comparison of SVF and P0 of subcutaneous and omentum fat



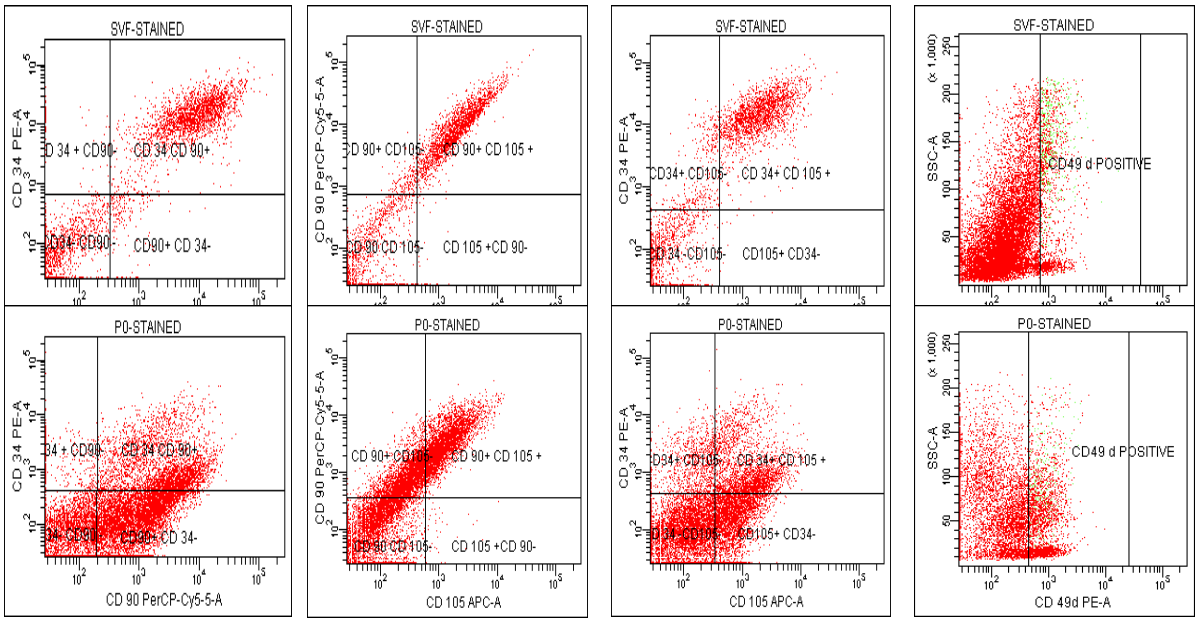


Figure 1. Flowcytometric enumeration of HSC and MSC in SVF and P0 cells of subcutaneous fat

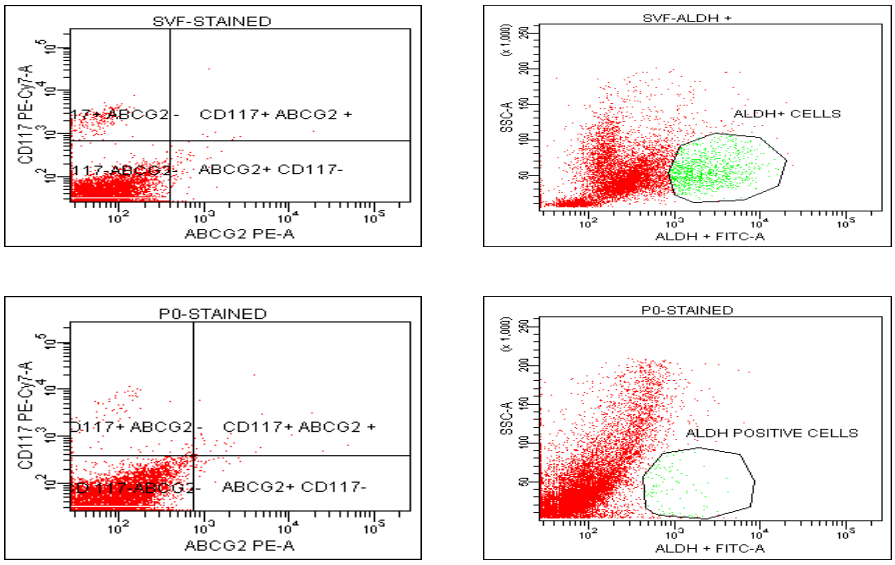


Figure 2. Flowcytometric enumeration of side population cells of SVF and P0 cells of subcutaneous fat

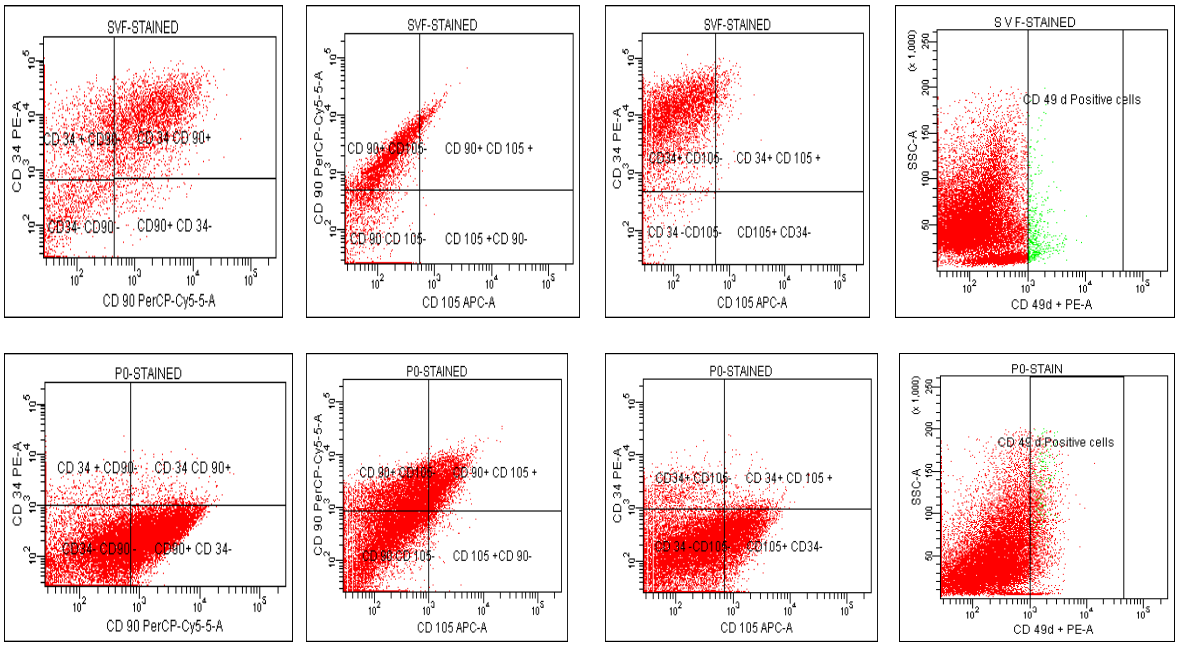


Figure 3. Flowcytometric enumeration of HSC and MSC in SVF and P0 cells of omentum fat

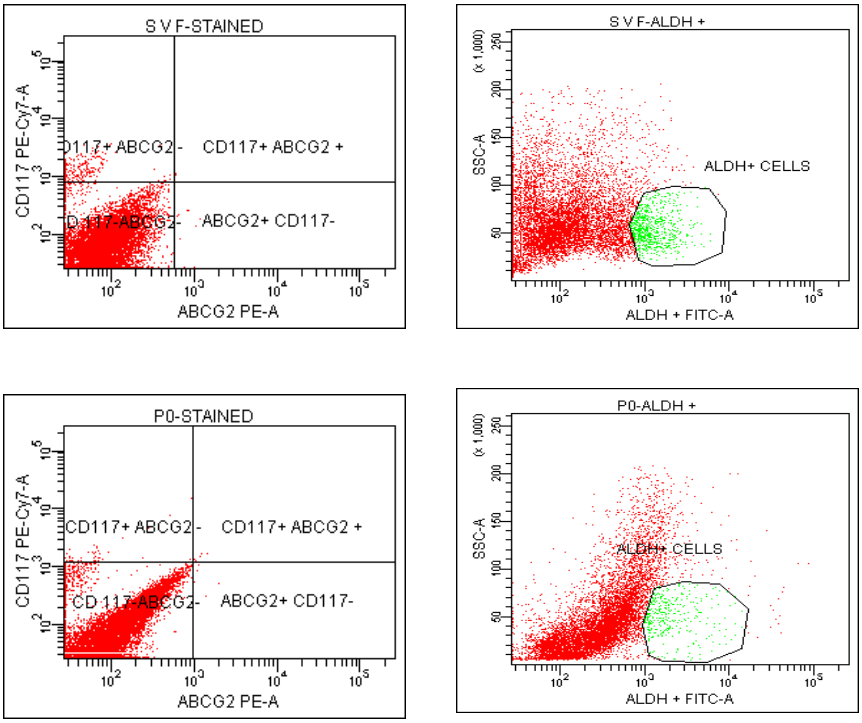


Figure 4. Flowcytometric enumeration of side population cells of SVF and P0 cells of omentum fat

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Identification of Phenol-Degrading *Nocardia* Sp. Strain C-14-1 and Characterization of Its Ring-Cleavage 2,3-Dioxygenase

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Abstract

An aerobic bacterial strain C-14-1 was isolated from an acrylic fiber wastewater. The strain was found to belong to *Nocardia* sp. according to morphological, physiological and its 16S rRNA gene sequence. This strain was able to degrade both alkanes and succinonitrile such as phenol. Catechol 2,3-dioxygenase (C23O) gene was found and amplified with the designed primers from the total DNA of C-14-1. The result of Southern blot indicated that there is only one C23O gene in the genome of C-14-1.

Keywords: Biodegradation, Broad-spectrum, Phenol, Catechol 2,3-dioxygenase gene

1. Introduction

Phenol is an aromatic molecule containing hydroxyl group attached to the benzenoid ring structure. The origin of phenols in the environment is both anthropogenic as well as xenobiotic (Hirayama, K. K., 1994; Bobdziewicy, J., 1998; Kumaran, P., 1997). Phenol is a major pollutant present in several types of industrial wastewater, such as that from coal refineries, phenol manufacturing pharmaceuticals, industries of resins, paints, dyes, petrochemicals, and textiles, and pulp and paper mills (Ahmed, 1995; Kumar, A, 2004; Bandhyopadhyay, K., 2001). It acts as a substrate inhibitor in the biotransformation (Hill, G. A., 1975). WHO has prescribed a concentration of 1 μg/L as the guideline concentration for drinking water. Thus, elimination of phenol effectively is necessary to preserve the environment and the health of human beings.

It is currently removed by costly and inefficient chemical or physical methods. Biological degradation has been utilized as an alternative, since it has low associated costs and leads to complete mineralization (Tay, S. T., 2005). The ability to degrade phenol and other phenolic compounds is widespread in microorganisms. Several bacterial strains belonging to the species of *Pseudomonas*, *Bacilli*, *Klebsiella*, *Ochrobactrum*, *Rhizobia*, etc. are reported for phenol degradation (Chitra, S., 1995; Balasankar, T., 2000).

As the complex composition of industrial wastewater, microorganisms with broad-spectrum degrading ability are urgently to be found to avoid antagonism and other adverse effect during biological degradation. In this paper, we report here the identification and characterization of phenol degrading bacteria isolated from the acrylic fiber wastewater. In previous study we get that this strain can degrade not only alkanes but succinonitrile. Physiological and biochemical features are used to characterize C-14-1, and phylogenetic analysis based on 16S rRNA gene is used to reveal genetic relationship of the isolate with other *Nocardia* strains. A catechol 2,3-dioxygenase is found and cloned from C-14-1 with designed primers firstly. A new way of studying bacteria broad-spectrum degrading ability and constructing genetically engineered bacterial can be provided through this paper.

2. Materials and methods

2.1 Chemicals

Most of the chemicals used were either from Sigma, USA or from Fluka Chemika, Switzerland. Phenol crystals (99%

purity) were obtained from Shanghai reagent factory. The experimental procedure for liquid phenol preparation was developed according to Sambrook et al. (Sambrook, J., 1989). Nutrient broth and other chemicals were obtained from commercial supplies.

2.2 Physiological and biochemical characteristics of C-14-1

Seven important physiological and biochemical features of C-14-1 were used to characterize the strain C-14-1. The colony character and bacterium configuration were observed by microscope. The analysis included dying, aerobic test, catalase activity, oxidation zymolysis of glucose, etc. All tests were done in duplicate.

2.3 16S rRNA amplification and phylogeny analysis of 16S rRNA gene

Strain C-14-1 was incubated in TSB and the genomic DNA was extracted using a DNA extraction reagent after fission by bacteriolysis. The 16S rRNA gene was amplified using primers FP (5′-GGTGTAGCGGTGGAATGCGCAGAT-3′) and RP (5′-CGAGCTGACGACAACCATGCACCAC-3′). 50 μL of PCR volume consisted of 10 pg of total DNA, 25 μL 2×Reaction buffer, 4μL of 2.5mM each dNTP, 1μL of 20mM FP, and RP, 0.5μL of 5 U LA Taq polymerase and adding ddH_2O making the volume up to 50μL. The PCR was conducted at 95℃ predenaturing for 5 min and the 30 cycles (94℃ denaturing 0.5 min, 58℃ annealing 0.5 min and 72℃ extending 0.5 min), finally 72℃ extending 7 min again. The PCR amplification product was purified using the PCR purification kit (geneworks). After purification, the PCR product was sequenced by Shanghai Sangon Company.

2.4 Cloning and sequencing of catechol 2,3-dioxygenase gene

PCR was performed to amplify the C23O structural gene. The sequences of the primers were designed on the basis of strain (GenBank ID AP006618) FP: (5′-CTGGGTGATGCCGTGCTT-3′) and RP: (5′-CTCTGGGAGGCCGAGAAAT-3′). 50 μL of PCR volume consisted of 10 pg of total DNA, 25 μL 2×Reaction buffer, 4μL of 2.5mM each dNTP, 1μL of 20mM FP, and RP, 0.5μL of 5 U LA Taq polymerase and adding ddH_2O making the volume up to 50μL. The PCR was conducted at 95℃ predenaturing for 5 min and the 30 cycles (94℃ denaturing 0.5 min, 58℃ annealing 0.5 min and 72℃ extending 0.5 min), finally 72℃ extending 7 min again.

2.5 Phenol degradation

The effect of the different substrate concentrations (0, 100, 200, 400, 600, 800, 1000mg/L) on the phenol degradation by C-14-1 was examined. The growth medium was a kind of inorganic medium incubated at 35℃, under a 220 rpm shaking rate and a pH of 7.0.

2.6 Analytical procedures

Growth of the organisms was recorded by monitoring the optical density (OD) of the culture in a 752-N UV spectrophotometer at 460 nm. Phenol was estimated spectrometrically using 4-aminoantipyrene as per standard procedure (APHA, 1992).

3. Results and discussion

3.1 Physiological and biochemical characteristics of C-14-1

The physiological, biochemical characteristics of C-14-1 were given in Table 1. It had several mycolic acids which were notable feature of *Nocardia.* sp. The colony was observed round shape by using microscope, Φ0.5~1.0mm, orange, smooth wet and low-raised surface. Bacterium configuration was hypha in medium Φ0.5~0.8μm, some branched, broken into globularity within 52h.

3.2 16S rRNA gene phylogeny of C-14-1

16S rRNA sequences analysis was a fast and accurate method to identify C-14-1 phylogeny position. Part-length (about 391bp) 16S rRNA genes were sequenced and shown in Fig. 1 (GenBank ID EU579437). We found that the strain C-14-1 was classified in the *Nocardia* genera, the similarities between C-14-1 and *N.amamiensis* (AB275164), *N.pneumoniae* (AB108780) and *N.inohanensis* (DQ659908) were 97.0, 97.0 and 96.0, respectively. Combined with the physiological and biochemical characteristic results, C-14-1 was identified to be *Nocardia* sp.

3.3 Detection of catechol 2,3-dioxygenase genes in C-14-1

A PCR product encoding the C23O gene from C-14-1 was successfully obtained with the expected size and cloned into the pGEM-T vector. A recombinant plasmid containing a 402bp insert with the correct orientation, which was transformed into E. *coli* DH5α for heterologous expression. Part-length (about 402bp) C23O genes were analyzed and sequenced and shown in Fig. 2 and Fig.3 (GenBank ID EU586325). The result hinted there might be some C23O genes in genomic DNA of C-14-1. In order to confirm how many copies of C23O were in C-14-1, southern blot with the labeled PCR product by Digoxin as the probe was carried out. Five kinds of restriction enzymes (*Kpn*I, *Nde* I, *Not* I, *Pac* I and *EcoR* I) were used to thoroughly digest genomic DNA of C-14-1. Probe labeling and efficiency determination and southern blot to locate the homologous DNA fragments in the genome of the strain C-14-1 were shown in Fig. 4

and Fig. 5. As shown in Fig. 5, the strain C-14-1 had only one C23O gene in its genomic DNA.

3.4 Effect of substrate concentration

C230 was known one of key enzymes in benzene ring refusion. C-14-1 was capable of using phenol as carbon source and its ring cleavage was found at 2,3-position in follow-up study. Seven different initial phenol concentrations were used (Fig. 6). It was shown that as the initial concentration of phenol increase the degradation rate increase to a value of 3.27 h^{-1} then started to decrease with further increasing the concentration of phenol. This was attributed to the fact that phenol was essentially toxic to microorganisms and cells were inhibited with further increase in the phenol concentration. The phenol degradation experienced two phases from the figure. The first was stagnant phase in which the phenol degradation was quite slow. In the second quick degrading phase, phenol was rapidly degraded. It conferred that microorganisms usually needed a period of time to adapt the reaction system. But in case the degrading reaction

begun after the adapting period, the strain could degrade the substrate quickly. This phenomenon was reported in some other studies (Wang, J. S., 1993).

4. Conclusion

The *Nocardia sp.* strain C-14-1 isolated from the acrylic fiber wastewater could well degrade phenol while it also had high strength alkanes and succinonitrile degrading ability. C-14-1 belonged to the *Nocardia* genus according to 16S rRNA gene analysis, and it was closely related to *N.amamiensis*. One catechol 2,3-dioxygenase gene was found in its genomic DNA. The phenol degradation rate was 100.0 and 42.2% with an initial concentration of 800 and 1000mg/L within 28h.

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Table 1. The physiological and biochemical characteristics of C-14-1

characteristics	C-14-1
Gram's stain	+
Aerobic	+
Catalase activity	+
Glucose oxidase	+ (produce alkaline)
Acid resisting dye	-
Colony character	Colony round in shape, Φ0.5~1.0mm, orange, smooth wet and low-raised surface, fringe hypha like root hair, no aerial hypha
Bacterium configuration	Hypha in medium Φ0.5~0.8μm, some branched, broken into globularity within 52h, amount of globularity and short haulm formed in anaphase

GGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAA
GCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTG
CCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
CGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATACACCGGACCGCCCCAGAGATGGGGTTTCCCTTG
TGGTCGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCG

Figure 1. The sequence of 16S rRNA fragment of C-14-1

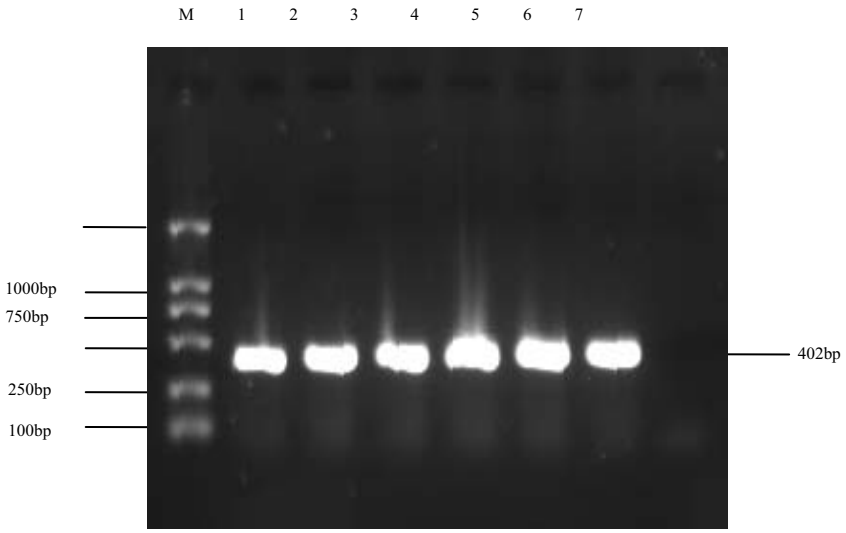

Figure 2. PCR identification photograph of the recombined plasmid pGEM-T-C14 C23O (M: DL2000 DNA Marker, Lane 1 to 6 are pGEM-T-C14 C23O PCR amplified DNA, 7: NTC)

CTGGGTGATGCCGTGCTTGTCGGGACCTGCCTCGATCTGAATGTCGTAGTCCCGGAACATCTCGACCGCGTCTGCGAGGTGCTGGCCGG
TGCCGTAGTAGAACGCGAGGTGGTGGAGCTTGCCGTGTCCGCCGGTCATGTCACGCATGCAGGCGACCTCGTGACCGAGCAGGTTCGA
ACTCATCCAGGCGCCGATCTCCACGCCGCCGTCCACCACGCGTTCGGTCGTGCGGAATCCGAGGTGCCGCTCGAACGAGTCCTTCACC
GCGGTCACGTCGGAGGACATGAGGTTCAGGTGATCGATCCGCTTGACCGGAATACCCTGGAGCGGCTTCTTCGACGGACGCGACAGGA
TCTTGCTCTGCAGCTCCTGCGGGGCGACGTATTTCTCGGCCTCAATCCC

Figure 3. The sequence of catechol C23O fragment of C-14-1

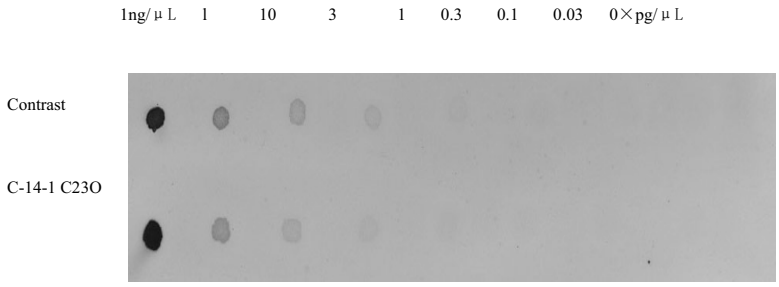

Figure 4. Result of probe labeling and efficiency determination

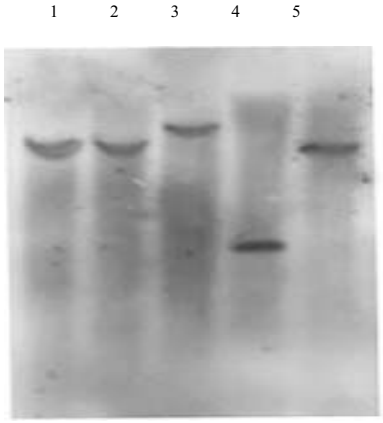

Figure 5. Southern blot to locate the homologous DNA fragments in the genome of the strain C-14-1 (The genomic DNA of strain C-14-1 were digested with the restriction enzymes *Kpn*I, *Nde* I, *Not* I, *Pac* I, *EcoR* I corresponding to lane 1, 2, 3, 4 and 5 respectively)

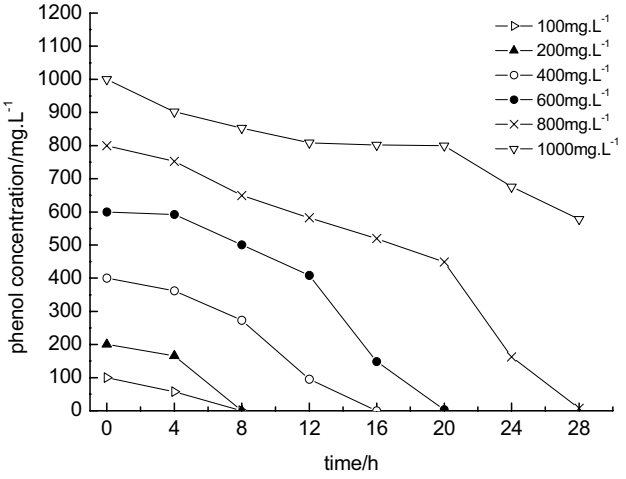

Figure 6. Phenol biodegradation of different temperature by C-14-1

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Culture and Identification of *Candida Albicans* from Vaginal Ulcer and Separation of Enolase on SDS-PAGE

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Abstract

Candida albicans were isolated from patients with clinical symptoms of vaginal ulcer. Culture test for vaginal swab and scrapings were conducted on Sabouraud's dextrose broth and Sabouraud's dextrose agar plate respectively. Hichrome candida agar culture was used for differential identification of *Candida.* Smears from vaginal scrapings were prepared for gram staining. The suspected strain of *Candida* was inoculated on corn meal agar medium for chlamydospore formation. The suspected strain of *Candida* was inoculated in human serum for germ tube formation. Carbohydrate assimilation and fermentation tests were also conducted. The selected *Candida* colony was inoculated in YEPD medium for subculture and the cultured organism was harvested. The organisms were homogenized, centrifuged and the supernatant was filtered. The filtrate was extracted in chloroform. The extract was centrifuged and the aqueous phase was dialyzed. The dialyzed crude enolase was subjected to SDS-PAGE. The Sabouraud's dextrose broth inoculated with vaginal swab showed turbid growth. The scraping from vagina showed typical smooth creamy white colonies with a characteristic yeast odour on Sabouraud's dextrose agar plate. On Hichrome candida agar the *Candida* growth appeared as glistening green colored. Gram stained smears from vaginal scraps showed appearance of fungus as yeast

budding. On corn meal agar the suspected *Candida* growth showed the formation of large, highly refractive, thick walled terminal chlamydospores. Germ tubes were seen as long tube like projections extending from the yeast cells on human serum inoculated with suspected strain of *Candida*. The carbohydrate assimilation tests were positive for dextrose, maltose, sucrose, galactose, xylose and trehalose, and negative for lactose, melibiose, ellobiose, inositol, reffinose and dulcitol. The carbohydrate fermentation tests showed positive for dextrose, maltose, galactose and trehalose, and negative for sucrose and lactose. SDS-PAGE for enolase from *C. albicans* showed a single polypeptide band of around 46 – 48 kDa.

Keywords: *Candida albicans*, Enolase, Vaginal-ulcer

1. Introduction

The yeast *Candida albicans* is commonly inhabits in oral and vaginal mucosa and gastrointestinal tract of human beings as one of the commensal organisms. It causes opportunistic infections in immuno-compromised patients, produces allergic reactions and rarely causes morbidity and mortality (Douglas, 1988). It also causes a variety of infections that range from mucosal candidiasis to life-threatening disseminated candidiasis (Dixon *et al*., 1996; O`Dwyer *et al*., 2007; Terrier *et al*., 2007).

The diagnosis of its deep seated infections remains a great challenge. One of the major reasons for the increase in *Candida* infections is the development of its resistant strains to azole drugs, such as fluconazole used in the prophylaxis and treatment of candidiasis (Diaz-Guerra *et al*., 1998; Shahid *et al*., 2006). The identification of invasive or disseminated candidiasis is based on clinical symptoms that are diffuse and not easily differentiated from those manifested by other infectious agents. Therefore considerable interest in the identification and characterization of antigens would be useful in the diagnosis and treatment of *Candida* infections.

It has been reported that in *C. albicans* and *Candida tropicalis* the cytosolic enzyme, enolase, was identified as an immuno-dominant antigen (Walsh *et al*., 199; Mitsutake *et al*., 1994; Angiolella, 1996). *Candida* enolase is a plasminogen- and plasmin-binding protein and the interaction of *C. albicans* enolase with the plasminogen system may contribute to invasion of the tissue barrier (Jong, 2003). Antibody to this bona fide cell wall protein is considered to be more predictive and specific as the cytoplasmic antigen is exposed only during invasive infection. Patients with localized candidiasis express only weak positive antibody titre (Van Deventer *et al*., 1993). In recent years *Candida* infection increases clinical awareness. Therefore, the present work was aimed to culture and identify *C. albicans* from patients with suspected invasive vaginal candidiasis, and separate enolase on SDS-PAGE to identify its molecular weight.

2. Materials and Methods

C. albicans strains were isolated from patients with clinical symptoms of vaginal ulcer and similar infections.

2.1 Collection and processing of vaginal samples

The suspected patients were used as subjects and the samples were collected and analyzed for *C. albicans.* The specimens were collected with meticulous care aseptically at the slit lamp.

2.2 Culture test from vaginal swabs

Sterile cotton swabs were prepared, gently smeared over the ulcers in the vaginal region and the swabs were immediately transferred to Sabouraud's Dextrose broth (pH, 5.6) containing dextrose (4 %), peptone (1 %) in double distilled water and incorporated with Chloramphenicol (5.0 mg). The culture tubes were incubated at 37^0 C for 24 – 48 hr.

2.3 Culture test from vaginal scrapings

Using a heat sterilized Kimura's platinum spatula of Bard Parker blade, vaginal scrapings were collected and the scrap material was streaked onto Sabouraud's dextrose agar plates as a fine C-streak.

2.4 Preparation of Sabouraud's dextrose agar plates

The Sabouraud's Dextrose agar consists of Sabouraud's dextrose broth (pH, 5.6) and 1.5 % agar. This was autoclaved and cooled to 45^0 C, and 5 ml of sterile defibrinated sheep blood was added to it aseptically. It was mixed thoroughly and dispensed into plates without air bubbles. The plates were incubated at 37^0 C for 24 – 48 hr. The suspected isolates of *Candida* were sub cultured onto Sabouraud's agar plates.

2.5 Differential identification of candida

Hichrome Candida agar (pH, 6.5) containing 1.5 % peptic digest of animal tissues, 0.1 % dipotassium hydrogen phosphates, 1.2 % chromogenic mixture, 0.05 % Chloramphenicol and 1.5 % agar in double distilled water was used for the presumptive identification of clinically important *Candida* species. The scraps were directly streaked on Hichrome Candida agar and incubated at 37^0 C for 48 hr.

2.6 Gram staining

Smears from the vaginal scrapings were prepared on slides cleaned with alcohol. They then heat-fixed;-staining was done by flooding the smears with crystal violet solution for 1 min and then with Gram's iodine for 1 min. After washing, the smears were decolorized with 95 % ethanol and counter strained with aqueous basic fuchsin. The slide was subjected to observation of *Candida* morphology under oil immersion objective lens (100 x) of a Bright Field microscope.

2.7 Test for chlamydospore formation

The suspected *Candida* cultures were inoculated on corn meal agar medium (pH, 7) containing 4% corn meal powder, 1 % Tween 80 and 1.5 % agar in double distilled water. The plates were incubated at 37^0 C for 48 - 72 hr.

2.8 Test for germ tube formation

The suspected *Candida* cultures were inoculated into 0.5 ml of human serum in a small tube and incubated at 37º C for 2 hr. After desired period of incubation, a loop-full of culture was placed on a glass slide and overlaid with a cover-slip. The preparation was examined for germinating blastospores.

2.9 Carbohydrate assimilation tests

The mainstay of yeast identification to the species level is the carbohydrate assimilation test, which measures the ability of yeast to utilize a specific carbohydrate as the sole source of carbon in the presence of oxygen. Sugars used for assimilation tests include dextrose, maltose, sucrose, lactose, galactose, melibiose, cellobiose, inositol (a form of sugar, carbocyclic polyol, cyclohexanehexol), xylose, raffinose, trehalose and dulcitol (or galactitol, a sugar alcohol, the reduction product of galactose).

Candida cultures were suspended in saline (NaCl) and to which 1.5 ml of basal medium containing 67.8 % yeast nitrogen base was added. This was then added to 13.5 ml of molten, cooled agar containing 2 % agar powder, mixed well, poured into Petri dish and allowed to solidify. After the medium was set, 20 % sugar-soaked filter paper discs were placed on the medium. The plates were incubated at 25^0 C for 10 – 24 hr and observed for the growth of *Candida.*

2.10 Carbohydrate fermentation tests

Fermentative yeasts recovered from clinical specimens produce carbon dioxide and alcohol. Production of gas rather than a pH shift is indicative of fermentation. Dextrose, maltose, sucrose, lactose, galactose and trehalose were used in the test.

The 5 ml of carbohydrate (pH, 7.4) containing 1 % peptone, 1 % sugar, 0.3 % beef extract and 0.5 % NaCl, 0.2 % Bromothymol blue in distilled water medium was dispensed in sterilized Durham tube and 0.2 ml of saline suspension of the test organism was added and incubated at 37^0 C for 10 days.

2.11 Subculture and harvesting of yeast cells

The selected organism was inoculated in yeast-extract-peptone-dextrose (YEPD) culture medium (pH, 7) containing 1 % yeast extract, 2 % peptone and 2 % dextrose, and incubated at 30^0 C for 24 hr in a mechanical shaker at 150 rpm. A 10 ml starter culture provided the basis for 500 ml *Candida* culture to be grown under similar conditions in a 2 l Erlenmeyer flask.

The *C. albicans* culture was harvested from the 2 l Erlenmeyer flask by centrifugation at 10,000 rpm for 15 min at 4^0 C. The cells were washed with distilled water and again collected by centrifugation. The supernatant was discarded and the cell pellets were re-suspended in protein-extraction buffer (pH, 6.5) containing 0.2 % Bis-Tris. The yeast cells were centrifuged as described above then re-suspended and pooled for further processing.

2.12 Cell fractionation

The organisms were suspended in a breaking buffer (pH, 6.8) containing 62.5 mM Tris-HCl buffer, 15 % glycerol, 1mM dithiotheritol and 20 mg of phenyl methyl sulfonyl flouride and broken by mechanical disruption in a Braun's homogenizer for 2 min with intermittent cooling in the presence of glass beads 0.45 mm in dia. After disruption of the cells, the glass beads and unbroken cells were removed by centrifugation at 500 x g. The homogenate was then centrifuged at 6000 x g for 20 min and the pellet was collected. The supernatant containing the soluble protein fraction was recovered and passed through a 0.45 μm filter membrane. The filtrates were then extracted with an equal volume of chilled chloroform. Following centrifugation at 4^0 C for 15 min at 2000 x g, the upper aqueous phase was aspirated and transferred to a dialysis tube. The crude protein fraction was dialyzed in column binding buffer (20 mM Bis-Tris, pH 6.5) for 24 hr. The dialysed crude enolase was subjected to Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (SDS – PAGE).

2.13 SDS - PAGE

SDS – PAGE was performed following the method of Laemmli (1970). Equal amount of sample loading buffer was added to the enolase samples (20 µg protein). The samples were boiled for 1 min and loaded on 12% SDS – PAGE. Protein standard of known molecular weight was also run parallel. The wells and tanks were filled with electrophoresis running buffer. The protein was allowed to isolate at 50 V DC in the stacking region and 100 V DC in the separating gel for about 3 hrs. The gel was stained overnight in Coomassie Brilliant Blue solution. The stained gel was de-stained in methanol – acetic acid solution until the gel background turns clear for visualizing the polypeptide band.

3. Results and Discussion

Candida is a dimorphic organism, meaning it can exist in two shapes and forms simultaneously. One form is a yeast-like state that is a non-invasive, sugar fermenting organism. The other is a fungal form that produces very long root-like structures, called rhizoids that can penetrate the mucosa and is invasive.

Healthy immune system prevents this yeast from becoming an infectious fungus. It is when our bodies lose their proper immune protection or the intestinal pH is altered unfavourably, that the organism can change from the yeast form to the fungal form. When this happens, the now-parasitic fungal form penetrates the gastrointestinal mucosa and breaks down the boundary between the intestinal tract and the rest of the circulation in our bodies. This allows partially digested dietary proteins to travel into the blood stream, where they exert a powerful antigenic (antibody-stimulating) assault on the immune system. It has been officially estimated that about 80% of the population may have candidiasis that is out of control.

The immune system is attacked by *Candida* as a result of the prolonged use of antibiotics, taking steroids or oral contraceptives on a regular basis, or due to high sugar diet. It is also known that *Candida* increases its numbers during periods of stress and lowered immune states.

Fungal infection of vagina is sometimes called a thrush. Candidiasis is also known as yeast infection is a common fungal infection that occurs when there is overgrowth of the fungus called *Candida* which is always present in the body in small amounts. However, when an imbalance occurs, such as when the normal acidity of the vagina changes or when hormonal balance changes, *Candida* can multiply. When this happens, symptoms of candidiasis appear. This may include depression, dry, itchy, flaky skin, anxiety, recurring irritability or mood swings, heartburn, indigestion, lethargy, food and environmental allergies, joint soreness, chest pain or other skin problems, recurring cystitis/vaginal infections, premenstrual tension and menstrual problems. Immune suppression, AIDS, diabetes and thyroid disorder may also cause candidiasis.

Vaginal thrush was characterized by typical white lesions developed on the epithelial surfaces of vulva, vagina and cervix. In the present study, the Sabouraud's dextrose broth inoculated with swab from the lesions of vagina showed turbid growth after incubation. On Sabouraud's dextrose agar, the scrapings from lesions of vagina showed typical smooth creamy white colonies with a characteristic yeast odour. Scrapings from the lesions of vagina were examined in the Gram stained smear. The fungus appeared as budding yeast cells and pseudomycelium was present in most cases (Fig. 1). On Hichrome candida agar, the scrapings from vagina produced glistening green color colonies (Fig.2). The suspected stains of *C. albicans* isolates were grown on the corn meal agar. It showed the formation of large, highly refractive and thick walled terminal chlamydospores (Fig. 3). When the suspected *C. albicans* was inoculated with human serum the formation of germ tubes was seen as long tube like projections extending from the yeast cells. There was no constriction at the point of attachment to the yeast cells. The germ tubes were formed within two hours of incubation in *C. albicans* (Fig. 4).

The carbohydrate assimilation tests for *C. albicans* are presented in table 1. It shows that the test was positive for dextrose, maltose, sucrose, galactose, xylose and trehalose. At the same time, the tests were negative for lactose, melibiose, cellobiose, inositol, reffinose and dulcitol. Similarly, the carbohydrate fermentation tests for *C. albicans* are presented in table 2. The tests for fermentation of dextrose, maltose, galactose and trehalose were positive but negative for fermentation of sucrose and lactose.

The SDS-PAGE profile of the crude sample prepared from *C. albicans* for enolase showed several polypeptide bands from 205 kDa to 29 kDa. Among these, the prominently resolved single polypeptide band of around 48 KDa was considered as enolase (EC: 4.2.1.11) (Fig. 5). This confirmation was based on the reports in the literature (Strockbine *et al.,* 1984; Walsh *et al.*, 1988, 1991; Savolainen *et al*., 1990; Sundstrom and Aliaga, 1992; Mason *et al*., 1993; Ito *et al*., 1995; Chang *et al*., 2002). The enolase from the pathogenic yeast *C. albicans* has been identified as an immunodominant antigen in disseminated infection and in allergic disease (Mitsutake *et al*., 1994). The *Candida* species such as *C. glabrata, C. tropicalis, C. krusei, C. parapsilosis* and *C. lusitaniae* are increasingly being identified as significantly causing infections in humans (Hazen and Hazen, 1992).

The cell wall of *Candida* is a dynamic and multilayered structure located external to the plasma membrane. The cell wall of *C. albicans* is composed of different types of carbohydrates (80 – 90 %): (i) Mannan or polymers of mannose

covalently associated with proteins to form glycoproteins, also referred to as mannoproteins, (ii) α-glucans that are branched polymers of glucose containing α-1,3 and α-1,6 linkages, and (iii) chitin, which is an un-branched homopolymer of N-acetyl-D-glucosamine (Glc-NAc) containing α-1,4 bonds (Gomez *et al*., 1996). The other constituents are proteins (6 - 25 %) and lipids (1 – 7 %) which are present as minor wall constituents (Shepherd, 1987). Yeast cells and germ tubes are similar in their cell wall composition, although the relative amounts of α-glucans, chitin and mannan may vary with morphology. Although glucans are present in greater abundance than mannan in *C. albicans*, they are immunologically less active (Poulain *et al.,* 1985).

The enzyme enolase was reported to be associated with glucan in the inner layers of the cell wall as well as in culture supernatants. Enolase seems to be the most immunodominant antigen in humans. In mice, enolase stimulates both humoral and cellular immune responses. The glycolytic enzyme, enolase catalyzes the reversible dehydration of 2-phospho-D-glycerate to high-energy phosphoenolpyruvate (Matthews *et al.,* 1984). Characterization of antigenic components in cytoplasmic extracts of *C. albicans* that were recognized in sera from patients with disseminated candidiasis has been reported (Strockbine *et al.,* 1984). Further, they reported that these patients had circulating antibodies directed against a 48 KDa protein antigen purified by anion-exchange chromatography from the *Candida* extract. Individuals colonized with *C. albicans* without evidence of candidiasis did not have detectable levels of antibodies to this antigen, which was subsequently identified as enolase (Franklyn *et al*., 1990). Circulating anti enolase antibodies may have potential value for the diagnosis of candidiasis (Mitsutake *et al*., 1994).

Antigenemia with the 48-KDa antigen has been reported in disseminated candidiasis in the absence of fungemia and correlated with deep tissue infection (Walsh *et al.*, 1988). *C. albicans* enolase antigenemia is a marker for deep tissue invasion even in the absence of fungemia (Walsh *et al*., 1991). Some major protein allergens of *C. albicans* have been identified by immunoblotting with anti-human IgE antibodies. For instance, in a study, serum samples from asthmatic patients reacted with fractions containing a 46 KDa protein (Walsh *et al*., 1991). The enzyme enolase from *Saccharomyces cerevisiae* and *C. albicans* acts as a major allergen and such allergen detected was believed to be enolase, a 46 KDa protein (Savolainen *et al*., 1990; Ito *et al*., 1995; Nittner-Marszalska *et al*., 2001; Kustrzeba-Wojcicka and Golczak, 2000; Chang *et al*., 2002). In *S. cerevisiae* the glycolytic enzyme, enolase has been localized as a 47 kDa protein (Pardo *et al*., 2000; Kim and Park, 2004).

The humoral and cellular immune responses to enolase were evident in germ-free mice colonized with *C. albicans*. The lymphocytes from intravenously challenged mice responded to enolase as an immunodominant humoral antigen (Del Prete, 1992). Although the cytoplasmic location of enolase appeared to be established, enolase was detected by radioimmuno precipitation in cell wall extracts obtained after digestion of the wall α- glucan network with α -glucanase, zymolyase (Stec and Lebioda, 1990; Sundstrom and Aliaga, 1992; Angiolella *et al.,* 1996). The release of enolase may be important in defining the selective stimulation of the host antifungal response during infection. It has recently been reported that anti-enolase antibodies may have an immunoprotective effect in mice. Cilofungin, a lipopeptide antibiotic affecting α -1, 3-D-glucan synthesis inhibited the incorporation of a 46 to 48 KDa glucan-associated protein, a cell wall associated form of enolase (Mason *et al*., 1993).

To summarize, *C. albicans* were isolated from patients with invasive candidiasis and characterized. Suspected *C. albicans* strains were selectively isolated on Hichrome Candida Agar and identified. The organism was grown on YEPD medium and harvested. The cell pellets were suspended in protein-extraction buffer. The cells were again suspended in the breaking buffer and broken by mechanical disruption with glass beads. The homogenate was centrifuged and the supernatant containing the soluble protein fraction was passed through the membrane filter. The filtrate was subjected to extraction with chloroform. The dialysed crude enolase cytoplasmic extract was subjected to SDS – PAGE along with standard markers and the *C. albican*s enolase was confirmed as a single polypeptide band of around 48 KDa.

Further, to investigate the immunological cross reactivity of enolase with appropriate antibodies and other biochemical studies, purification of enolase is required. Such type of investigation is very much needed to study the pathology of *C. albican*s. Immunological relevance and interspecies relationship of the *Candida* enolase antigens can also be investigated.

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Table 1. Carbohydrate assimilation tests for *C. albicans*

Species	Substance	Detection
C. albicans	Dextrose	+ ve
	Maltose	+ ve
	Sucrose	+ ve
	Lactose	- ve
	Galactose	+ ve
	Melibiose	- ve
	Cellobiose	- ve
	Inositol	- ve
	Xylose	+ ve
	Reffinose	- ve
	Trehalose	+ ve
	Dulcitol	- ve

Table 2. Carbohydrate fermentation tests for C. albicans

Species	Substance	Detection
C. albicans	Dextrose	+ ve
	Maltose	+ ve
	Sucrose	- ve
	Lactose	- ve
	Galactose	+ ve
	Trehalose	+ ve

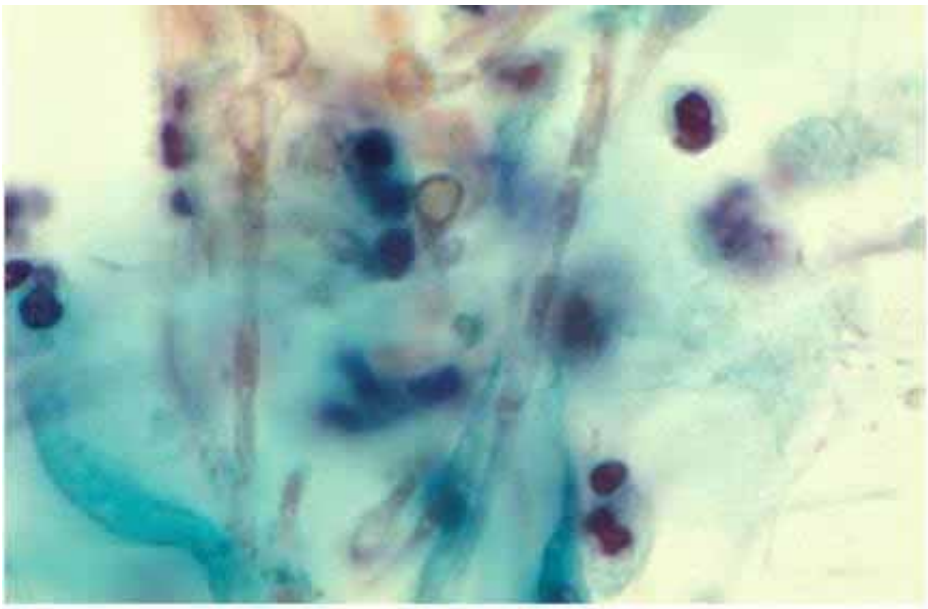

Figure 1. Budding yeast cells with pseudohyphae of C. albicans

Figure 2. Glistening green colour colonies produced by C. albicans on Hifhrome canaida agar

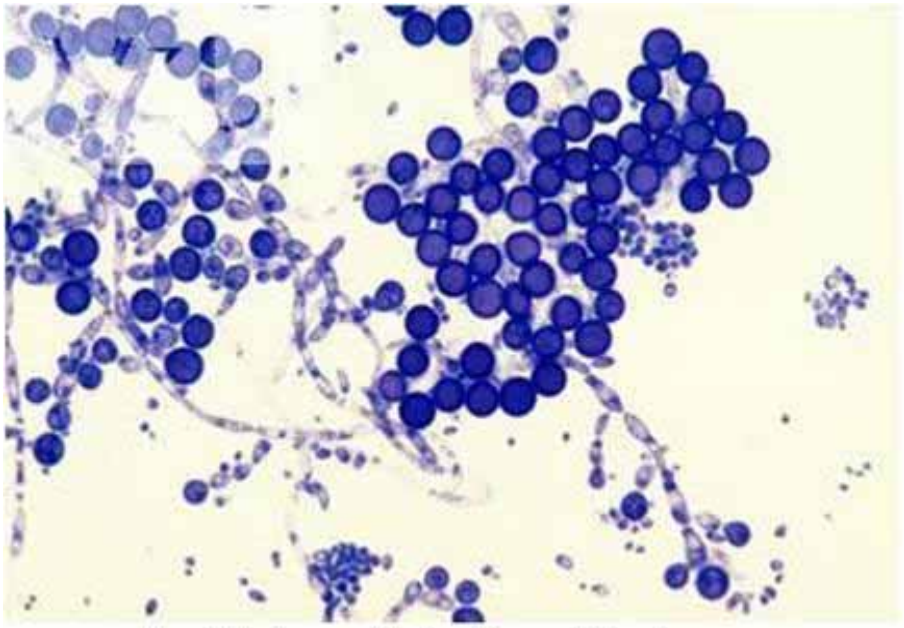

Figure 3. C. albicans with thick walled terminal chlamydospores

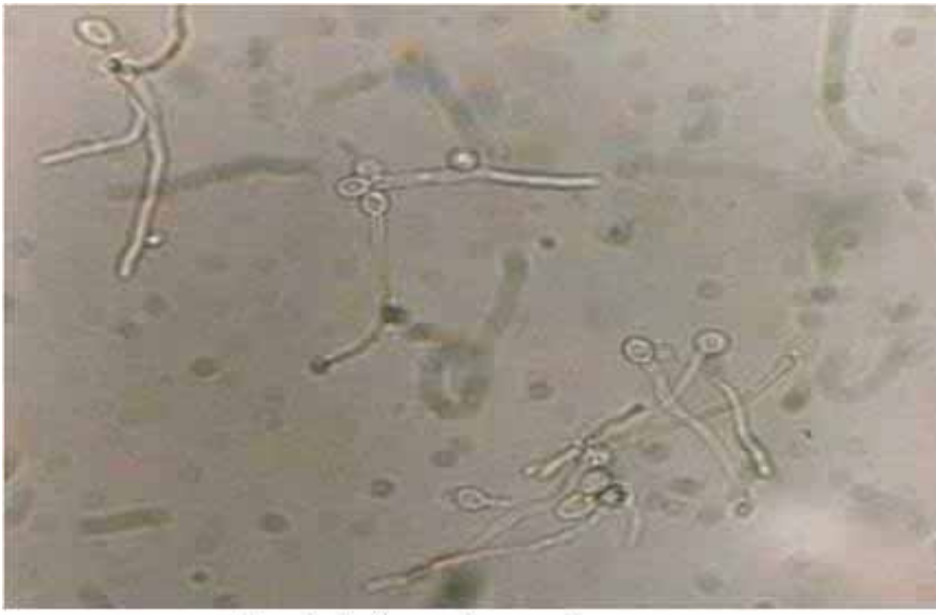

Figure 4. C. albicans with germ tubes

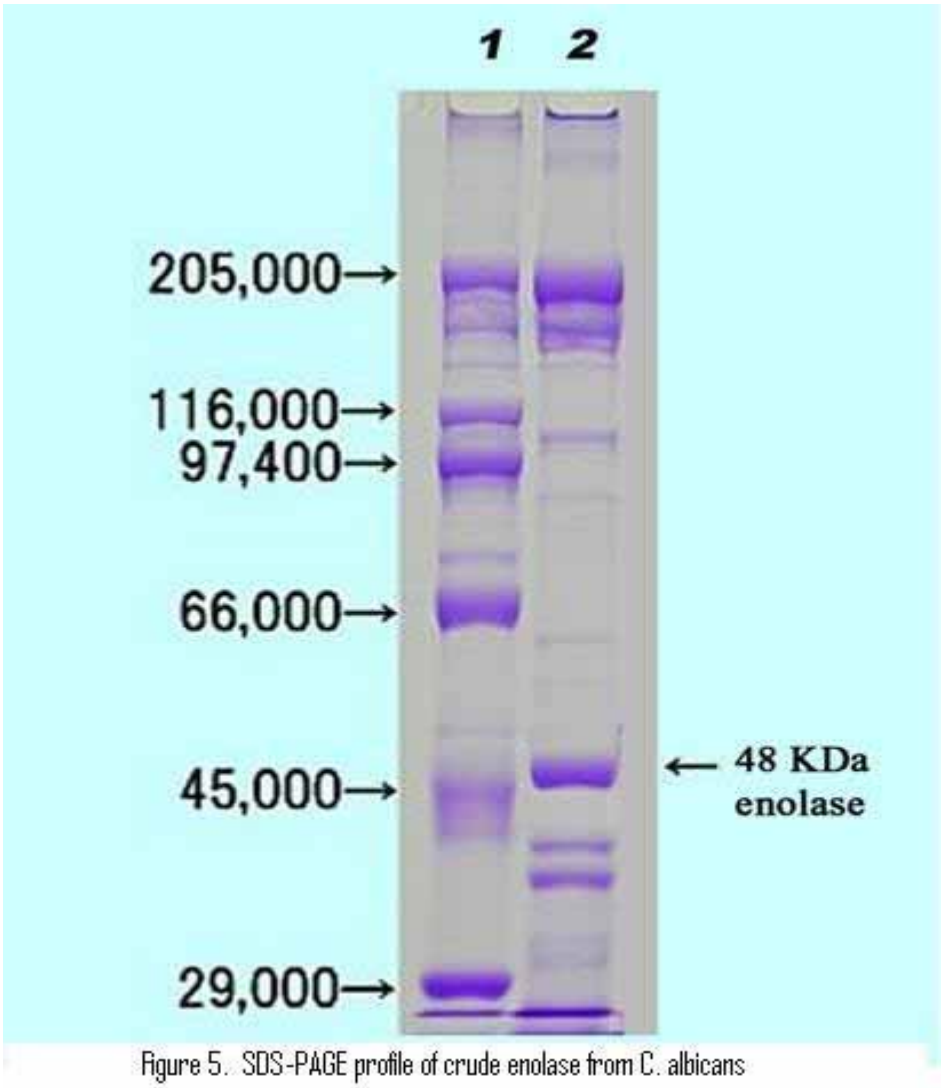

Figure 5. SDS-PAGE profile of crude enolase from C. albicans
(1, standard protein markers; 2, crude enolase sample)

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Cytoplasmic and Combining Ability Effects on Agro-Morphological Characters in Intra and Inter Crosses of Pima and Upland Cottons (*G.Hirsutum* and *G. Barbadense*)

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Abstract

Combining ability and heterosis were determined in a population obtained from the full diallel crossing of four different cotton genotypes (G. hirsutum and G. barbadense) for agro morphological traits and yield. High variation was observed for characteristics among parents and the F1 combinations. So, selection could be done for improved yield, yield components and agro morphological traits. Barbadense 5539 and Termeze14 (G. barbadense) had positive GCA for height, bolls/plant and sympodia branch/plant, Inverse, Sahel and Sepid (G. hirsutum) had negative GCA for these characteristics. G. barbadense genotypes showed negative GCA for monopodia branch/plant, sympodia branch length and boll weight, Inverse, G. hirsutm genotypes was observed positive GCA for this traits. The GCA: SCA ratios for the studied traits were higher than one indicating the presence of additive genetic effects for most of the characteristics studied, except for sympodia branch length.

Keywords: Cotton, General combining ability (GCA), Specific combining ability (SCA), Intra and Inter specific crosses, Maternal effects

1. Introduction

Cotton (Gossypium spp) the worlds' most important natural source of fiber is comprised of about 50 diploid and tetraploid species. Commercial cotton fiber is produced from only four species: two diploids G. arboretum L. (n=13, A2A2) and G. herbaceum L. (n=13, A1A1) and two tetraploids G. barbadense (n=26, AAD2D2 genome) and G. hirsutum L. (n=26, AAD1D1 genome (Vafaie-tabar et al. 2004).

Upland cotton, *G. hirsutum*, dominates the worlds' cotton fiber production, representing 90% of the production. Compared with the upland cotton, the second most cultivated species, *G. barbadense*, has superior fiber length, strength, and fineness. *G. hirsutum* varieties, however, are usually early- maturing and higher yielding (Lacape et al.2005).

Upland cotton (*Gossypium hirsutum* L) is the most extensively cultivated of the four cultivated Gossypium species, and, as such, it has been the target of numerous genetic studies and breeding efforts. The level of genetic diversity is low in *G. hirsutum*, especially among agriculturally elite types, as revealed by all means of assessment (Gutiefrrez et al. 2002; Ulloa and Meredith 2000; wendel et al. 1989).

Gossypium barbadense (L.) is the only 52- chromosome relative of upland cotton (*G. hirsutum*, 2n=52) that is cultivated. It is valued for its fiber length and quality, whereas upland cotton is more valuated for its high yield (Saha et al. 2006).

Most genetic traits useful for cotton improvement are influenced by several genes. These are called quantitatively inherited traits (Shappley et al., 1998). The identification and characterization of genes controlling traits of use in plant improvement has long been a focus of scientists in the agricultural community. Cotton is the most important textile fiber crop and the worlds' second-most important oil-seed crop after soybean (Poehlman and Sleper, 1995). It is grown commercially in the temperate and tropical region of more than 50 countries, including the United States, India, China, central and South America, The Middle East, and Australia (Fryxell, 1979; Smith, 1999).

Increasing diversity is therefore essential to genetic improvement efforts. Each of the three major approaches to increasing genetic diversity (mutagenesis, germplasm introgression, and transformation) has advantages and disadvantages. Interspecific germplasm introgression is particularly attractive in that it utilizes abroad germplasm base, can be targeted to one or more specific traits or genes or modulated to include thousands of genes or even entire genomes. However, the biological and technical challenges of introgression increase as the phyletic distance between the donor and recipient genome increases (Saha et al. 2006).

Diallel crossing technique in cotton has been used by cotton breeders. Baloch et al. (1995) revealed the importance of specific combining ability for yield, 100- seed weight and lint percentage, and general combining ability for boll number per plant and lint percentage. Wilson (1991): Tang et al.(1993) and Nadeem et al. (1998) reported significant general and specific combining ability effects for lint yield, lint percentage, seed cotton weight per boll and boll number per plant.

Hybrid vigor in cotton has been observed in interspecific crosses as well as in crosses between varieties within the species. Fryxell et al. (1958), Hutchinson et al. (1938), Marani (1967), Stroman (1961), and Ware (1931) in particular showed that crosses between *G. barbadense* and *G. hirsutum* were much more productive than either parent. Because of the differences in the characteristics of the lint of the two species, it frequently has objectionable qualities in the hybrid. This problem is less likely to arise in intraspecific hybrids where considerable hybrid vigor has also been shown.

In a molecular analysis of *G. hirsutum* introgression into Pima (*G. barbadense*) cotton, 7.3% of the alleles of Pima cultivars were found to be derived from *G. hirsutum* (Wang et al., 1995). These alleles were not randomly distributed within the *G. barbadense* genome, since nearly 60% of the total introgression was found within five specific chromosomal regions accounting for less than 10% of the genome.

Transfer of reniform nematode resistance from *G. Longicalyx* into *G. hirsutum* requires introgression of genes from the unique diploid F genome of *G. Longicaly*x into either the A or D sub genome of allotetraploid *G. hirsutum*. As bridges, two synthetic tetraploid triple species hybrids, referred to as HLA and HHL, were developed (Bell and Robinson, 2004). Robinson et al. (2007) was studied two trispecies hybrids of *G. hirsutum*, *G. longicaly*x and either *G. armourianum Kearney* or *G. herbaceum* L. to introgress high resistance to the nematode from *G. Longicaly*x into *G. hirsutum*. Introgression was pursued from 28 resistant BC1 plants, each of which was backcrossed four to seven times to G. hirsutum to derive agronomically suitable types.

The objective of this study was to estimate parental general combining ability effects, to compare performance among F1 hybrids, and to identify those superior for yield and agro morphological traits.

2. Material and Method

During the summer of 2005, four different cotton genotypes, Thermez 14 (line 2) and barbadense 5539 (line 18) of G. barbadense species, Sepid (line 22) and Sahel (line 13) of G. hirsutum species, were crossed to produce F1 seeds. These genotypes display a continuous spectrum of morphological traits between the two parental species (Ulloa et al., 2000). Parents and F1 seed was planted in the spring of 2006. Four parents and 12 hybrids were planted in randomized complete blocks. Plots consisted of four, 10m- long row with 80 cm apart. Soil was a silt- loam. Plant density was about 33000 plants ha-1. Weed control, irrigation, and insect control were standard practices for production of cotton in the Hashemabad cotton research station, Gorgan, Iran. Boll weight was determined from 20 hand-harvested bolls, just prior to first harvest from each plot. Seed cotton yield was determined by hand harvest. Plant height was measured from ground level to the top of the plant at harvest time. Monopodia and sympodia branch length, monopodia and sympodia branches/plant was measured prior to first harvest.

The data for each measurement was tabulated and analyzed by Fisher's analysis of variance. The diallel analysis was

used to evaluate traits that had significant variation among the parents. Griffing-type diallel analysis was applied to estimate the GCA and SCA effects.

3. Results

Preliminary analysis of variance indicated that parents and their hybrids were significantly different from each other for all investigated traits in the study, which enable the diallel analysis to be run (Table 1).

3.1 Female parents different

The four parents used in this study varied significantly for each components and agro morphological traits except for sympodia and monopodia branch length (Table 2). Termeze 14 had the highest values in height characters. About boll/plant, barbadense 5539 and Termeze 14 had the highest value. The maximum monopodia branch number/plant was for Sahel and Sepid cultivars. Termeze 14 and barbadense 5539 had the highest values in sympodia branch/plant. The monopodia and sympodia branch length were not significantly difference in female parents. Sahel and Sepid (G. *hirsutum* spesies) had the weightiest boll. These, also, had the highest values for yield.

3.2 Male parent's difference

The genotypes used for male parents were significantly different for each yield components and agro morphological traits except for boll/plant and boll weight characters. Barbadense 5539 had the highest value for height. *G. hirsutum* species, Sahel and Sepid, had the maximum monopodia branch/ plant and minimum sympodia branch/plant. The highest sympodia branch length was for Sahel cultivar. Barbadense 5539 had the lowest monopodia branch length. Sepid had the highest value for seed cotton yield.

3.3 F1 performance

The data in Table 3 were significantly difference for each yield components and agro morphological traits for F1 hybrids used in this study. Sahel×barbadense 5539 hybrid had the highest value for height (170 Cm). Sahel×Sahel had the lowest height (114 Cm). Bolls/plant were significantly different among the genotypes, where barbadense 5539×Sahel (37.5), barbadense 5539×Termeze 14 (38.3), Termeze 14×barbadense 5539 (35.4) and Termeze 14×Termeze 14 (35.1) crosses had many bolls, while Sahel×Sepid (19.13) hybrid had significantly lower numbers of boll/plant. Among the crosses, Sepid×Sepid had the highest values for monopodia branch number/plant, also, Sahel×barbadense 5539 (20.1), barbadense 5539×barbadense5539 (19.8), barbadense 5539×Termeze 14 (19.7) and Termeze 14×barbadense 5539 (20.1) crosses had the highest sympodia branch number/plant. Sympodia branch length in Termeze 14*Sahel crosses was the highest (62.9 Cm). Sahel×Termeze 14 (98.5 Cm) and Termeze 14×Sahel (102.7 Cm) had the highest value for monopodia branch length. Sahel×Sepid (128.0 gr) and Sepid×Sahel (133.1 gr) crosses had the weightiest boll and Termeze 14×Sepid crosses had the lowest boll. Sepid×Sahel crosses had the highest yield/plot (4977 gr) and Termeze 14×Sahel had the lowest yield/plot (2195 gr).

3.4 Agro morphological GCA and SCA

Analysis of variance for genotypes indicated the presence of significant differences among genotypes (Table 4). Combining ability mean squares for the characteristics are presented in Table 4. Significant GCA mean squares for yield components, height, bolls/plant, monopodia branches/plant, sympodia branches/plant, monopodia and sympodia branch length, boll weight and seed cotton yield indicated that additive genes controlled most of the characteristics. GCA mean square values were higher compared to the mean squares for SCA except for sympodia branch length (Table 4).

Results for GCA effects are given in Table 6. Sahel had negative GCA effects for height, bolls/plant, sympodia branches/plant and yield. Sahel, known to have big bolls, had positive and significant GCA effects for boll weight, monopodia branches/plant, sympodia and monopodia branch length. GCA effects for barbadense 5539 on height, bolls/plant and sympodia branches/plant were positive. GCA effects for sympodia branches/plant, sympodia and monopodia branch length, boll weight and yield were negative at Hashemabad research station (Table 6). The GCA effect for Sepid cultivar was positive for monopodia branches/plant, sympodia and monopodia branch length, boll weight and yield. Negative GCA effects were observed for bolls/plant, height, sympodia branches/plant. Positive GCA effects were shown by Termeze 14 genotype for height, bolls/plant, sympodia branches/plant and monopodia branch length while the values for monopodia branches/plant, sympodia branch length, boll weight and yield were negative.

SCA effect estimates for height, bolls/plant, sympodia branches/plant, monopodia branches/plant, sympodia branch length, monopodia branch length, boll weight and yield are presented in Table 7. SCA effects for some characteristics indicated variation among F1 hybrids. For height, three combinations had positive SCA effects. Four combinations had positive SCA effect for bolls/plant, sympodia branches/plant, sympodia and monopodia branch length. For monopodia branches/plant, five combinations had positive SCA effects. Sahel×Sahel, Sahel×Sepid, barbadense 5539×barbadense 5539, barbadense 5539×Termeze14 and Termeze14×Termeze14 had positive SCA effects for boll weight. Sahel×Sahel, Sahel×barbadense5539, Sahel×Sepid, Sahel×Termeze14, barbadense5539*barbadense5539, barbadense5539×Sepid had the positive SCA effects for seed cotton yield (Table 7).

3.5 Cytoplasmic effects

Results for cytoplasmic effects are given in Table 8. Sahel×barbadense 5539 and barbadense 5539×Sepid combinations had positive effect. For height, positive maternal effect was showed at barbadense5539×Sepid crosses. Barbadense5539×Termeze 14 combinations had positive maternal effect for bolls/plant. Maternal effect was positive for monopodia branches/plant at barbadense5539×Termeze14 crosses. About sympodia branches/plant Sahel×barbadense5539, barbadense5539×Sepid and Sepid×Termeze14 had positive maternal effects. Positive cytoplasmic effects were observed for sympodia branch length at barbadense5539×Sepid crosses. The observed maternal effect for boll weight, Sahel×Termeze14 and barbadense5539×Termeze14 combinations were positive. For seed cotton yield, Sahel×barbadense5539 and Sahel×Termeze14 had the positive cytoplasmic effect (Table 8).

3.6 Heterosis estimates

Heterosis values for the combination varied from negative to positive (Table 5). Height heterosis was positive for all of the combinations. Sahel×Termeze14 had high positive heterosis. Heterosis values for bolls/plant were positive for most of the combinations except for Sahel×Sepid and Sahel×Termeze14, and Sahel×barbadense5539 combination had the highest heterosis (6.83). The heterosis value for monopodia branches/plant for all combinations except for Sahel×Termeze14 was negative. Heterosis estimates recorded on combinations for sympodia branches/plant varied from negative to positive. Sahel×barbadense 5539 had the highest heterosis value, Also Sahel×Termeze14 had the highest heterosis value for sympodia and monopodia branch length, When *G. hirsutum* and *G. barbadense* species were crossed, heterosis was positive. In this study, Sahel×Sepid had the maximum heterosis for boll weight. Heterosis values for seed cotton yield were positive for most of the combinations, except for Sahel×Termeze14 and Sahel×barbadense5539. The high heterosis value were obtained in Sahel×Sepid (345.83, G. *hirsutum*×*G. hirsutum*) and Sepid×Termzeze14 (758.33, *G. hirsutum*×*G. barbadense*).

4. Discussion

High variation was observed for characteristics among parents and the F1 combinations. So, selection could be done for improved yield, yield components and agro morphological traits. GCA values obtained for Sahel, barbadense5539, Sepid and Termeze14 indicated the possibility of good combining from these parents for the some traits. Barbadense5539 and Termzeze14 (*G. barbadense*) had positive GCA for height, bolls/plant and sympodia branches/plant, inverse Sahel and sepid (*G. hirsutum*) had negative GCA for these characteristics. *G. barbadense* genotypes were showed negative GCA effect for monopodia branches/plant, sympodia branch length and boll weight, inverse *G. hirsutum* genotypes were observed positive GCA for these traits. Positive GCA for yield was observed only in Sepid. Positive SCA effects observed for same crosses. Sahel×barbadense5539 crosses had the highest positive SCA for bolls/plant. Highest positive SCA was observed for boll weight in Sahel×Sepid. In Sahel×Sahel, highest positive SCA was showed for yield.

Significant SCA mean squares observed for boll weight was reported by Echekwu and Alaba (1995). The performance of some combinations indicated the possibility of improvement of these traits. Griffing (1956) and Machado et al. (2002) reported that crosses with high SCA values from parents with highest SCA in a population should be efficient in selection in segregation population. The high and significant positive GCA were observed in crosses for seed cotton yield, lint yield, seed/boll, bolls/plant and boll weight (Lukonge, 2005).

The GCA:SCA ratios for the studied traits were higher than one indicating the presence of additive genetic effects for most of the characteristics studied except for sympodia branch length. According to Ashraf and Ahmad (2000), high additive genetic variation for these characteristics suggested a possibility of improvement in these characteristics. Therefore normal recurrent selection would be required to accumulate the additive genes in order to increase seed cotton yield (Lukonge, 2005).

Positive heterosis for height was observed for all of combinations. Positive heterosis for boll weight was showed in Sahel×Sepid, barbadense5539×Sepid and Sepid×Termeze14. Sambamurthy et al. (1995) reported that in tetraploid cotton, boll weight and boll number for intraspecific hybrids are the major components of heterosis in yield and this usually observed in *G. hirsutum* crosses and not for *G. barbadense*. The highest positive heterosis for yield was observed in Sepid×Termeze14 and Sahel×Sepid. Xian et al. (1995) and Zhang and Zhang (1997) reported high heterosis for seed cotton and lint yield. Sahel×barbadense5539 had the highest reciprocal effects for height and yield. For bolls/plant, barbadense5539×Sepid had the high positive reciprocal effects. High positive reciprocal effects for boll weight was showed in Sahel×Termeze14.

Gossypium hirsutum and *G. barbadense* differ significantly in their agronomic and fiber traits (Percey et al, 2006).*G. hirsutum* had the higher yield potential and *G. barbadense* had the best fiber quality. Interspecific hybridization and introgression, *G. hirsutum* and *G. barbadense*, has led to improve lines with the higher yield and the best fiber qualities. Efforts to improve *G. hirsutum* or *G. barbadense* through introgression have been hindered by genetic breakdown in segregating interspecific breeding populations (Stephens, 1949). Genetically stable lines have been developed after

multiple cycles of breeding and selection (Tatineni et al., 1996; Cantrell and Davis, 1993). We consider commercial interspecific hybrids (F1) to cultivate in cotton farms in different region of Iran. We hope to improve and develop stable lines of interspecific crosses (*Gossypium hirsutum* and *G. barbadense*) and intraspecific crosses (*G. hirsutum* × *G. hirsutum*, and *G. barbadense* × *G. barbadense*) after lengthy cycles of selection.

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Table 1. Mean squares of yield components and agro morphological traits

source	Degrees of freedom	height	Bolls /plant	Monopodia Branches/ plant	Sympodia branches/ plant	Sympodia branch length	Monopodia branch length	Boll weight	yield
Replication	2	347.781 ns	16.101 ns	1.061ns	0.503ns	83.341ns	1595.236ns	780.667 ns	1964756.438**
Female	3	1028.921**	447.130**	3.096*	19.683**	8.430ns	618.349ns	4979.304**	3736243.583**
Male	3	941.856**	44.601 ns	15.149**	24.987**	126.701*	3207.435**	882.517 ns	634808.694ns
Female* male	9	712.598**	31.606 ns	0.511ns	4.242ns	881.942**	1079.927ns	1933.988**	1779542.713**
Error	30	184.258	29.541	0.739	3.242	28.202	537.399	507.764	250667.638

**: significant at 0.01 level, *: significant at 0.05 level and ns: non significant

Table 2. Means of yield components and agro morphological traits for female and male parents

	height	Bolls/plant	Monopodia Branches/plant	Sympodia branches/plant	Sympodia branch length	Monopodia branch length	Boll weight	yield
Female								
Sahel	137.08bc	24.33b	2.417a	16.42b	43.37a	69.98a	90.04a	3482b
Barbadense5539	147.28ab	35.73a	1.433b	19.08a	41.92a	58.28a	58.64b	3215b
Sepid	129.97c	24.38b	2.583a	16.70b	41.40a	74.20a	99.25a	4359a
Termeze 14	149.98a	33.98a	2.100ab	18.32a	42.03a	72.47a	61.41b	3147b
Male								
Sahel	136.25b	28.32a	3.000a	16.47b	46.37a	78.98a	85.29a	3497ab
Barbadense5539	153.37a	31.00a	0.817	19.37a	39.53b	45.08b	65.41a	3498ab
Sepid	133.27b	27.62a	3.133a	16.42b	43.13ab	80.32a	81.22a	3876a
Termeze 14	141.43b	31.50a	1.583b	18.27a	39.68b	70.55a	77.42a	3333b

* Means within columns followed by the same letter(s) are not different at 0.05 probability level

Table 3. Means of yield components and agro morphological traits for hybrid combinations

	height	Bolls/plant	Monopodia Branches/plant	Sympodia branches/plant	Sympodia branch length	Monopodial branch length	Boll weight	yield
Sahel×Sahel	114.0g	22.5cde	2.933abc	14.7cd	32.3d	67.0abcde	101.2ab	4440ab
Sahel× Barbadense5539	170.6a	31.5abcd	0.933defg	20.1a	53.2abc	51.7bcde	58.6bcd	2960efgh
Sahel× Sepid	115.1fg	19.1e	3.400ab	13.8d	31.8d	62.7abcde	128.0a	4205abc
Sahel× Termeze14	148.7abcd	24.1bcde	2.400abcd	17.1abcd	56.1abc	98.5a	72.3bcd	2325gh
Barbadense5539× Sahel	144.5abcde	37.5a	2.267bcde	18.4ab	57.6ab	65.0abcde	45.5cd	2378fgh
Barbadense5539× Barbadense5539	151.9abcd	32.9abc	0.733efg	19.8a	25.3d	44.7cde	43.0cd	3260cdef
Barbadense5539× Sepid	146.2abcd	34.3ab	2.133bcdef	18.4ab	59.9ab	85.7abc	65.2bcd	3630bcde
Barbadense5539× Termeze14	146.5abcd	38.3a	0.600fg	19.7a	24.9d	37.7de	80.9bc	3592bcde
Sepid× Sahel	125.7defg	22.3de	3.533ab	15.5bcd	32.7d	81.2abcd	133.1a	4977a
Sepid× Barbadense5539	133.8cdefg	24.2bcde	1.133defg	17.5abc	52.8bc	58.5abcde	80.8bc	4132abc
Sepid×Sepid	120.3efg	22.6cde	4.000a	15.3bcd	33.3d	81.5abcd	97.8ab	4050abcd
Sepid×Termeze14	140.0bcdef	28.5abcde	1.667cdefg	18.5ab	46.9c	75.6abcd	85.3bc	4278ab
Termeze14× Sahel	160.7ab	30.9abcd	3.267abc	17.3abc	62.9a	102.7a	61.4bcd	2195h
Termeze14× Barbadense5539	157.2abc	35.4a	0.467g	20.1ab	26.9d	25.5e	79.2bc	3638bcde
Termeze14× Sepid	151.5abcd	34.5ab	3.000abc	18.2ab	47.6c	91.3ab	33.9d	3618bcde
Termeze14×Termeze14	130.5defg	35.1a	1.667cdefg	17.7abc	30.8d	70.4abcd	71.2bcd	3137defg

* Means within columns followed by the same letter(s) are not different at 0.05 probability level

Table 4. Mean squares for yield and agro morphological GCA, SCA and GCA: SCA ratio for cotton genotypes

source	d.f.	height	Bolls/plant	Monopodia Branches /plant	Sympodia branches/plant	Sympodia branch length	Monopodia branch length	Boll weight	yield
Rep	2	347.781ns	16.101ns	1.061ns	0.466ns	83.341ns	1595.236ns	71.183ns	2.956e+006**
genotypes	15	821.714**	117.310**	3.956**	11.464**	556.192**	1413.113*	1451.711**	1.918e+006**
GCA	3	1727.389**	381.946**	15.664**	44.465**	88.716*	3233.130**	3469.292**	3.663e+006**
SCA	6	856.122**	42.899ns	0.721ns	4.802ns	1316.205**	1497.564*	1778.966**	2.726e+006**
Recip	6	334.469ns	59.403ns	1.336ns	1.627ns	29.916ns	418.653**	115.666ns	238401.389ns
Error	30	184.258	29.541	0.739	3.373	28.202	537.399	220.758	241021.840
GCA: SCA		2.02	8.9	21.7	9.2	0.07	2.2	2	1.3

GCA= General combining ability, SCA= Specific combining ability, df= degree of freedom

Table 5. Mean mid-parent heterosis for yield and agro morphological traits

Female	male	height	Bolls /plant	Monopodia Branches /plant	Sympodia Branches /plant	Sympodia branch length	Monopodia branch length	Boll weight	yield
Sahel	Barbadense 5539	24.63	6.83	-0.23	2.0	26.60	2.53	-23.17	-1207.50
Sahel	Sepid	3.23	-1.87	0	-0.33	-0.57	-2.30	31.07	345.83
Sahel	Termeze14	32.43	-1.30	0.53	1.03	27.93	31.90	-19.37	-1528.33
Barbadense 5539	Sepid	3.90	1.50	-0.73	0.43	27.07	9.00	-10.65	36.67
Barbadense 5539	Termeze14	10.67	2.87	-0.67	1.17	-2.13	-25.93	9.72	227.50
Sepid	Termeze14	20.30	2.60	-0.50	1.87	15.20	7.47	2.92	758.33

Table 6. General combining ability (GCA) effects of yield and agro morphological traits

Genotypes	height	Bolls/plant	Monopodia Branches/plant	Sympodia branches/plant	Sympodia branch length	Monopodia branch length	Boll weight	yield
Sahel	-4.41	-3.28	0.58	-1.19	2.69	5.75	6.47	-114.69
Barbadense5539	9.25	3.76	-1.01	1.60	-1.45	-17.05	-12.52	-153.85
Sepid	-9.46	-3.61	0.72	-1.07	0.09	8.52	13.44	573.02
Termeze 14	4.63	3.1	-0.29	0.65	-1.32	2.78	-7.38	-304.48

Table 7. Specific combining ability (SCA) effects for yield and agro morphological traits

Female	Male	height	Bolls/plant	Monopodia Branches/plant	Sympodia branches/plant	Sympodia branch length	Monopodia branch length	Boll weight	yield
Sahel	Sahel	-18.25	-0.51	-0.35	-0.58	-15.22	-13.23	4.55	1024.06
Sahel	Barbadense5539	11.65	4.45	-0.10	1.20	11.99	0.93	-15.46	487.19
Sahel	Sepid	-6.80	-2.02	0.03	-0.73	-12.72	-11.04	26.91	437.81
Sahel	Termeze 14	13.40	-1.92	0.42	0.11	15.95	23.34	-16.00	428.02
Barbadense5539	Barbadense5539	-7.70	-4.26	0.62	-1.03	-14.00	10.03	10.87	300.73
Barbadense5539	Sepid	-0.86	-0.52	-0.22	-0.19	15.52	11.89	-11.64	100.31
Barbadense5539	Termeze 14	-3.09	0.33	-0.30	0.02	-13.50	-22.86	16.24	-183.64
Sepid	Sepid	-1.82	0.21	0.42	-0.21	-9.09	-4.25	-12.85	-545.10
Sepid	Termeze 14	9.49	2.33	-0.23	1.13	6.29	3.40	-2.42	-741.35
Termeze 14	Termeze 14	-19.80	-0.74	0.12	-1.26	-8.74	-3.88	2.18	-966.14

Table 8. Reciprocal effects for yield and agro morphological traits

Female	male	height	Bolls /plant	Monopodia Branches /plant	Sympodia branches /plant	Sympodia branch length	Monopodia branch length	Boll weight	yield
Sahel	Barbadense5539	13.03	-3.00	-0.67	0.83	-2.20	-6.63	-3.63	128.33
Sahel	Sepid	-5.33	-1.6	-0.07	-0.83	-0.43	-9.23	-2.53	-385.85
Sahel	Termeze 14	-6.03	-3.40	-0.43	-0.07	-3.37	-2.13	5.47	65.00
Barbadense5539	Sepid	6.20	5.03	0.50	0.43	3.53	13.63	-7.82	-250.83
Barbadense5539	Termeze 14	-5.33	1.43	0.07	-0.17	-0.97	6.13	0.85	-23.33
Sepid	Termeze 14	-5.73	-3.00	-0.67	0.13	-0.37	-7.83	-2.08	-73.33

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DNA Damage in Workers Occupationally Exposed to Lead, Using Comet Assay

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Abstract

Lead is one of the most clinically important heavy metals, because it induces a broad range of physiological, biochemical, and genetical dysfunctions. Occupational exposure to lead leads to increased frequency of genetic system in human beings. In the present study the DNA damage among the workers occupationally exposed to lead was analyzed using peripheral blood lymphocyte by comet assay. The result of this study shows increased levels of DNA damage among experimental subjects. The habit of cigarette smoking among the workers has a synergistic effect on inducing DNA damage.

Keywords: Lead exposure, Comet assay, DNA damage, smoking

1. Introduction

Mutagenesis is involved in the pathogenesis of many neoplasias. Occupational exposure may contribute to the development of pernicious illnesses, many times through mechanisms that involve genetical changes. In order to evaluate the possible impact of environmental and occupational exposition on health, it is essential to identify the effects of exposure through epidemiological studies.

Continuous efforts have been made to identify genotoxic agents, to determine conditions of harmful exposition and to monitor populations that are excessively exposed (Maluf and Erdtmann, 2000).

Lead (Pb) is an abundant, globally well-distributed, dangerous and important environmental chemical. Pb has been found to be capable of eliciting a positive response in an extraordinarily wide range of biological and biochemical test, which include the tests for enzyme inhibition, fidelity of DNA synthesis, mutation dislocation, chromosome aberrations, cancer and birth defects (Johnson, 1998). Determination of blood lead concentration is a widely accepted biological marker of exposure to lead (Lanphear et al., 2000; Mielzyn´ska et al., 2001). Also the cytogenetic studies in workers occupationally exposed to Pb produced inconsistent results (Johnson, 1998). Recently, the results of some in vitro and in vivo tests detecting the genotoxic effects of Pb have been reported (Vaglenov et al., 2001). In other in vivo study, significant increase in mean tail length of DNA was observed at all time intervals after treatment with Pb nitrate when compared to control (Devi et al., 2000).

During the last few years, there has been a great interest in developing, rapid and simple tests to identify the effects of exposure to environmental agents that can affect the health of individuals due to DNA damage. In this observe, present study considered whether individuals working with Pb exposure more DNA damage with the increase in the exposure period and smoking habits using by the Comet assay also known as single cell gel electrophoresis (SCGE) and it is a very sensitive method for measuring DNA damage (Singh et al., 1988).

Therefore the focal aim of present study was DNA damage in peripheral blood lymphocytes it can be used as a biomarker of health outcome, measuring genetic damage due to exposure that results from non-repaired primary lesions in Pb exposure. Although Pb exposure has been extensively investigated, to the greatest of our knowledge, this is the first to perform a Genetic study in this region.

2. Material and methods

2.1 Demographic profile of selected area

Coimbatore, the Manchester of South India is located in the western region of Tamilnadu, bordered by the panaromic Western Ghats. The total population of the Coimbatore district is 42.25 lakhs (21, 56,280 males and 20, 67,817 females).

Due to the existence and predominance of population the silent toll of human lives due to numerous diseases is on the rise.

2.2 Subject Recruitment and Sample Collection

The study was conducted on 30 male (83.33%) and female (16.67%) workers aged 20–50 (mean age±SD: 33±6.51) years who had been employed in the recycling and manufacture of automotive batteries in surroundings of coimbatore city (Tamilnadu). Of these workers, 14 (46.67) were smokers (no more than 20 cigarettes/day) and 16 (53.33) were non-smokers. The control groups consisted of 30 healthy male (83.33%) and female (16.67%) aged 20-50 (32.9±6.37) years with no history of exposure to clastogenic and/or aneugenic agents and of socioeconomic level also similar to that of the subjects exposed to lead. Blood lead level also estimated in both control and experimental subjects.

At the time of blood collection (3 ml/individual) the workers signed a term of informed consent and replied to a questionnaire elaborated to determine the profile and habits (age, time of work, salary, type of diet more commonly used, type of housing, use of drugs and medications, etc.) of the study population. The study procedures used in the present study were approved by the Institutional ethical committee.

2.3 Sample collections

Peripheral blood samples (V = 5 ml) were collected under sterile conditions by venipuncture into heparinized tubes for comet assay (Singh, 1988).

Blood samples were collected into sodium–heparin Vacuette tubes for setting up vacutainers for determining the concentration of lead in the blood.

2.4 Lead in blood (PbB) determination

The levels of lead in whole blood were determined by electrothermal atomic absorption spectrophotometry according to Stoeppler and Brandt (1978). Vortex mixed blood (200 µl) was added to 800 µl of 5% HNO3 in a pre-cleaned 2.2-ml Eppendorf tube. The mixture was vortexed and left for 24 h in the refrigerator for better deproteinization. After centrifuging at 10000 rpm for 15 min, the supernatant was transferred to the Perkin-Elmer polystyrene autosampler cups. Then, 20 µl of the solution was automatically injected into the pyro-coated graphite tube with a L'vov platform. Lead in the sample was vapourized at the optimized sequential dry-atomize transverse-heated graphite atomizer furnace programme developed in the laboratory. The atomic absorption signal of lead was measured in the absorbance-peak area mode using the Zeeman effect for background correction (Perkin-Elmer 4100ZL). The amount of lead in the blood samples was calculated by reference to matrix-matched calibration plots.

2.5 Single cell gel electrophoresis (SCGE) assay

The comet assay was conducted under alkali conditions according to Singh et al. (1988). All chemicals were obtained by Sigma. Two microlitre of whole blood were suspended in 0.5% low melting agarose and sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel-layer. After the solidification of 0.6% agarose layer the slides were immersed in lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and DMSO 10%) at 4 °C. After 1 hr, the slides were placed in the electrophoresis buffer (0.3 M NaOH, 1 mM Na2EDTA, pH 10) for 20 min at room temperature to allow for DNA to unwind. The buffers were then chilled and the electrophoresis was performed at 300 mA and 19 V in a horizontal electrophoresis platform for 20 min. The slides were neutralized with Tris–HCl buffer (pH 7.5) and stained with 10% ethidium–bromide for 10 min. Each slide was analyzed by using Leitz Orthoplan epifluorescence microscope. For each subject 50 cells were analyzed by automatic digital analysis system Comet assay II (Perceptive Instruments Ltd., Suffolk, Halstead, UK), determining tail length and tail moment (tail length×tail % DNA/100). DNA damage was further quantified by visual classification of cells into categories of 'comets' corresponding to the amount of DNA in the tail according to Anderson et al. (1994).

3. Statistical analysis

All calculations were performed using MINITAB RELEASE II Software package for windows. Mean values and standard deviations (S.D.) were computed for the scores and the statistical significance (P/0.05) of effects (smoking) was determined using analysis of variance (ANOVA). Simple linear regression analyses were performed to assess the association between endpoints and independent variables.

4. Results and discussion

The effect of occupational exposure to Pb, on the level of DNA damage in leucocytes of study group was assessed by the comet assay. A total 60 subjects corresponding to 30 experimental, and 30 controls were recruited for this study. With regard to serum Pb levels, experimental subjects showed maximum value of 5.28±0.25, and control value was recorded as 3.28± 0.64 respectively.

Table 1 and 2 represents the age, the number of smokers and years of exposure between the two groups involved in this study. The mean age of the control subjects was 32.9±6.37 and that of experimental was 33±6.51. The exposed groups displayed significantly higher levels of DNA damage than controls. The ranges of the MTL (mean tail length) and the MTM (mean tail moment) were 2.22 ±0.47 µm and 0.76±0.26 in workers, respectively, while the MTL and the MTM were 0.91±0.54 µm and 0.31± 0.45in controls, respectively. There was significant difference between workers and controls for MTL and MTM ($P < 0.01$).

Smoking exposure the lymphocytes of the exposed workers expressed higher DNA migration than of controls. The smokers had MTM (0.71±0.27 and 2.33±0.29) and MTL than the non-smokers (0.70±0.26 and 2.25:0.34). Data shows a significant effect of Usage on MTL and MTM in the smokers. A significant increase of MTL and MTM in smokers was observed in the exposed workers. A clear and statistically significant increase in DNA migration was found in the study group when compared with the control groups as analyzed by ANOVA. Among the study group, significantly more were observed, the presence of cells demonstrating greater DNA damage than among control subjects

The present study indicates that workers occupationally exposed to Pb, in the particular conditions of exposure of this collective evaluated, show clear evidence of genotoxic activity in their lymphocytes. These workers were not apparently exposed to other suspicious genotoxic agents at the workplace. Danadevi et al. (2003) detected a significant increase in DNA breaks in workers exposed to Pb, as determined by the comet assay.

Although, environmental exposure to Pb has decreased considerably in countries that have banned leaded product, it is still a major environmental health problem in many countries that continue to use Pb, or where people are exposed to deteriorated residential lead-based paint or Pb glazed ceramics used for storage and preparation of food (WHO, 1995). Lead readily passes the placenta, and the central nervous system is particularly sensitive during development (Lidsky and Schneider, 2003).

Pb is known to be a toxin affecting both the nervous and haematopoietic systems (Goyer, 1993). Its genotoxic potential has also been shown, although exact mechanisms are not explained (IARC 1980). Pb and inorganic Pb compounds are classified by International Agency for Research on Cancer in group 2B as possibly carcinogenic to humans. Our research revealed increased levels of Pb in blood concentration in the exposed group compared to the controls. Although, some studies indicated that gastrointestinal Pb absorption is enhanced at low body iron stores (Goyer, 1993), several recent studies speak against a common transport mechanism for iron and Pb (Bannon et al., 2003; Barany et al., 2005).

To our knowledge, there are three polymorphic genes identified that potentially can influence accumulation and toxicokinetics of Pb in humans. (Onalaja and Claudio, 2000).

The workers occupationally exposed to Pb were monitored in our investigation for genotoxic effects by the comet assay, the study group revealed a statistically significant increase in the level of DNA damage compared with the controls ($P < 0.01$). The similar results were obtained in other investigations. The study of Danadevi et al. (2003) indicated that significantly more cells with DNA damage (44.58%) were observed in the study group than in the controls (27.49%).

It was observed in the investigation reported by Fracasso et al. (2002) that Pb exposed workers have significantly elevated levels of DNA breaks compared to the unexposed group. Similarly, Palus et al. (2003) found that the frequency of SCEs and the incidence of lymphocytes with DNA fragmentation in Pb exposed group were slightly, but significantly increased ($P < 0.05$) as compared with controls. Also the results were supported by some in vitro and animal experiments using comet assay (Wo´znika and Blasiak, 2003; Valverde et al., 2002; Devi et al., 2000). Valverde et al. (2002) carried out an experiment to explore the capacity of lead to interact with a cellular DNA, by employing a variant of the comet assay.

Smoking-related DNA adducts have been detected by a variety of analytical methods in the respiratory tract, urinary bladder, cervix and other tissues. In many studies the levels of carcinogen-DNA adducts have been shown to be higher in tissues of smokers than in tissues of nonsmokers (Nakayama et al., 1984). On terms of biological activity, cigarette smoke and its conductors have been shown to form adducts with DNA protein and to induce chromosome aberrations. Nakayama et al., (1985) reported DNA strand break in smokers due to the effect of electrophilic substances in tobacco. The “slower” response of peripheral lymphocytes of smokers as compared with non-smokers found in this analysis may be an expression of influence of Pb with cigarette smoke components on cellular immune response in man.

Although former cigarette smokers were more likely than those who had never smoked to have a smokeless tobacco lesion, we found little evidence for an independent effect of cigarette smoking and alcohol consumption on the presence of these lesions. (Balachandar et al., 2008; Sasikala et al., 2003). Jayakumar and Sasikala (2008) reported cigarette smoking routine has a synergistic effect on inducing DNA damage among the jewellery workers are occupationally exposed to nitric oxide.

DNA damage significantly increased with increase in years of exposure. Pinto et al. (2000) reported that cytogenetic damage in painters exposed to Pb was associated with occupational exposure time. Smoking had an insignificant effect

on the level of DNA damage in the study group of our investigation. The lack of smoking influence on the comet assay results of this study could be due to less number of nonsmokers in the study group. These results agree with those presented by Fracasso et al. (2002), who found that smoking did not significantly affect comet assay values in Pb exposed workers.

In conclusion, even though the information available about the genotoxic and carcinogenic effects of Pb exposure are contradictory, the present results show that workers occupationally exposed to Pb showed a significantly higher DNA frequency in peripheral blood lymphocytes and smoking inhibits significant effect than controls. These observations support the need for preventive action that will improve conditions in the job environment and in micronutrient status since several studies have indicated that an increase in genotoxic effects is associated with an increased cancer risk.

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Table 1. Data showing the general characteristics of control subjects

Controls	Sex	Age	PbB (μg/dl)	MTL (μm)	MTM
1	M	32	3.45	0.96	0.49
2	M	28	3.69	1.29	0.14
3	M	23	3.16	1.36	2.47
4	M	27	3.47	1.45	0.98
5	M	41	3.43	1.18	0.39
6	M	38	3.47	0.29	0.21
7	M	26	3.43	0.89	0.15
8	M	41	3.24	0.90	0.26
9	M	33	3.42	0.81	0.61
10	M	39	3.39	0.77	0.13
11	F	29	2.78	0.34	0.16
12	M	26	3.21	0.77	0.16
13	M	43	3.06	1.03	0.24
14	M	29	3.17	3.39	0.13
15	M	35	3.41	0.71	0.17
16	M	26	3.18	0.91	0.17
17	M	32	3.01	0.74	0.16
18	F	42	3.12	0.62	0.14
19	M	30	3.28	0.96	0.18
20	M	25	3.24	0.87	0.19
21	M	31	2.87	0.74	0.25
22	M	45	3.15	0.95	0.17
23	F	32	3.75	0.49	0.14
24	M	27	3.29	0.90	0.14
25	M	37	3.32	0.78	0.15
26	M	44	3.26	0.92	0.17
27	M	28	3.41	0.71	0.23
28	F	30	3.32	0.43	0.17
29	F	30	3.34	0.74	0.29
30	M	38	3.75	0.62	0.35

F, female; M, male; MTL, mean tail length; MTM, mean tail moment; PbB, Blood lead level.

Table 2. Showing the Blood lead level and DNA damage in experimental subjects

Workers	Sex	Age	Sex	Smoking	Exposure Period (yrs)	PbB (µg/dl)	MTL (µm)	MTM
1	M	32	M	S	4	5.29	2.52	0.67
2	M	28	M	NS	2	5.42	1.51	0.69
3	M	24	M	NS	3	5.28	2.01	0.62
4	M	27	M	S	3	5.39	1.94	0.40
5	M	41	M	S	3	5.17	2.43	0.53
6	M	38	M	NS	3	5.42	1.88	0.48
7	M	25	M	NS	3.5	5.81	1.84	0.82
8	M	42	M	NS	2	5.26	2.28	0.73
9	M	33	M	S	2	4.87	2.09	1.24
10	M	39	M	NS	3	5.55	1.58	0.76
11	F	28	F	NS	2.5	5.23	2.77	1.05
12	M	27	M	S	3	5.12	2.19	0.98
13	M	43	M	NS	2	5.03	1.66	0.65
14	M	29	M	S	2.5	5.08	2.76	0.49
15	M	35	M	NS	3	5.06	3.43	0.79
16	M	26	M	S	3	5.20	2.16	0.55
17	M	32	M	NS	3.5	5.01	3.02	0.39
18	F	41	F	NS	4	5.63	2.32	0.74
19	M	29	M	S	3	5.21	2.47	1.15
20	M	25	M	S	2.5	5.01	2.06	0.53
21	M	31	M	S	4	5.09	2.48	0.76
22	M	46	M	S	3	5.42	2.68	0.77
23	F	31	F	NS	2.5	5.62	1.57	0.58
24	M	27	M	S	2	5.42	2.51	0.86
25	M	37	M	NS	2.5	5.68	2.27	1.31
26	M	45	M	S	4.5	5.81	2.52	0.45
27	M	29	M	NS	3.5	4.99	2.13	1.35
28	F	31	F	NS	4	5.05	2.49	0.78
29	F	30	F	NS	2	5.01	1.32	1.07
30	M	39	M	S	3.5	6.10	1.82	0.63

F, female; M, male; MTL, mean tail length; MTM, mean tail moment; PbB, Blood lead level.

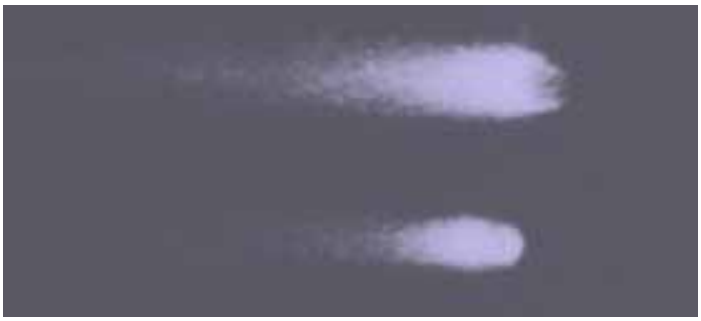

Figure 1. Experimental subjects

Figure 2. Control subjects

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In Silicon Cloning and Analysis of a *LACS* Gene from *Glycine Max* (L.)

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Abstract

Long chain acyl-coenzyme A synthetases (LACSs) activate free fatty acids to acyl-CoA thioesters, and play important roles in the biosynthesis and degradation of lipids. In this study, a *Glycine max*(L.) *LACS* gene, designated as *GmLACS*, has been isolated through *in silicon* cloning. The gene is 2,219 bp with an open reading frame (ORF) of 1,989 bp, which encodes a LACS with 662 amino acid residues, with the isoelectric point of 6.42 and the calculated molecular mass of 65.6 kDa. Sequence analysis showed that GmLACS possessed typical domains of LACSs. Real-time quantitative PCR data analysis suggested that *GmLACS* was hightly expressed in leaves and young pods.

Keywords: *Glycine max*(L.), *GmLACS*, *Silicon* cloning, Real-time quantitative PCR

1. Introduction

Acyl-coenzyme A synthetases (ACSs) are generally classified by their specificities for fatty acids of varying chain length. The Commission on Enzymes of the International Union of Biochemistry has classified these enzymes as acetyl-CoA synthetase (EC 6.2.1.1), medium-chain ACS (EC 6.2.1.2) and long-chain ACS (EC 6.2.1.3). A unifying feature of all acyl-CoA synthetases is the presence of an "AMP-binding domain signature" (PROSITE PS00455)(Watkins, P.A., 1997, pp.55-83). In all of LACSs, long chain acyl-coenzyme A synthetases (LACSs) play a key role in metabolization of fatty acid (Shrago, E., 2000, pp.290-293). LACS esterifies free fatty acids to acyl-CoAs, a key activation step that is necessary for the utilization of fatty acids by most lipid metabolic enzymes(Bradford, M.M., 1976, pp.248-254). LACS catalyzes the formation of acyl-CoAs by a two-step mechanism. In the first step, the free

fatty acid is converted to an acyl-AMP intermediate, called an adenylate, through the pyrophosphorolysis of ATP. The activated carbonyl carbon of the adenylate is then coupled to the thiol group of CoA, releasing AMP and final product, acyl-CoA (Groot, P.H., 1976, pp.75-126). Another special molecular characteristic is that eukaryotic LACSs contain a linker domain with the length about 30 to 40 amino acid residues(Shockey, J.M., 2002, pp.1710-1722). The linker domain does not exist in the other acyl-CoA synthetases. Though the detailed utility is unknown, the linker domain seems to be necessary for eukaryotic LACSs' function(Iijima, H., 1996, pp.186-190). The length of linker domain is also important for the activity of LACSs. Two LACS-like proteins At4g14070 and At3g23790 in *Arabidopsis thaliana* have high identity with AtLACSs, but they do not encode LACS activity. The abnormal length of their linker domain, about 70 amino acid residues, is a putative reason for their non-LACS function(Shockey, J.M., 2002, pp.1710-1722).

This important class of enzymes affect prominently in several fatty acid-derived metabolic pathways, including phospholipid, triacylglycerol, jasmonate biosynthesis and fatty acid β-oxidation. Oil accumulation in oilseeds has significant economic interest for food, feed, cosmetics and detergents etc., and triacylglycerol (TAG) is the main component of plant oil(Shen, B., 2006, pp.377-387). Fatty acyl-CoA thioesters and glycerol 3-phosphate are substrates for acyltransferases to synthesize TAG via Kennedy cycle. During the processes, LACSs play a pivotal role by providing fatty acyl-CoA and link fatty acid *de novo* synthesis and TAG assembly(Ohlrogge, J.B., 1997, pp.109-113). Another important role LACSs played is in fatty acids transport. This process has been studied in detail in bacteria, yeast (*Saccharomyces cerevisiae*), and mammalian cells. *Escherichia coli* contains a single LACS, encoded by *FadD* gene, which was proved to transport the fatty acids(Black, P.N., 1992, pp.25513-25520). LACS also initiates the process of fatty acid β-oxidation. In oilseeds, carbon reserves are stored as triacylglycerol (TAG). With the onset of germination, lipases release free fatty acids from the TAG molecules(Hills, M.J., 1986, pp.671-674; Lin, Y.H., 1986, pp.346-356). LACS activates the free fatty acids to acyl-CoAs that enter the β-oxidation pathway in the glyoxysomes of the germinating seedling.

In *Arabidopsis thaliana*, it has been established that nine *LACS* genes exist and were named *LACS1-9*. Nine *LACSs* could be classified into 3 distinct clades. *LACS1* is supposed to be involved in the syntheses of lipids and *LACS2* is supposed to be involved in the pathway of cutin synthesis(Schnurr, J., 2004, pp.629-642). *LACS6* and *LACS7* are localized in peroxisome, and both of them are involved in peroxisomal fatty acid β-oxidation(Fulda, M., 2004, pp.394-405). In *Ricinus communis*, three *LACS* genes have been cloned. *RcLACS2* is likely to be a peroxisomal ACS isoform. *RcLACS4* is supposed to be involved in the syntheses of lipids(He, X., 2007, pp.931–938). *Capsicum annuum* also has been found *GaLACS*. In this paper, we found a *LACS* gene from *Glycine max* through *in silicon* cloning, designated *GmLACS*. Sequence analysis indicated that the deduced protein possessed AMP-binding motifs and a linker domain.

2. Materials and methods

2.1 Bioinformatics analysis

Blast in NCBI(http://www.ncbi.nlm.nih.gov/) was used for genes searching in network. DNA and amino acid sequence manipulation was performed with EditSeq program of DNAStar 5.0 package. Domain prediction was done at ExPASy Proteomics Server(http://au.expasy.org/). Sequence alignments were carried out by GeneDoc. Phylogenetic tree was constructed using MEGA4.0 and TreeView was used for exhibition of phylogenetic tree.

2.2 Plant materials

Glycine max(L.)*cv* Willimas was utilized for expression analysis of *GmLACS* at different reproductive stages.

2.3 RNA extraction

RNA samples extracted from different soybean tissues including leaf, root, flower and pod. RNA samples were extracted by plant Trizol reagent (Invitrogen Biotech Co. Ltd., U.S.). All steps were carried out following the instruction of manufacturer. RNA samples were DNase treated with DNA-free(TaKara, Japan) according to the manufacturers directions. RNA concentrations were determined using spectrophotometer (GeneSpecIII, U.S.) at absorbance 260 nm. Aliquots of RNA (free of genomic DNA) were diluted to 50 ng uL^{-1} in RNase-free water and stored at −70℃ until use. To verify RNA integrity, 500 ng of total RNA of each sample was examined on a 1% agarose gel following electrophoresis and staining with ethidium bromide.

2.4 RT-PCR assay of GmLACS expression profiles

2μg total RNAs were used for the first strand cDNA synthesizing with M-MLV Reverse Transcriptase (Takara, Japan) according to the manufacture's protocol. Real-time reverse transcription-polymerase chain reaction(RT-PCR) was performed with the iCycler using the SYBR Green RT-PCR kit (Takara, Japan) to quantify *GmLACS* in different tissues. *GmLACS* gene specific primers *GmLACS* (5′-AGCTGGGGTTAAGCGACTG -3′ and 5′-CCTATTGAAGCACCATGCCATA -3′) were designed in the non-conservative regions. Primers used for the housekeeping gene soybean actin (5′-GAGCTATGAATTGCCTGATGG-3′ and

5′-CGTTTCATGAATTCCAGTAGC-3′) were designed by Byfield (Byfield, G.E., 2006, pp.840–846) based on the GenBank accession number U60500(Monizde, S.M., 1996, pp.1198-1212). Real-time quantitative polymerase chain reactions with the *GmLACS* and actin primers produced the following amplicons: *GmLACS*, 138 base pairs (bp); and actin, 188 bp. A typical reaction done in duplicate, contained 10 uL of 2X SYBR Green PCR master mix, 250 nM each primer, and 1uL RT mix, followed by 95°C for 5 min and then 40 cycles of 15 s at 94°C, 30 s at the annealing temperature 56°C, and 30 s at 72°C. A melt curve analysis over a 10°C temperature gradient at 0.05°C s^{-1} from 78 to 88°C was done after amplification to verify that a single product was produced in each reaction.

3. Results

3.1 In silicon cloning of GmLACS

Arabidopsis LACS1-9 (GeneBank Acc. No. AAM28868-AAM28876) were used as querys to blast *Glycine max* (L.) database, two *Glycine max* cDNAs(GeneBank Acc. No. AK245419 and AK245622) with high sequence identity were obtained. From this group, one cDNA(AK245419) which share 75% identity with *AtLACS4*, 74% identity with *AtLACS5* and 73% identity with *AtLACS3* was selected. It is a 2,219 bp long fragment. Sequence aligning with *GmLACS* sequence suggested that the whole fragment contained intact ORF of 1,989 bp, designated as *GmLACS*. Translated by DNAStar software, *GmLACS* was predicted to encode a protein of 662 amino acid residues (Fig. 1), with the theoretical pI of 7.11 and calculated molecular weight of 74.06 KDa.

3.2 Sequence analysis of GmLACS cDNA

Sequence alignment detected that the *GmLACS* ORF shared 74% sequence identity with *AtLACS5* (GeneBank Acc. No. AF503755) and the deduced amino acid sequence shared 74% sequence identity with AtLACS5 (GeneBank Acc. No. AAM28872). The deduced peptide sequence of GmLACS shared high identity with other LACSs from plants which was 81%, 81%, 78%, and 75% sequence identity with RcLACS4 (GeneBank Acc. No. ACB30545), RcLACS1(GeneBank Acc. No. ABC02880), CaLACS (GeneBank Acc. No ACF17663), and AhLACS (GeneBank Acc. No. ACC91252), respectively. GmLACS shared much lower identity with LACSs from mammal and microbe (data not shown).

Multiple sequence alignment of GmLACS and some other LACSs from plants showed that there was some considerable conserved amino acid sequences existed in the form of blocks. Three blocks appeared among these proteins, and three AMP-binding motifs–I[MCV]TSG[TS][ST]GXPK, GYGXTE and GW[FL][HK]TG– orderly located in Block I- III. The conserved tyrosine residue at the position 481 on GmLACS was assumed to be involved in adenylate formation (Fig. 2. A). Multiple sequence alignment of central sequences of GmLACS, AtLACS1, AtLACS2, RcLACS4 and *Arabidopsis* LACS-like protein At4g14070 demonstrated that GmLACS contained a linker domain of 31 amino acid residues as well as other LACSs (Fig. 2. B), and this length of the linker domain suggested it might encode LACS activity.

3.3 Molecular evolution analysis

A neighbor-joining phylogenetic tree was generated to exhibit the distances among GmLACS and other plant LACSs(Fig. 3). Bootstrap analysis was performed for the reliability of phylogenetic tree. The phylogenetic tree demonstrated that the LACSs derived from a common ancestor and diverged into two separate clades. AtLACS3, AtLACS4, AtLACS5, AtLACS6, AtLACS7 and AtLACS8 were in the first clade; RcLACS4, GmLACS, CaLACS, AhLACS, AtLACS1, AtLACS2 and AtLACS9 were in the second clade. The phylogenetic tree revealed that there was remarkable species specificity among LACSs. The sequence homology among those proteins was probably coincident with their function or subcellular location. A phylogenetic tree was drawn based on the deduced amino acid sequence and other LACSs (Fig. 3). In the phylogenetic tree, it showed that GmLACS had a higher homology with the second clade, especially with the RcLACS4 in the second clade.

3.4 Analysis of GmLACS expression profile

To investigate the *GmLACS* expression profile in different tissues, total RNA from root, leaf, flower and pod tissues were used as templates to detect the transcription of *GmLACS* by real-time quantitative PCR. The actin gene was used as the internal control to ensure that the amount of RNA used is equal. The results from real-time PCR assay indicated that *GmLACS* genes were expressed in all tissues tested, but the patterns is quite different, stronger expression exhibited in leaves and young pods (Fig. 4). The most distinctive result was the high accumulation of *GmLACS* in young pod. Based on the high sequence similarity of GmLACS to RcLACS4 which was supposed to be related with the syntheses of lipids, *GmLACS* was likely to be involved in the syntheses of lipids (Fig. 3).

4. Discussions

The long chain acyl-coenzyme A synthetases (LACSs), such as AtLACS1, AtLACS2, AtLACS6 and AtLACS7, play essential roles in the biosynthesis and degradation of lipids in model plant *Arabidopsis thaliana*(Shockey, J.M., 2002, pp.1710-1722; Fulda, M., 2004, pp.394-405; Schnurr, J., 2004, pp.629-642). But their functions in *Glycine max*(L.) are

not reported. In this study, we successfully identified a novel gene, named as *GmLACS*, which might be involved in lipids metabilasim in soybean. We obtained a complete cDNA of *GmLACS* from *Glycine max*(L.) using *in silico* cloning. Sequence analysis indicated that GmLACS belonged to AMP-binding super-family and contained a linker domain of 31 amino acid residues as well as other LACSs, it is suggested GmLACS presumably encoded LACS activity. GmLACS was highly homologous to RcLACS4, an gene involved in the syntheses of lipids, in the second clade in phylogenic tree. (He, X., 2007, pp.931–938). Real-time quantitative PCR analysis showed that the *GmLACS* was strongly expressed in leaves and young pods. This indicated that GmLACS may be involved in the syntheses of lipids in soybean seed development like *AtLACS1* or *AtLACS2* play roles in cutin biosynthesis. Based on the sequence similarity of GmLACS to RcLACS4, *GmLACS* is likely to be involved in the syntheses of lipids (Fig. 3). To indentify its functional characterization, more evidences need to get.

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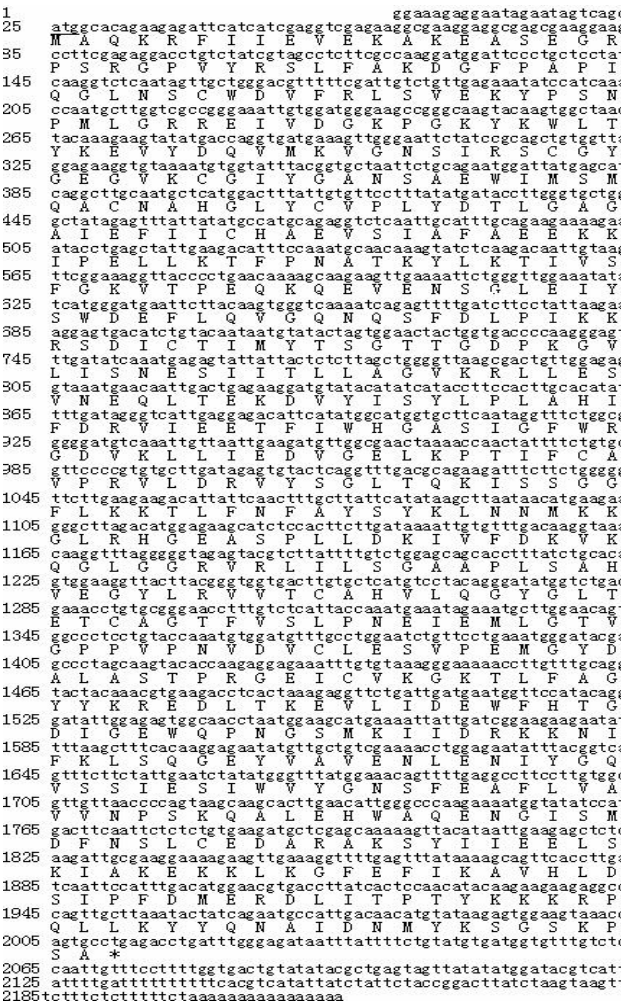

Figure 1. The nucleotide sequence and deduced amino acid sequences of GmLACS

First line: nucleotide sequence. The initiation codon (ATG) is underlined. The stop codon (TGA) is asterisked. Second line: deduced amino acid sequence.

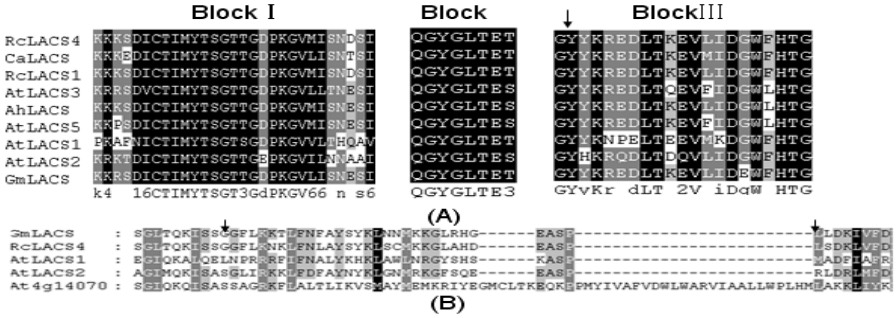

Figure 2. Alignment of GmLACS with other LACSs

(A). Multiple amino acid sequence alignment of three blocks located in GmLACS with other homologous plant LACSs. Black shading presented strictly conserved residues, and gray presented less strictly conserved residues. The conserved tyrosine which was indicated with black arrow was assumed to be involved in the adenylate formation. RcLACS1, RcLACS4 (*Ricinus communis* LACS1, LACS4, ABC02880, ACB30545), CaLACS(*Capsicum Annuum*LACS, ACF17663), AtLACS1,AtLACS2, AtLACS3,AtLACS5(*Arabidopsis thaliana* LACS1, 2, 3, 5, AAM28868, AAM28869, AAM28870, AAM28872, AhLACS (*Arabidopsis halleri*LACS, ACC91252). (B). Comparison of the central sequences of At4g14070, AtLACS1, AtLACS2, RcLACS4 and GmLACS. Black triangles indicated the rough borders of the linker domain.

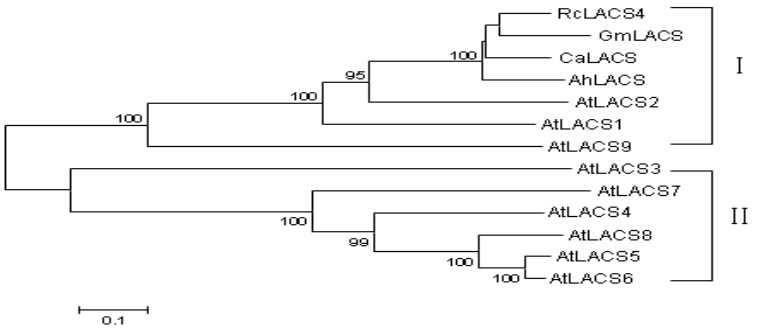

Figure 3. Phylogenic analysis of *Glycine max* LACS, *Ricinus communis* LACS4, *Capsicum Annuum* LACS, *Arabidopsis halleri* LACS and *Arabidopsis thaliana* LACSs

Protein sequences of LACSs were aligned by ClustalX and Treeview. The bars stand for evolutionary distance. Bar = 0.1.

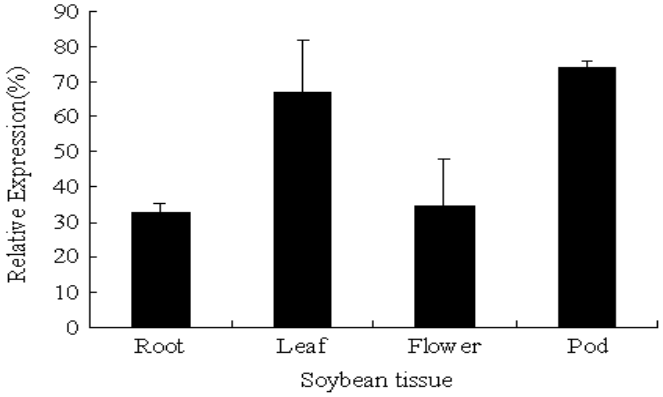

Figure 4. Expression of *GmLACS* genes in the soybean plant

Aliquots of total RNA were analyzed for expression of the gene in different tissues by real-time quantitative PCR using *GmLACS* gene-specific primers. Values were normalized to actin and represent mean ± SE ($p < 0.05$).

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Spectrum of ATP7B Gene Mutations in Pakistani Wilson Disease Patients: A Novel Mutation Is Associated with Severe Hepatic and Neurological Complication

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Abstract

Wilson disease (WND) is an autosomal recessive disorder caused by mutation in ATP7B gene that impairs copper metabolism. ATP7B is involved in the transport of copper into the plasma protein ceruloplasmin and copper excretion out of the liver. Defects in ATP7B lead to excess of copper in various organs primarily in liver. The diagnosis of WND is more complex due to variations in its biochemical and clinical features and the broad range of disease onset. The objective of the present study was to establish molecular analysis system for screening of Wilson disease in Pakistani population. Three mutations were identified; with one being is a novel mutation never reported before.

Keywords: ATP7B, Neurological Disorder, Ceruloplasmin, Wilson Disease, Pakistani population

1. Introduction

Wilson disease (WND) is an autosomal recessive disorder of copper metabolism described by Wilson in 1912. Patients with WND usually suffer from hepatic, neurologic and psychiatric complications. Most frequently findings associated with WND are low serum ceruloplasmin level, high concentrations of copper in liver and the presence of Kayser–Fleischer (KF) ring (Sternlieb, 1980, 1990). The worldwide prevalence of WND was reported to be 1 in 30,000 with a carrier ratio of 1 in 90 (Scheinberg and Sternlieb, 1984). The symptoms appear between ages 5 to 35 (Schoen and Sternlieb, 1990) but it can vary from 2-years old to 72 years (Ala et al., 2005; Beyersdorff et al., 2006; Wilson et al., 2000). It is generally believed that WND is caused by defect in ATP7B gene located on q14.3 band of chromosome 13 which was cloned in 1993 (Bull et al., 1993; Yamaguchi et al., 1993; Tanzi et al., 1993). ATP7B gene consists of six copper binding domains, eight transmembrane domains and ATP loop that transports copper into bile. ATP7B is a copper-transporting P-type ATPase involved in transporting copper into the secretory pathway for incorporation into apoceruloplasmin and excretion of copper into the bile (Bartee et al., 2007; Ferenci, 2004; Hellman et al., 2002). Mutation in ATP7B gene can impair the protein function leading to accumulation of copper in liver, kidney and cornea. The diagnosis of WND is much complex due to variations in biochemical and clinical features and broad range of disease onset. Therefore, molecular diagnosis plays a pivotal role in pre-symptomatic diagnosis of WND and effected individuals can get treatment in time to prevent further progression of disease.

This study was performed to screen patients with WND through molecular genetic testing for ATP7B gene mutation and establish a molecular diagnostic system for detection of pre-symptomatic WND. This may serve as a very useful approach for early introduction of therapeutic intervention in order to check the progression of the disease.

2. Materials and Methods

This study was approved by the ethical committee of National University of Sciences and Technology. Informed consent was obtained from 11 WND patients included in this study. There were (5) males and (6) females with the mean age 6.3. $\pm$ 1.35 (hepatic manifestation), 15±5.6 (hepatic & neurological complications) and 19-yeras (neurological manifestation). Each of these patients had a score of at least 3 according to a scoring system based on clinical and biochemical parameters. The patients were diagnosed on the basis of clinical features such as presence of hepatic disturbance, typical neurological symptoms, low serum ceruloplasmin concentration, high urinary copper level and presence of the KF ring. The molecular genetic analyses were also performed for detection of mutation in the ATP7B gene. The WND patients and their family members were screened through molecular genetic testing. The healthy individuals were included as control for the confirmation of mutation. Genomic DNA was extracted from peripheral blood of patients as well their family members and healthy individuals by standard phenol/chloroform extraction method. The exons 2, 3, 8, 13, 14 of ATP7B gene were used for PCR and direct sequencing (forward and reverse strands) on CEQ8000 Genetic Analyzer (Beckman Coulter). The mutations in WND patients were confirmed in repeated experiments and through comparison with their parents and controls.

3. Results

A total of 11 WND patients were clinically examined. All patients had shown variations in clinical features and biochemical analysis. Eight patients had a hepatic manifestation confirmed through ultrasonoghraphy and liver biopsy, 1 showed severe neurological symptom and 2 had both neurological and hepatic complications (Table 1 & 2). All patients were receiving chelation and oral zinc treatment. The mutation analysis had confirmed the defect in entire ATP7B gene in three patients. Three variants were identified at exon 2 & 3 with one novel never reported before. The insertion (c.815-816insT) at exon 2 was resulted in premature protein truncation with creation of stop codon 10bp downstream (Figure-1d). This novel frameshift mutation might have produced functionless protein. This patient-1 was presented with severe hepatic & neurological complications of WND. The patient was died within six month after disease onset. Her sister was earlier died of WND. The patient's parents were found normal in both biochemical and genetic analysis. The sibling was found negative in KF ring and urinary copper analysis but ceruloplasmin level was at border range and under investigation through mutation analysis. This case shows the importance of molecular genetic testing for diagnosis of Wilson disease.

The non-sense mutation Cys271X (Figure-1e) in exon 2 was previously reported (Hao et al., 1998, Gupta et al., 2005). The patient having this non-sense mutation had severe neurological symptoms like dystonia, dysarthria and arthralgia. Behavioral and psychiatric changes were also developed in later stage of treatment. This nonsense mutation was resulted in the premature truncation of protein at amino acid residue 271. The c.G1366C (Figure-1f) resulted in valine transition to leucine on exon 3 was detected as nonpathogenic variation as described previously (Gupta et al., 2007).

4. Discussion

Early diagnosis of WND disease is very crucial to prevent its progression. Late onset of Wilson disease is also creating hinders in proper diagnose and treatment. We have identified several cases where late onset of disease caused a sudden death of patient (personal communication). Investigation of genotype–phenotype correlations in WND is impeding by a variety of factors. The frequency of most mutations is low and initial symptoms of WND may be nonspecific and will not be easily recognized, resulting in a considerable diagnostic delay and imprecise clinical data (Caca et al., 2001). Therefore molecular genetic testing is a powerful tool for pre-symptomatic diagnose and proper treatment of this disease. Our clinical data has confirmed these findings. In our study, two patients were presented with severe hepatic symptoms but KF ring was absent. Similarly, one patient had neurological type of Wilson disease but developed KF ring at the age of 20 years.

The two frameshift mutations have resulted in premature protein truncation and expected as disease causing mutations. The fulminant hepatic failure and early symptoms in WND are related to nonsense or frameshift mutations that encode a truncated ATP7B protein having significant functional loss and being responsible for the seriousness of the disease (Okada et al., 2000). There are several factors involved in the pathogenesis of WND (Schilsky, 2005). Both insertion and non-sense mutations found here were diseases causing affected copper domain 3. The insertion of T at nucleotide position 816 although does not change the amino acid, but resulted in premature termination.

All patients examined in this study had strong hepatic and neurological symptoms as compare to control. We have concentrated specifically on exon 8 & 14. The occurrence of most common mutation H1069Q (Olivarez et al., 2001) and R778L (Mak et al., 2008) are reported on these exons. The frequencies of R778L mutation have been reported 14.6% in Japanese patients (Nanji et al., 1997), 37.5% in Korean patients (Kim et al., 2000), 30% Chinese patients (Fan

et al., 2002). Both mutations were not detected in our patients. The onset of WND in neurological and hepatic patient was same as reported earlier that hepatic disorders are major symptoms during childhood and neuropathy becomes evident during adolescence (Shimizu and Nakazono, 1999; Ferenci, 2001; Roberts and Schilsky, 2003).

Our report is the first local study to elucidate the genotype of ATP7B gene in Pakistani WND patients. The detection of either novel or common mutations will help to assess their impact on disease severity and functional characterization will open the way to understand the mechanisms of protein dysfunction for particular mutation of ATP7B. This study will help in screening heterozygote carrier to prevent progression of disease in a family or population.

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Table 1. Serum ceruloplasmin levels and Urinary copper level of WND patients (p1-p11). The patients were presented with hepatic, neurological or with both complications

Patients	Ceruloplasmin (mg/dl)	Urinary Copper ug/24hrs
p1	15	1200
p2	10	1800
p3	12	700
p4	9.7	800
p5	18	2000
p6	16	1800
p7	20	2100
p8	6.8	1796
p9	15	1600
p10	20	1000
p11	20	500
Controls (30)	35 (average)	80 (average)
Normal	<20mg/dl*	>100µg/24h *

*(Sternlieb 1990)

Table 2. Hematological Analysis in Wilson disease patients

Patients	Hb	Total Bilirubin	AST	ALT	ALP	Serum Albumin
P1	7.9g/dl	4.4mg/dl	215U/L	30U/L	304U/L	2.4g/dl
P2	10.5g/dl	4.5mg/dl	160 U/L	91 U/L	2264 U/L	2g/dl
P3	8.7 g/dl	30 mg/dl	200 U/L	40 U/L	350 U/L	1.7g/dl
P4	6.7 g/dl	26.8 mg/dl	945 U/L	160 U/L	150 U/L	2g/dl
P5	5.5g/dl	26mg/dl	200U/L	100 U/L	300 U/L	2.3g/dl
P6	10.5g/dl	2mg/dl	40U/L	45 U/L	95 U/L	4.4g/dl
P7	4.5 g/dl	1.9 mg/dl	215 U/L	20 U/L	300 U/L	2g/dl
P8	8.9 g/dl	1.8 mg/dl	1400 U/L	40 U/L	569 U/L	2.3g/dl
P9	5.2 g/dl	2.9 mg/dl	50U/L	122 U/L	272 U/L	2.2g/dl
P10	5 g/dl	30 mg/dl	450 U/L	200 U/L	350 U/L	2.2g/dl
P11	10.2 g/dl	2.4 mg/dl	85 U/L	85 U/L	312 U/L	2.5g/dl
Normal	**13–17 g/dl**	**0.3–1.2 mg/dl**	**7–45 U/L**	**7–45 U/L**	**98–279 U/L**	**3.5-5.5g/dl**

Note. Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine, aminotransferase; ALP, alkaline phosphatase;

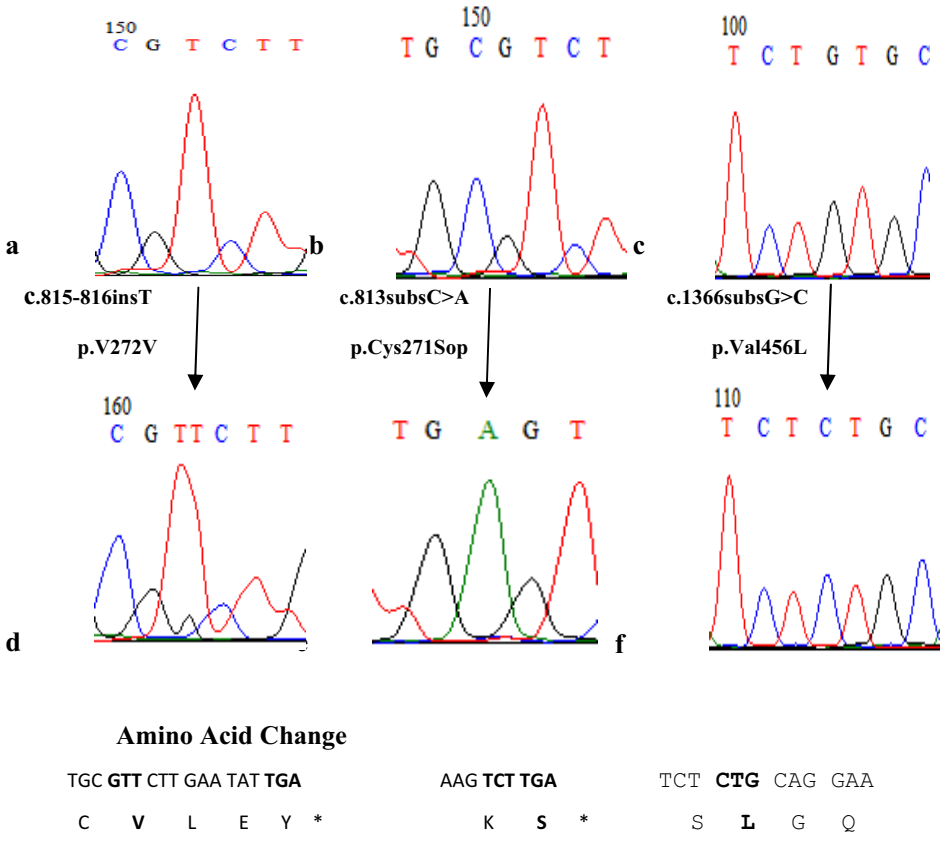

Figure 1. **a-c**: The DNA sequence of controls. **d-f**, The DNA sequence of the patients shows a insertion of T, substitution of C>A,**G>C** (as indicated by an arrow). The DNA sequencing of patients and controls was performed with forward and reverse primers for the confirmation of sequence change.

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Study Progress of Intergeneric Hybridization between Chicken and Quail

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Abstract

In the present paper, we reviewed intergeneric hybridization between chicken and quail from four aspects, such as morphology, molecular genetics, sex identification of earlier embryos and prospects, summarized the recent research progress from the scholars at home or abroad, and aimed to attract more and more attention to intergeneric hybridization between chicken and quail, offer more space and room for other researchers to consider and develop, and lay a foundation for the exploitation, protection and utilization of China genetic resources.

Keywords: Chicken, Quail, Intergeneric hybridization

1. Introduction

Protein human being need principally came from products of meat, eggs, milk and so on, and poultry meat topped the list of domestic meat. Along with economic globalization and market economization, poultry meat has attracted more and more attention. Cases that new products obtained by distant hybridization have been used for produce and life are not seldom. There are not only precedents of offspring between horse and donkey, namely mule in the earlier stage, but also new strains of liger with scientific and ornamental values at present.

Intergeneric hybridization between chicken and quail had been tested successfully in USA (Hua, 1983, PP. 35-36), Japanese (Ahmad, 1989, PP. 2-5) and Malaysia (Liu, 1986, PP. 101-108) successively, and A successful straight crossing test was performed (chicken ♂× quail ♀ hybridization) in experimental station of Shihezi University in China on Oct. 1992, which filled a domestic gap in this field (Wang, 1979, PP. 1-5). Through distant hybridization approach, hybrids combined with excellent characteristics from parents, such as better suitability, and some biological functions for growth, development, propagation and so on. Hybrids not only possessed the characteristics of rapid growth and large body type of male parent, but also early maturity and delicious flesh of female parent. Therefore, it was a perfect flesh product over parents. Accordingly, research and development on hybrids of poultry would be of profound scientific significance with market prospects.

2. Morphology studies

Chen et al(2005, PP. 36-37) have measured body size and carcass traits of adult quail, layers, black-bone chicken and

hybrids, and applied one-way analysis of variance to reveal that hybrids were different from both of chicken and quail, and were intergeneric hybrids between those two. Through SSR multiple comparative law, it was revealed that each characteristic of hybrids was more similar to female parent of quail. Differences between the same samples resulted from excessive variance which enveloped the variance between hybrids and quail due to multiple comparison. According to the calculation methods of Qiu, each adulthood poultry habitus index, namely the percentage of organ weight to live weight or carcass weight was measured, indicated their carrying genetic and biological information, in agreement with the result of Zhao et al(2005, PP. 22-23) using one-factor analysis of variance, which fully reflected hybridization efficiency.

Yu et al(2003, PP. 25-28) had assayed the weight, relative growth speed, absolute growth speed, accumulative growth speed of quail, layer, black-bone chicken and hybrid ranged from 1 to 10 weeks. Results showed that growth-development law of hybrids possessed not only the rapid growing of male parent, but also early maturity of female parent, exceeding both parents, which was in agreement with the results of Zhao et al(2005, PP. 22-23) and Liao et al(2006, PP. 4634-4636).

F. Minvielle have investigated plumage color heredity of hybrids, and found that plumage color of hybrids integrated the color of parents when plumage color of parents was dominant inheritance while didn't express recessive plumage color when plumage color of parents was recessive inheritance. Research of Li et al (2003, PP. 11-13) showed that hybrids between chicken and quail inherited principal characters of parents in body size, and also showed some novel properties. Plumage color of hybrids between chicken and quail was mostly white or reddish brown. When black-bone chicken used as male parent, plumage and bone color of hybrid offspring showed a uniform variation, viz. dark brown plumage black shank, and black and white plumage yellow shank. Therefore, solid foundation could be established for poultry identification through positioning control of plumage color genes.

3. Molecular genetics studies

Hybrids chromosome number was 2n=78, and its sex determination were ZZ(♂) and ZW(♀). As for sex determination of silkworm, male was ZZ, and female was ZW, with balanced sex linked lethal. If hybrid poultry had lethal genes in Z chromosome, it would be responsible for female embryo death, which was not yet convinced at present.

Yoshihiro(1982, PP. 53-54) reported that embryos of fertilized eggs died after 3-5 days, and died totally after 5-8 days, which revealed that combination with different sperm eggs couldn't obtain rational incubation, and could possibly result in hybrid sterility or unisexual sterility due to chromosome recombination and partnership of different poultry. Zhao et al (2005, PP. 10-15) had found that most hybrid embryos died during the early stage of growth, and death period centralized after 2.8 to 4 days. Among them, majority was female with less death during the late stage.

Straight crossing combination has been made successfully between mountain dark-bone chicken black plumage lines(♂), or parents of Hongbao yellow plumage broiler and quail(♀) in Sichuan Agriculture University. Combination between new roman layers (♂) and Japanes(♀), silky black-bone chicken(♂) and quail(♀) successfully had their hybrids respectively in the experimental station of Shihezi University in Sep. 1999. Results convinced the feasibility of straight crossing among these five poultry species.

Until now, reports of reverse crossing combination (quail ♂× chicken ♀) were not documented yet. Zhao et al(2003, PP. 13-15) had studied the genital tracts of female layers and quails, and results showed that structure, pH value, conductance and internal secretion of female chicken genital were different from quail's, but this difference had no affection on the performance and survival of sperms. Less time for the combination of heterogeneous germ cells was one of the causes for reverse crossing failure. Whether for straight crossing or reverse crossing, germ cells among different genus could all combine, but female genital tract didn't block completely the passing and capacitating of sperms in heterogeneous poultry.

Song found that the karyotype of No.1 and No.2 chromosome in muscovy duck and domestic duck was responsible for hybrid sterility. Sun et al(2007, PP. 4152-4153) had studied the morphology of hybrid chromosome, and found that discrepancy among No.1, No.4, No.6, and No.8 chromosomes could possibly lead to sterility resulting from the failure of good match during the period of chromosome meiosis. Phenomena, namely robertsonian ectopia and closely related interspecific karyotype inversion were quite universal in bird chromosomes. Stock considered that inversion could prevent abroad interspecific hybridization, which might be responsible for sterility.

Liao et al (2006, PP. 4634-4636) had calculated mitochondrial DNA (mtDNA) size of each poultry by using Lab Works TM Software. For instance, chicken was 19.1 kb, quail was 16.9 kb and hybrid was 16.9 kb. Li et al (2003, PP. 185-189) have analyzed the mtDNA of chicken, black chicken, quail and their hybrids, and their genetic effect, and results indicated that mtDNA of hybrids was completely identical to their female parents, and quite different from their male parents. All these results suggested that extranuclear genomes of hybrids and male quail showed a high consistence-maternal inheritance.

Differentiation of bird embryo gonads was affected by sex hormones at the early stage. Estrogen was associated with

ovary differentiation, genital tracts feminization and external genital organs. Jane (1997, PP. 182-190) reported that estrogen receptor(ER) expression was earlier than gonad differentiation, and estrogen played a permanent role in bird gender differentiation. Aromatase was expressed during the process of bird gonad differentiation, and gonads at both sides could respond to estrogen before sex differentiation. ER expression of female embryos was higher than that of male ones in sex differentiation, and remained such situation after differentiation period. Gonads distributed in both sides of female and male embryos with more in the left sides. All the facts indicated that estrogen expression was mediated and regulated by ER. Research of Tang had detected ER related antigen mRNA expression, and this gene expressed ER protein. Craig(1997, PP. 295-302) had found that ER expression site was only limited to gonads within 5.5 days of chicken embryo growth, and ER expression level was higher in female embryo than in male one during the sex differentiation period (5.5-6.5days). Taken together, according to incubation period, it could be speculated that hybrid sex differentiation occurred in 3-5days or so of incubating, in line with the time of embryo early death, namely that early embryo death was associated with its sex differentiation and ER expression level. Study of Li et al(2007, PP. 1311-1315) indicated that hybrid sex differentiation occurred approximately in 2.75-3.50days, which was in agreement with the results of Liao et al and Yu et al.

Qiao et al and Liao et al(2008, PP. 1311-1315; 2008, PP. 907-912) had investigated Bcl-2 and P53 expression in early embryos, examined the early growth of male and female hybrid embryos at different time points, and results showed that there was no statistical difference among different days. It suggested that Bcl-2 and P53 played a significant role in the growth and development of chicken early embryos, in line with Tang's observations that embryo death peak occurred after 69-72h. As seen from his research, it might be related to the disorders appeared in the cell apoptosis signal transduction pathway. All these researches laid a solid foundation for searching cell apoptosis causation during embryo period.

4. Studies of early embryo sex identification

Intergeneric hybridization between chicken and quail, similar to other distant hybridizations, also faced the issues such as heterogeneous germ cell incompatibility, hybrid offspring sterility and so on. Yoshihiro (1982, PP. 53-54) had found the first filial generation brood embryo after 3-5 days' incubation had males and females, while the following embryos were totally males through cytologic examination at the subsequent stages and females all died in his investigation of sexual proportion among hybrids between chicken and quail, which was in agreement with the reports of many researchers in China.

With the rapid development of functional genomic technology, it was possible to reveal embryo early death mechanism resulted from intergeneric hybridization incompatibility between chicken and quail from the angel of gene. Before seeking for approaches to reveal the real causation for early embryo death, it was very important to figure out the gender of early embryo hybrid. In order to overcome the hybridization incompatibility between chicken and quail, discovering lethal gene became one of the premises for important studies.

Currently, excellent effects have been acquired using repeats, SRY, ZFX, ZFY and so on in mammalian Y gene to undertaken sex identification. Ogawa had cloned a section of non-repeated DNA fragment (EE0.6) from chicken W chromosome gene pool. Ellergren have separated chicken highly conservative CHD-W gene which could be used as an extensive genetic marker to identify all non ratites.

Hori had found Wpkci/ASW gene located in the end of W chromosome which was highly conservative in the evolution of non ratites, highly expressed in the female embryo of early growth, and had a 65% homology with ckPKCI gene located in acrocen-tric long arm of chromosome Z. Study of Shunsuke indicated that Wpkci and ckPKCI formed heterodimer, and inhibited biological function of chPKCI in vitro, which confirmed the deduction of Hori-Wpkci gene could possibly play a special role in deciding the gender of non ratites.

In order to establish a simple, rapid, reliable and stable method to identify the gender of hybrid early embryo, Liu et al(2007, PP. 9-12) selected embryos during the period of centralized death (2.50-5.00d) as samples, using RT-PCR, and preliminarily estimated that hybrid embryo with two particular bands was female while male with no bands. CDNA fragments of double genes, viz. Wpkci and β-actin were amplified from unknown gender hybrids between chicken and quail. Three bands of 490bp, 402bp and 296bp displayed female while one band of 490bp displayed male. In order to validate its reliability and stability, further experiment using single Wpkci gene to identify the gender of hybrid embryo was performed, and results were in agreement with previous investigation.

Qiao et al (2008, PP. 1311-1315) further validated the investigation results, and first used single Wpkci gene to identify the gender of hybrids between chicken and quail. After χ^2 test, the proportion of male and female embryos was unbalanced, and male was significantly more than female. Mortality of early male was greatly significantly higher than that of female, in line with previous studies.

5. Prospects

Intergeneric hybridization between chicken and quail was the typical representative of animal distant hybridization, and

its hybrids integrated their parents' characteristics such as body form, meat-producing, flesh and so on. In the present trail of intergeneric straight crossing between chicken and quail, fertilization and hatching rate was low, and hybrids obtained were all male of unisexual sterility. Sperm egg of reverse crossing combination could fused, but until now no reports on their hybrids was available, which was the inhibitory factors for people to understand, develop scientific research, and implement large extension and production.

Mechanism investigation of hybrids during the period of embryo and early fertilization would be the critical procedure to solve those issues. If successful, it could offer important theory basis and excellent animal model for the researches of animal distant hybridization.

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A Rapid and Simple Method for *Brassica Napus* Floral-Dip Transformation and Selection of Transgenic Plantlets

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Abstract

The floral-dip method of transformation by immersion of inflorescences in a suspension of Agrobacterium was applied in the *Brassica napus* transformation, but it involves a number of relatively time-consuming and laborious steps, including manipulating an *Agrobacterium tumefaciens* culture and aseptic procedures for the selection of plant lines harboring antibiotic-selection markers. It calls for a long time to prepare the buffered media. To circumvent these bottlenecks, we have developed a rapid and simple method. We find that *Brassica napus* can be transformed by dipping directly into an *Agrobacterium tumefaciens* culture supplemented with surfactant, eliminating the need for media exchange to a buffered solution. We report a method of transformant selection by soaking seeds with antibiotic. These methods save time and money, and reduce the possibility of contamination.

Keywords: *Brassica napus*, floral-dip, *Agrobacterium Tumefaciens*, Antibiotic, Identify

1. Introduction

Transformation of plants usually requires sophisticated regeneration methods that imply technical abilities. The first *Arabidopsis* transformation method required tissue culture and regeneration(DIRK VALVEKENS 1988); however, in planta transformation protocols have been developed to eliminate these steps (Seok So Chang 1994; Vesna Katavic 1994). *Arabidopsis* transformation mediated by vacuum infiltration of inflorescences with an *Agrobacterium* suspension was first introduced by Bechtold et al. (Bechtold N 1993). This method has been widely used in preference to tissue culture techniques as it directly produces transformed seed and negates lengthy and complicated tissue culturing procedures. This transformation

method was further modified by Clough and Bent(Bent 1998) who demonstrated that the method was just as effective without vacuum infiltration. But the tall plants (such as *Brassica napus*) were difficult to use this method, then the floral dip method was used. The floral dip method of transformation by immersion of inflorescences in a suspension of *Agrobacterium tumefaciens* is the method of choice for *Brassica napus* transformation. These methods allow plant transformation without the need for tissue culture (Bechtold N 2000; Clough SJ 1998), but the growth of the *Agrobacterium tumefaciens* strain in liquid culture calls for pelleting the culture and resuspending in a buffered media. This typically takes one hour after the time spent preparing the buffered media (Amanda M Davis 2009). Progeny seed are harvested and selection is applied to the resultant seedlings as they germinate. However, Selection for kanamycin- and hygromycin B-resistance in MS medium is a lengthy process. During this lengthy period, fungal contamination may be a problem. Fungal contaminants may be present in seed stocks generated from floral dipping, because sucrose is used in the dipping medium and plants will have been, at least initially, kept in a warm damp environment providing ideal conditions for fungal growth. During seedling selection, fungal contaminants may deplete antibiotic present in the selection medium, such that non-transformants are able to remain green. Crowding of seedlings on an agar plate may allow root growth above the medium and so result in a delay in bleaching of non-transformants due to decreased concentrations of selection agent. To address these problems, we developed a robustly effective in the generation and selection of transgenic *Brassica napus* first, we provide a description of a bacterial-growth media that supports direct dipping and plant transformation after the trivial addition of surfactant, thereby eliminating the need to exchange bacteria from growth media to a buffer. Second, we provide an improvement method on current selection as it allows quicker identification of transformed seedlings: transformed seedlings are easily discernable from non-transformants this current protocols.

2. Materials and methods

2.1 Plant material and growth condition

Brassica napus cv. “Ningyou 16” was used as the transformation recipient plant, and sow in experiment farm in October, 2007. *Brassica napus* cv. “Ningyou 16” rapeseed plant at bud stages were transformed through *Agrobacterium*–mediated gene transfer method of floral-dip in April, 2008.

2.2 Agrobacterium strain and plasmids

Transformations of *Brassica napus* were performed by the floral dip method (Clough SJ 1998) using *Agrobacterium tumefaciens* strain LBA4404. The following binary plasmids were used: pCAMBIA2200 which confers kanamycin resistance via the nptII gene and pCAMBIA1300 which confers hygromycin B resistance via the hpt gene. The initial bacterial cultures were grown in 5ml of LB liquid medium with corresponding antibiotic at 28℃ and 220 rpm, then an inoculum transferred into 500 ml LB liquid medium at 28℃ and 220 rpm for two more days (Ming-Hsan Chung 2000).

2.3 Plant transformation

The (Main racemes of) rapeseed plants were transformed at bud stages (the initial blossom stage). The plant racemes were submerged in a 20ml beaker with Agrobacterium medium containing 3% sucrose, 0.1% Silwet L-77, 2 ng/l 6-BA and 8 mg/l acetosyringone (Clough SJ 1998).The inflorescence of rapeseed submerged were covered with sulphate paper bags to keep moist for 24h (Conny Bartholmes 2008).

2.4 Plant selection

Seeds were soaked with kanamycin monosulphate solution at a concentration of 300 mg/l or hygromycin B at a concentration of 100mg/l, after 24-36 hours incubation, seeds are sown in the soil. After one week, kanamycin-resistant Brassica napus seedlings and kanamycin-susceptible seedlings had significant difference (figure 1).

2.5 Transgenic detection by PCR

Genomic DNA was extracted with the CTAB method (Rogers 1988) from leaves of randomly chosen plants transformed with target gene and non-transformed plants as a negative control. PCR analysis was performed using the two primers of target genes. All PCR products were visualized on a 1.0% agarose gel containing ethidium bromide. The expected length of the amplified product is correct.

3. Results

3.1 Transformation protocol

The Agrobacterium medium containing Silwet L-77, sucrose, 6-BA and acetosyringone was sufficient for floral-dip transformation. The transformation rates (about 2-3%, data not shown) reported here were similar to the transformation yield of the traditional floral-dip protocol (Amanda M Davis 2009; Bent 1998; Ming-Hsan Chung 2000). We now consider the need to exchange growth media to a buffered solution to be entirely eliminated.

3.2 Selection of kanamycin-resistant transformants

After being soaked in water containing 300mg/l kanamycin, rapeseeds were sown in the soil, When wild-type and

kanamycin-resistant seedlings growing in the soil were examined after 2 weeks, kanamycin-resistant transformants accumulate chlorophyll and grow photoautotrophically. Transformants had green, open, expanded cotyledons. Non-transformants failed to accumulate chlorophyll; they had pale, closed and unexpanded cotyledons (Figure 2). This selection regime produced green transformants that were easily distinguished from yellow non-transformants in the soil.

To test the reliability of the selection protocol, PCR was applied to detect the candidate transgenic plants. We have confirmed the simple of this rapid selection method with different B. napus. The rapid selection method, as described in the protocol, has been used successfully to identify transformants seedlings (data not shown).

3.3 Selection of hygromycin-resistant transformants

Seedlings grown in the presence of hygromycin B displayed a different morphology to those grown in the presence of kanamycin. After 2 weeks in the soil containing 100mg/l hygromycin B, hygromycin-resistant transformants had long hypocotyls of approximately 4.5–5.0 cm, whereas non-transformants had short hypocotyls (2.5–3.0 cm) (Figure 3). Additionally, in contrast to seedlings grown on kanamycin, all seedlings grown in the soil containing hygromycin B were green after 2 weeks. However, selection of hygromycin-resistant seedlings from non-transformants was easily achieved by selecting those with elongated hypocotyls.

To confirm the phenotypes of seedlings grown in the presence of hygromycin B, wild-type controls were grown alongside previously characterized hygromycin-resistant lines obtained following transformation with pCAMBIA1300 which confers hygromycin B resistance via the hpt gene. The majority of characterized: hygromycin-resistant seedlings had long hypocotyls following rapid selection. The rapid selection method, as described in the protocol, has been used successfully to identify transformants seedlings (data not shown). PCR analysis was performed using the primers of target genes. All PCR products were visualized on a 1.0% agarose gel containing ethidium bromide. The expected length of the amplified product is correct (figure4).

4. Discussion

We have developed a method that dips and transforms plant directly after the trivial addition of surfactant and distinguishes kanamycin- and hygromycin B-resistant seedlings from non-resistant seedlings through soaking seeds in antibiotics solution. We provide a description of a bacterial-growth media that supports direct dipping and plant transformation after the trivial addition of surfactant, thereby eliminating the need to exchange bacteria from growth media to a buffer. It reduced the risk of seedling loss by pathogen infection. The protocol produces easily identifiable. We also provide an improvement methods on current selection as it allows quicker identification of transformed seedlings: transformed seedlings are easily discernable from non-transformants this current protocols. Kanamycin-resistant seedlings can be distinguished from non-transformant seedlings by the presence of chlorophyll in the expanded cotyledons. However, all seedlings grown in the presence of hygromycin B have green cotyledons, and the selection is based on hypocotyls length. Hygromycin-resistant seedlings have extended hypocotyls in the simple selection method, whereas non-ransformant seedlings have short hypocotyls. The different effects of hygromycin B and kanamycin on hypocotyl elongation may be related to the target of these antibiotics. Hygromycin B inhibits cytosolic protein synthesis (Cabanas MJ 1978), whereas kanamycin inhibit plastid protein synthesis (Gray JC 1984) and glutamine synthase activity (Tachibana K 1986), respectively. This suggests that cytosolic, but not plastid, protein synthesis is required for hypocotyl elongation in the dark. The length of hypocotyls extension in the dark is proportional to the amount of lipid reserve mobilized (Penfield S 2004) and thus remaining reserves may influence the extent of chlorophyll synthesis on transfer of seedlings to the light. non-transformed seedlings grown on hygromycin B have short hypocotyls and are able to synthesize chlorophyll in the light, whereas non-transformed seedlings grown on kanamycin have long hypocotyls and are unable to make chlorophyll (Samuel J Harrison and Cottage 2006).

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Figure 1. kanamycin-resistant *Brassica napus* seedlings and kanamycin-susceptible seedlings had significant difference after sowing one week

Transformants had green, open, expanded cotyledons. Non-transformants had pale, closed and unexpanded cotyledons. Size bars 1 cm.

Figure 2. Simple selection of kanamycin-resistance Brassica napus seedlings.

Simple selection of kanamycin-resistant Brassica napus seedling after simple selection procedure(left); kanamycin-susceptible seedling after simple selection procedure (right). Size bars 1 cm.

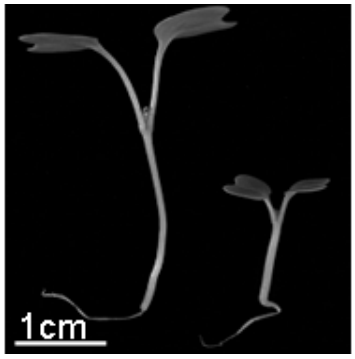

Figure 3. Simple selection of hygromycin-resistance Brassica napus seedlings

Hygromycin-resistant Brassica napus seedling after simple selection procedure (left); hygromycin-susceptible seedling after simple selection procedure (right). Size bars 1 cm.

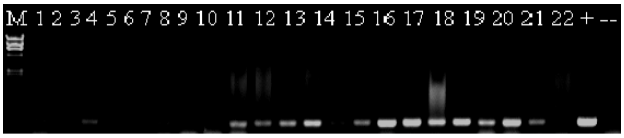

Figure 4. The fragments are amplified by PCR with the primers of target gene from genomic DNA of kanamycin-resistance Brassica napus seedlings and hygromycin-resistance Brassica napus seedlings

The “M” was marker. The lanes 1, 3-6 and 9-22 showed the amplification of the 375 bp DNA fragment of the target gene. The lanes 2, 7 and 8 showed that they were non-transgenic. The lane “+” was PCR product with plasmid as template. The lane “-” was PCR product with genomic DNA of non-transgenic plant as template.

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An Investigation of the Prevalence of Upper Limb Neuropathies in Different Types of College Musicians by Use of Neurometrix Device

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Abstract

In general, people who perform repetitive motions are often vulnerable to repetitive strain injuries. Because musicians must execute the same motion over and over again while practicing and performing their music, they are an example of a group that often develops these repetitive strain injuries. More specifically, musicians are known for developing neuropathies in their upper limbs, with carpal tunnel syndrome and cubital tunnel syndrome being most common. However, because of varying playing postures, all musicians may not be at equal risk for developing these two neuropathies, so the purpose of this study was to identify which musician group has the highest risk of developing median and ulnar neuropathies. Results of this study show that the prevalence of carpal tunnel syndrome and cubital tunnel syndrome is fairly low, and that tingling is one of the first signs of these diseases. Also, brass players may have the highest risk of developing carpal tunnel syndrome, as only this musician group had a significant p-value when its incidence rate of carpal tunnel syndrome was compared to the incidence rate found in the general population. Finally, the results suggest that there may be a negative correlation between performing a warm-up routine and experiencing tingling. Therefore, music schools should teach all students, and especially brass players, to be aware of tingling and to take appropriate preventative measure such as warming-up in order to keep the incidence rates of carpal tunnel syndrome and cubital tunnel syndrome as low as possible.

Keywords: Carpal tunnel, Musicians, Occupational exposure, Nerve conduction studies

1. Introduction: Clinical parameters of Carpal Tunnel Syndrome and Cubital Tunnel Syndrome

1.1 Etiologies of Carpal Tunnel Syndrome

In many instances in which a person is required to perform the same physical action over and over again, a certain amount of stress may be put onto the overused body parts, which can result in a repetitive strain injury/cumulative trauma. These injuries can affect anyone who performs these repetitive types of motions, but are especially prevalent in typists, carpenters, electronics assembly line workers, textile workers, butchers, grocery checkers, packers, and musicians, as these groups of people perform repetitive and sometimes forceful motions almost every day (Cummings et al. 1989). In particular, a number of musicians tend to develop upper limb neuropathies as a common type of repetitive strain injury, with carpal tunnel syndrome and cubital tunnel syndrome being two of the most common types of neuropathies.

1.2 Physiologic Changes of Carpal Tunnel Syndrome

Carpal tunnel syndrome is caused by an abnormal amount of pressure being placed on the median nerve as it passes through the carpal canal of the wrist (the canal walls are composed of the carpal bones and the transverse carpal

ligament, which enclose the median nerve and nine flexor tendons). This can either occur through "factors that increase the volume of the contents of the carpal canal" (intrinsic carpal tunnel syndrome), or through factors that decrease the actual size of the canal (extrinsic carpal tunnel syndrome) (Kerwin 1996). If this excessive amount of pressure occurs regularly, it can cause damage to the median nerve by removing part of the myelin sheath surrounding the nerve, which then slows the conduction velocities of both motor and sensory electrical impulses that are being sent through that nerve.

1.3 Presenting Symptoms

The first symptoms of carpal tunnel syndrome include pain, weakness, or paresthesias in the first three digits and in the radial half of the ring finger, and the symptoms often increase in severity at night as many subjects tend to sleep with their wrists in a flexed or extended position, which increases the pressure inside the carpal canal (Strickland 2001). If the condition is then left untreated, demyelination of the nerve can continue and can severely limit use of the hand, as the damaged median nerve will not dependably conduct an electrical impulse to the neuromuscular junction, resulting in limited muscular contraction and eventual atrophy of the innervated muscles.

1.4 Diagnosis

Carpal tunnel syndrome can be diagnosed in a couple of ways, the first of which is provocative tests including Tinel's test and Phalen's test. Though these types of tests are somewhat useful, they should not be the only diagnostic technique used, as Gerr and Letz (1998) found that sensitivity for the Tinel's test can range from 26% to 79%, and that sensitivity for the Phalen's sign can range from 10% to 88%. Therefore, the best way to diagnose carpal tunnel syndrome is through nerve conduction studies. During these studies, the medical professional is often most interested in distal motor latency, which is the amount of time it takes for the nerve impulse to travel from the site of the stimulus (such as some specified point along the median nerve) to the innervated muscle (such as the thenar muscles). A person who is affected with carpal tunnel syndrome will usually exhibit a longer than normal distal motor latency due to the damaged myelin sheath of the median nerve.

1.5 Presenting symptoms and Physiology of Cubital Tunnel Syndrome

The second common upper limb neuropathy found in musicians is cubital tunnel syndrome, which involves entrapment of the ulnar nerve at the elbow. Similar to carpal tunnel syndrome, cubital tunnel syndrome is caused by excessive pressure on the ulnar nerve as it passes through the cubital tunnel on the outside edge of the elbow. This excessive pressure can damage the myelin sheath and slow conduction velocities, which can prevent appropriate contraction of the innervated muscles.

1.6 Physiology of Cubital Tunnel Syndrome

Also similar to carpal tunnel syndrome are the primary symptoms of cubital tunnel syndrome. The initial symptoms of cubital tunnel syndrome include pain, weakness, and paresthesias in the little finger and ring finger, as these are the digits that are innervated by the ulnar nerve. If left untreated, the myelin sheath will likely suffer an increasing amount of damage, which can again reduce activity at the neuromuscular junction and cause atrophy of the innervated muscles as well as "clawing of the fourth and fifth digits" (Robertson 2005)

1.7 Diagnosis of Cubital Tunnel Syndrome

Cubital tunnel syndrome is usually diagnosed via nerve conduction studies involving F-waves. To do this, a specific section of the ulnar nerve is electrically stimulated, which causes electrical impulses to travel both orthodromically and antidromically. The orthodromic impulse will of course reach the innervated muscle first, and the muscle contraction that results creates the M-wave. At the same time, the antidromic impulse first travels to the spinal cord and will then reverse directions and eventually reach the neuromuscular junction, where it will cause another muscular contraction that will produce the F-wave. Because the impulse that produces the F-wave must travel through the cubital tunnel during its journey to and from the spinal cord, the latency, amplitude, and duration of the resulting F-wave can be examined for abnormalities that are characteristic of cubital tunnel syndrome.

2. Risk Exposure of different Musicians to these Syndromes

Now knowing the causes and diagnostic techniques of these two neuropathies, it is possible to examine the playing postures of four different types of musicians in order to see which musicians may be especially at risk for one or both of these disorders.

2.1 Brass Players

Brass players are one type of musician, and include people who play the trumpet, tuba, trombone, and French horn. In order to play these instruments, the musician does not usually need to have an especially strong grip on the instrument, nor is this gripping hand in an awkward position. The other hand, known as the valve hand, may deviate slightly from

neutral position, but overall is still fairly free to move. This neutral positioning probably means that brass players would not be at a high risk of developing either of these two neuropathies.

2.2 Woodwind Players

A second group of musicians are the people who play woodwind instruments such as the clarinet, saxophone, and flute. Musicians who play the flute are required to engage in extension and ulnar deviation of the left hand, which could put them at a higher risk for carpal tunnel syndrome. Additionally, playing the saxophone and clarinet often requires wrist flexion and high levels of thumb stress, which could also lead to carpal tunnel syndrome.

2.3 Percussionists

A third group of musicians are percussionists, such as those people who play piano or drums. The instruments are often played with a great deal of force and often require flexion of the wrist when playing the instrument, which could put excess pressure on the median nerve and increases this group's risk of developing carpal tunnel syndrome. In fact, Gohl (2006) found that sixteen percent of university pianists exhibited evidence of carpal tunnel syndrome, which is indeed higher than the two to three percent incidence rate that is found in the general population (Atroshi 1999).

2.4 String Instruments

A fourth group of musicians are those people who play string instruments such as violin, viola, cello, and bass. These musicians are often required to hold the instrument quite firmly with a flexed wrist, which could put them at higher risk for carpal tunnel syndrome. In addition, violins and violas are also held in place by the chin, which forces the neck into a flexed position, and can therefore cause damage to the median nerve as it branches off from the brachial plexus. Also, because violinists and violists must constantly flex the left elbow to support their instrument, this arm could be at a higher risk of developing cubital tunnel syndrome. In a study of young adult violinists, Bowie et al. (2000) found that thirty-five percent of the subjects showed early electrophysiologic signs of carpal tunnel syndrome, which is again higher than the incidence rate of the general population.

2.5 Hypothesis related to groups

Therefore, knowing the playing requirements of each type of musician, it is hypothesized that the type of instrument played (brass, woodwind, percussion, or string) may be a determining factor in the amount of nerve damage present on the median and ulnar nerves of Kennesaw State University music students. In addition, it is predicted that because the string group usually has the most strained playing postures, this group will be most likely to have nerve damage, while the group with the least strained playing posture (the brass group) will be least likely to show signs of median or ulnar nerve damage.

In order to test this hypothesis, Kennesaw State University music education or music performance majors were randomly selected to participate in the study. They were asked to fill out a survey that will give the researchers background information about each subject's medical history, health habits, and musical habits. After this, the health of both the median and ulnar nerve was assessed for each subject using the NeuroMetrix NC-Stat electrodiagnostic system, and the resulting data for each group was analyzed.

3. Methods

3.1 Musician Type Findings

For this experiment, the independent variable was the musician type. It was applied by asking each subject to state which instrument is his or her most major instrument. Based on the answer to this question, the subject was placed into one of four major groups: brass, woodwind, percussion, or string. The dependent variable was the health of both the median and ulnar nerves. This was measured using the NC-Stat system, using distal motor latency as one of the most important pieces of data describing the health of the median nerve and F-wave latency as one of the most important pieces of data describing the health of the ulnar nerve. In addition, the number of median and ulnar nerve "flags" was examined, with a flag being defined as any result deemed by the NeuroMetrix system to be abnormal (these abnormalities can range from being borderline abnormal to severely abnormal).

3.2 Age Contribution to Findings

In addition, a number of variables were also controlled during this experiment, the first of which was the age of the subjects. Because all of the subjects were undergraduate students in college, they were all around the same age. This is important because neuropathies generally tend to increase in severity in older people, so if the subjects were not all around the same age, one musician group could show less healthy nerves simply because it had a larger number of older individuals.

3.3 Measuring Device standardization

The same NC-Stat system was used to measure each subject. This ensured that differences in machine sensitivity did not bias the information. In addition, because the NC-Stat system corrects for the temperature of the subject, it ensured

that some subjects did not have longer latency periods just because their body temperature was colder than other subjects' body temperatures. Finally, after the data was collected from each subject, it was compared to a large database of national averages, so this database (provided by NeuroMetrix) acted as the control population.

3.4 Data Gathering and Measurment of Subjects

In order to carry out the actual experiment, the test subjects were selected from the group of music education and music performance majors at Kennesaw State University. To do this, a researcher went to the music building, discussed the study with available music students, and obtained email addresses for those students who were interested in participating. An email was then sent to the interested students describing the study in more detail and informing the students about how they could make an appointment time.

3.4.1 Survey

When the subject arrived for his appointment, he was given a booklet that included an informed consent as well as a survey. The first portion of the survey inquired about physical and mental health history, while the second part of the survey asked the participant to answer questions regarding his or her major musical instrument, musical history, daily/weekly musical routines, and music related medical problems. It is important to note that if the participant asked for clarification, "tingling" was defined as a sensation of pins and needles or numbness in the fingers and "warm-up" was defined as any stretching or other motions that were done prior to playing one's instrument.

3.4.2 Nerve Measurements

After the survey was completed, the forearm of the student was cleaned and the electrodes of the right median nerve biosensor were applied. This biosensor was then connected to the NC-Stat system, the subject information was entered, and then the test was started. During the test, the NC-Stat system sends electric impulses of gradually increasing strength through the appropriate nerve, which makes the innervated muscles contract. This muscular contraction is recorded by sensory electrodes, the data is stored in the machine, and is later compared against the control database discussed earlier. The same process was then repeated for the left median nerve, the right ulnar nerve, and finally the left ulnar nerve.

3.5 Data Analysis and Statistics

Then, after the data was collected, each subject was placed into one of four musician groups: brass, woodwind, percussion, or strings. General descriptive statistics such as the proportion of results that were outside the reference range and the distribution of results in terms of percentile groups were then performed. More detailed descriptive statistics were also performed in order to compare reported tingling, electrophysiological slowing, and reported warm-up in each of the musician groups. Chi-square tests were also performed in order to evaluate the incidence rate of carpal tunnel syndrome in each of the musician groups versus the incidence rate in the general population.

Finally, in order for the obtained data to support the study's hypothesis that the type of instrument played is a determining factor on the amount of median or ulnar nerve damage seen in college musicians, at least one of the chi-square tests comparing the incidence of carpal tunnel in the general population to the incidence rate in a certain musician population would have to produce a statistically significant p-value (less than 0.05). In addition, in order for the prediction that string players are at the highest risk for nerve damage to be supported, this group would have to show the most electrophysiological evidence of slowing (for instance, having the greatest proportion of members who were outside the reference range) or other abnormalities (having the greatest average number of median and ulnar flags). Finally for the data to support the second prediction that brass players are at the lowest risk for nerve damage, this group would have to show the least electrophysiological evidence of slowing or other abnormalities.

4. Results

4.1 Relationship between this group and the general population

Before looking at the differences between musician groups, it was first important to note the general trends that were found in this population of musicians. First, the overall incidence rate of carpal tunnel syndrome in the musician population was not significantly different from the incidence rate in the general population, as a chi-square test comparing these rates returned a p-value of 0.2092. Additionally, **figure 1** shows that only a small proportion of musicians had values outside of the reference range, which again was the criteria used to diagnose someone as having electrical evidence of nerve compression and carpal tunnel syndrome or cubital tunnel syndrome.

In addition, **figure 2** shows the gradation of the test results, and again highlights the fact that most musicians do not have abnormal conduction rates, and that a large proportion of musicians are at or above the fiftieth percentile when compared to the average population. When examining this figure, it is important to remember that the only people who could be neuroelectrically diagnosed as having carpal tunnel syndrome or cubital tunnel syndrome are subjects found in the lowest percentile group.

4.2 Findings of Measured Nerve Latency vs Symptomatic Subjects

A second general trend shows that a large proportion of musicians who experience tingling in their fingers do not have any significant electrophysiological signs of carpal tunnel syndrome. In **figure 3,** over sixty percent of musicians reported tingling, but of that sixty percent, only 16.7% had a right DML below the twentieth percentile, and only 16.7% had a left DML below the twentieth percentile.

Also, for all musicians who reported tingling, the average percentile for DML-R and for DML-L was greater than 50%, therefore showing that the average musician who reports tingling does not have any evidence of objective changes in his nerve studies.

4.3 Measuring Reported symptoms vs Instrument Played

In addition, a number of results were calculated regarding different categories of musicians. **Figure 4** shows that symptomatically, percussion players had the most reported subjective symptoms (as all percussion players reported tingling), while string players had the least reported subjective symptoms (with only twenty percent reporting tingling).

4.4 Measurement of DML in each studied instrument group

In addition to looking at different symptoms between groups, the researchers also looked at electrophysiological differences between groups. **Table 1** highlights these differences, and shows that brass musicians had the most problems, while all of the other groups had no electrophysiological confirmations of carpal tunnel syndrome or cubital tunnel syndrome.

4.5 Comparing Study group prevalence vs general population.

Chi-square tests were also used to compare the prevalence of carpal tunnel syndrome in the general population to the prevalence in each of the musician groups, and as shown in **table 2,** only the brass group showed a statistically significantly higher rate than that found in the general population.

4.6 Severity of the Measured Nerve changes in the Brass Player Group

Also, as **figure 5** and **figure 6** show, brass players had the highest average number of median and ulnar nerve flags (flags were determined by the NC-Stat system and represented mild to severe abnormalities in any of the electrical values).

4.7 Affect of Warm Up on presence of Symptomatology in all groups

Finally, the researchers examined the correlation between performing a warm-up and the prevalence of symptoms. As **figure 7** shows, musicians who perform a warm-up routine prior to playing are less likely to report experiences of tingling than those who do not warm-up prior to playing.

Figure 8 also shows a similar negative correlation between warming-up and reports of tingling, especially for the brass and string groups.

5. Discussion

5.1 Trends in Data

5.1.1 Low incidence of Disease

By examining the above data, a number of general trends can be identified. First, it seems that there is not a large prevalence of carpal tunnel syndrome or cubital tunnel syndrome in this population of college musicians. Figure 1 shows that only a small proportion of students had values outside of the reference range for DML-R, DML-L, F-wave R or F-wave left, and all of the values that were outside the reference range are from three students who each had evidence of slowing in more than one nerve. Figure 2 also shows a low prevalence of electrical signs of carpal tunnel syndrome or cubital tunnel syndrome, as most musicians were not even below the tenth percentile and a large number of musicians were at or above the fiftieth percentile.

5.1.2 First Sign of Carpal Tunnel is usually tingling

Secondly, it seems that there is no earlier sign or symptom of carpal tunnel syndrome than a subject's reports of tingling. Figure 3 shows that most musicians who report tingling do not have any electrical slowing in the median nerve. Because sensations of tingling appear to be more sensitive than electrophysiological evidence, musicians should generally have the ability to identify the start of a problem without expensive nerve testing.

5.1.3 Subjects did not demonstrate Failure to report symptoms

Also, it also seems that there is no failure to report symptoms. If students who had signs of electrical slowing had failed to report symptoms, this may have shown the effect of a professional pressure to play without complaints in order to get more jobs or better jobs. However, all students that had values outside of the reference range also reported having tingling in their fingers, so it appears that students are not afraid to report symptoms.

5.1.4 Further Analysis

Brass Players had more abnormal studies which does not substatiate "unnatural position theory" as etiology of Carpal tunnel Syndrome or Cubital Tunnel Syndrome in this population

In addition to these general conclusions, a number of more specific conclusions can be made regarding this experiment's original hypothesis. This experiment tested the hypothesis that the type of instrument played may be a determining factor in the amount of nerve damage present in the median and ulnar nerves of Kennesaw State University musicians. In order for this hypothesis to be supported, one category of musician would have to show a significantly greater amount of electrical slowing than other categories of musicians. As table 1 shows, only brass players had values outside of the reference range for any of the four electrical values (DML-R, DML-L, F-wave right, and F-wave left). In addition, only brass players had a statistically significant p-value when the incidence rate of carpal tunnel syndrome in that category of musicians was compared to the average incidence rate of 2.5% in the general population. Figure 5 also shows that brass players had the highest average number of flags, again indicating more electrical evidence of nerve damage. Therefore, because brass players showed more electrical evidence of nerve damage than any other musician category, the researchers fail to falsify the hypothesis that the type of instrument played may be a determining factor in the amount of nerve damage present.

It was originally predicted that because string players generally have the most strained playing posture, this group would have the most nerve damage, and because brass players have the least strained playing posture, this group would have the least nerve damage. However, as stated above, the brass group was the only group to show a statistically significantly greater prevalence of carpal tunnel syndrome when compared to the general population. Also, 62.5% of brass players had experienced tingling while only 20% of string players had experienced tingling.

5.1.5 Causes of Discrepancies in Hypothesis

These results are basically opposite of what the researchers had originally predicted, but a closer look into personal care habits may explain this unanticipated trend. First of all, 100% of the string players reported that they perform a warm-up routine prior to playing their instrument, but only 12.5% of brass players reported performing such a warm-up routine. This may mean that there is some sort of negative correlation between performing a warm-up routine and experiencing tingling. In addition, all string players reported exercising more than two times per week, while only 16.7% of brass players reported exercising more than two times per week. This suggests that there may also be some sort of negative correlation between exercising and experiencing tingling.

6. Conclusions and Reccomendations

When analyzing the overall experimental design of this study, it seems that the experiment did adequately test the hypothesis that the type of instrument played may be a determining factor in the amount of nerve damage present in the median and ulnar nerves. The independent variable was of course the type of instrument played (brass, percussion, woodwind, or string), and was determined by the researchers after analyzing each participant's response to the survey question "Instrument that you now think is your major instrument?" There were a number of dependent variables. Two of the most important dependent variables were the distal motor latency of the median nerves and the F-wave latency of the ulnar nerves. These were measured by the NC-Stat system, so they were fairly trouble-free except for the fact that a few ulnar sensors did not work properly, so a few of the F-wave latencies could not be computed. Other dependent variables included reports of tingling, warm-up, and weekly exercise, and the respondents simply circled the appropriate multiple choice answer, so this was also fairly trouble-free (some subjects did not see the multiple choice answers, but could still be put into an appropriate category fairly easily). The experimental design also included a number of control variables. The body temperature of the subject, gender, height, and weight was taken into account by the NC-Stat system as it was calculating the electrical values, so these variables were adequately controlled. Also, almost all subjects were either in their late teens or early twenties and the NC-Stat system also helped to adjust for age, so this was fairly well controlled. However, if may be important to note that one of the brass players who had electrophysiological signs of both carpal tunnel syndrome and cubital tunnel syndrome was thirty-seven years old, which was in fact older than most of the other subjects.

Also, when looking at similar studies, these results appear to correspond nicely with other findings. For example, Logue (2005) found that in a study of fourteen university cellists, "all subjects were found to have normal nerve conduction studies of the median and ulnar nerves in both extremities when compared with a chart of normal values." Therefore, Logue's findings are very similar to this study's findings, as both showed no abnormal conduction in string players. Also, Gohl (2006) had similar findings in a study of nineteen university pianists, as she found that all subjects had normal conduction values when compared to a chart of normal values. However, when she compared median and ulnar latencies in the same and opposite hand, she found that three of the nineteen pianists (16%) showed early evidence of median neuropathy. Finally, Bowie (2000) also had similar findings in a study of twenty young adult violinists. Bowie found that all subjects had normal median and ulnar conduction values when compared to a chart of normal values.

However, like Gohl, Bowie found that when the median nerve conduction was compared to ulnar nerve conduction in the same extremity, seven of the twenty violinists (35%) showed evidence of early median neuropathy.

Therefore, the results of our study tend to agree with previous research. In addition, because these results suggested negative correlations between performing a warm-up routine and experiencing tingling and between exercising more than two times per week and tingling, it may be interesting to do further study in order to see if these correlations are indeed significant In conclusion, it seems that tingling is usually the first symptom of early carpal tunnel syndrome and cubital tunnel syndrome, so music schools should teach students to be aware of these symptoms and take the proper preventative measures. Also, it appears that brass players may have the highest risk of developing carpal tunnel syndrome or cubital tunnel syndrome, so these musicians should be especially aware of early symptoms and take the necessary precautions to help prevent these neuropathies.

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Table 1. Number of musicians who had values outside of the reference range, therefore confirming electrical evidence of carpal tunnel syndrome or cubital tunnel syndrome. 30 musicians were tested for DML-R, 29 for DML-L, 24 for F-wave Right, and 25 for F-wave Left.

	DML-R	DML-L	F-wave Right	F-wave Left
Brass	1	2	3	2
Percussion	0	0	0	0
String	0	0	0	0
Woodwind	0	0	0	0

(overall, this represents one brass player with an abnormal DML-R, DML-L, and F-wave right, one brass player with an abnormal DML-L, F-wave right, and F-wave left, and one brass player with an abnormal F-wave right and F-wave left)

Table 2. Results of chi-square tests comparing the incidence of carpal tunnel syndrome in the general population to the incidence in each musician population. Data represent 8 brass musicians, 8 percussion musicians, 5 string musicians, and 9 woodwind musicians.

Musician Group	P-value
Brass	3.94E-5
Percussion	0.5271
String	0.6171
Woodwind	0.5145

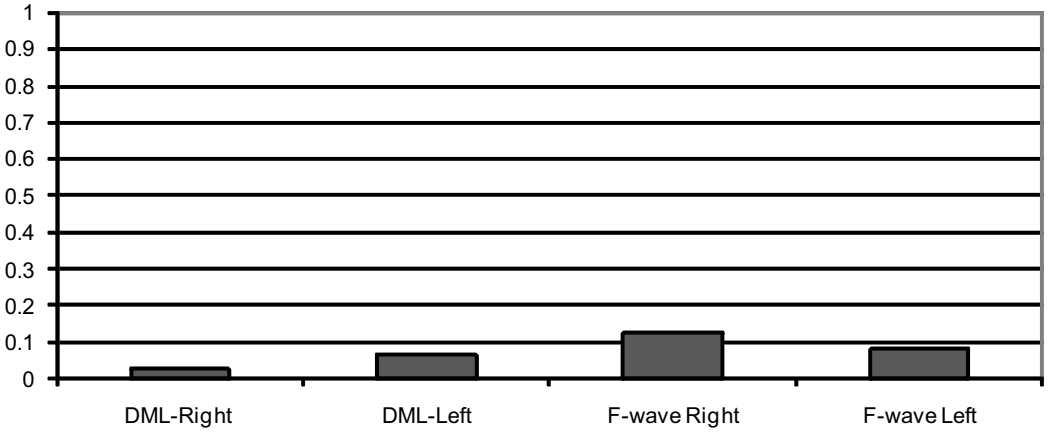

Figure 1. Proportion of all musicians who had values outside of the reference range. Data describe 30 musicians for DML-R, 29 musicians for DML-L, 24 musicians for F-wave right, and 25mu sicians for F-wave left.

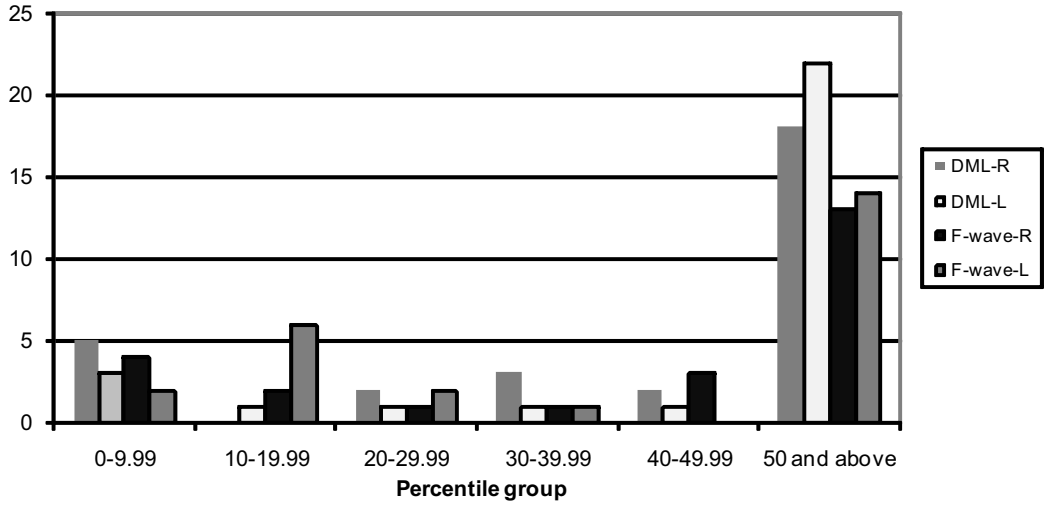

Figure 2. Distribution of percent tiles for all musicians. Data represent 30 musicians for DML-R, 29 for DML-L, 24 for F-wave right, and 25 for F-wave left. Higher percentiles represent faster conduction and therefore healthier nerves.

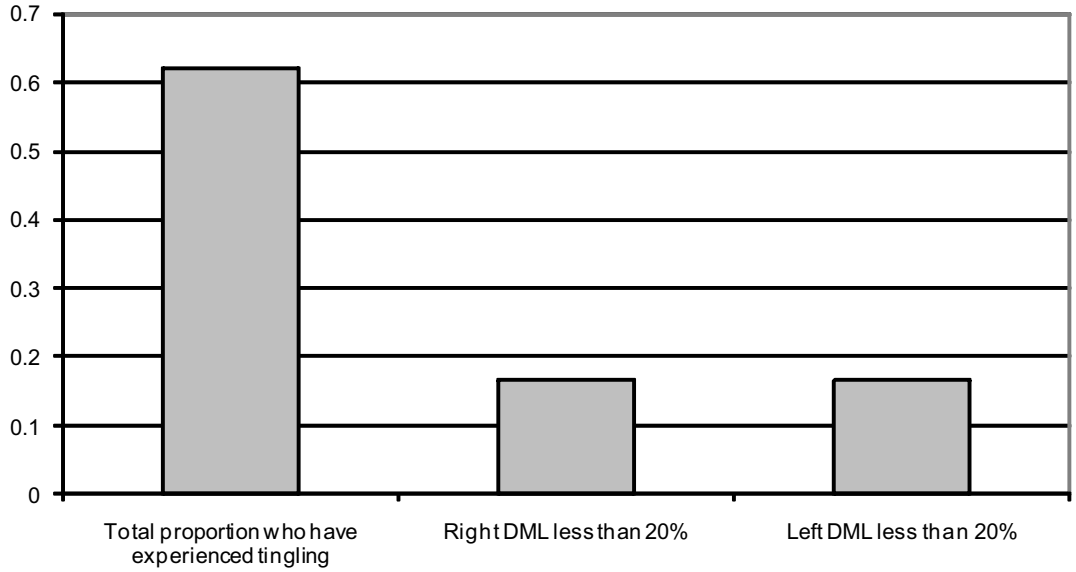

Figure 3. Proportion of musician who have experienced tingling, and of those musicians, the proportion that actually had electrophysiologic values below the 20th percentile. Data represent 29 total musicians.

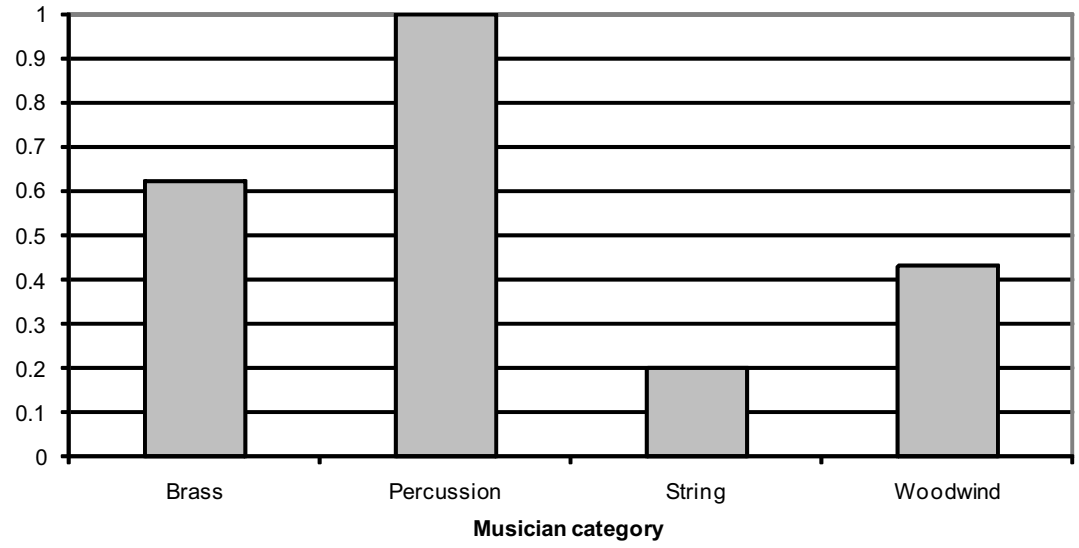

Figure 4. Proportion of musicians who have had reports of tingling in their fingers. Data represent 8 brass musicians, 8 percussion musicians, 5 string musicians, and 8 woodwindmusicians.

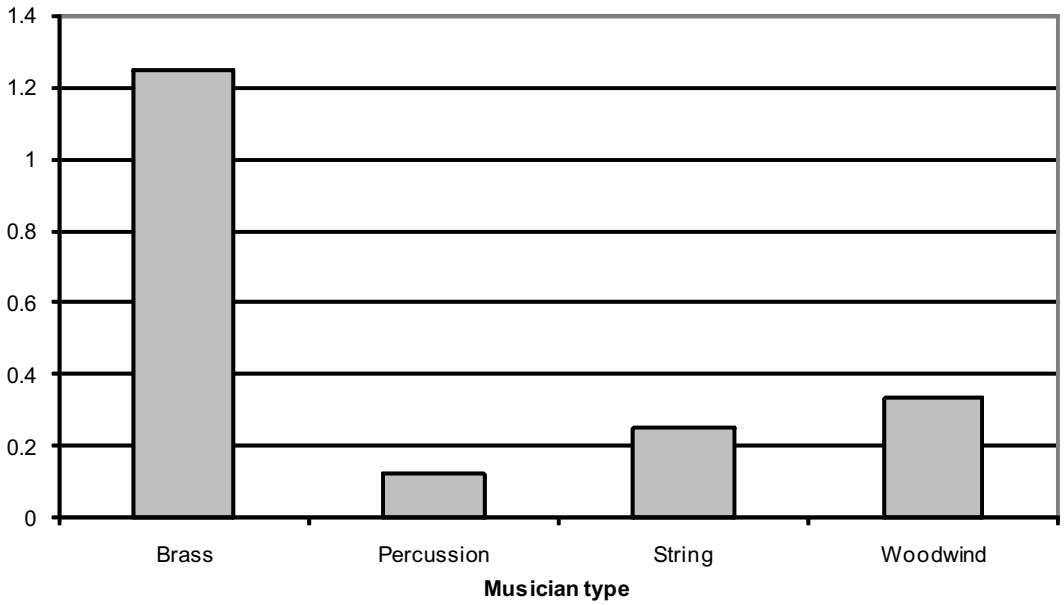

Figure 5. Average number of median nerve flags per musician. Data represent 8 brass musicians, 8 percussion musicians, 5 string musicians, and 9 woodwind musicians.

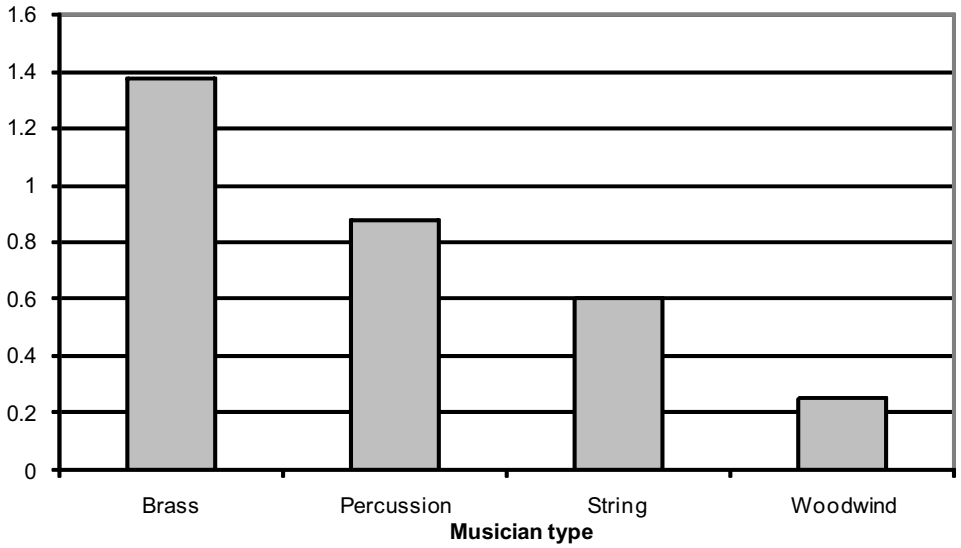

Figure 6. Average number of ulnar flags per musician. Data represent 7 brass musicians, 8 percussion musicians, 5 string musicians, and 8 woodwind musicians.

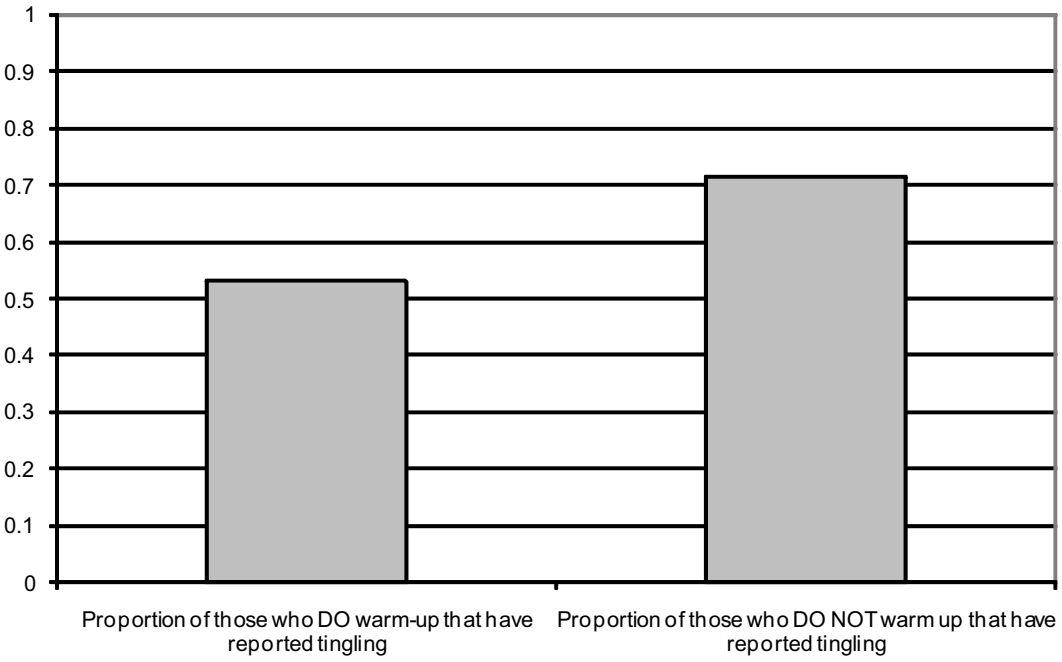

Figure 7. The correlation between performing a warm-up routine and reported experiences of tingling. Data represent survey results of 29 musicians.

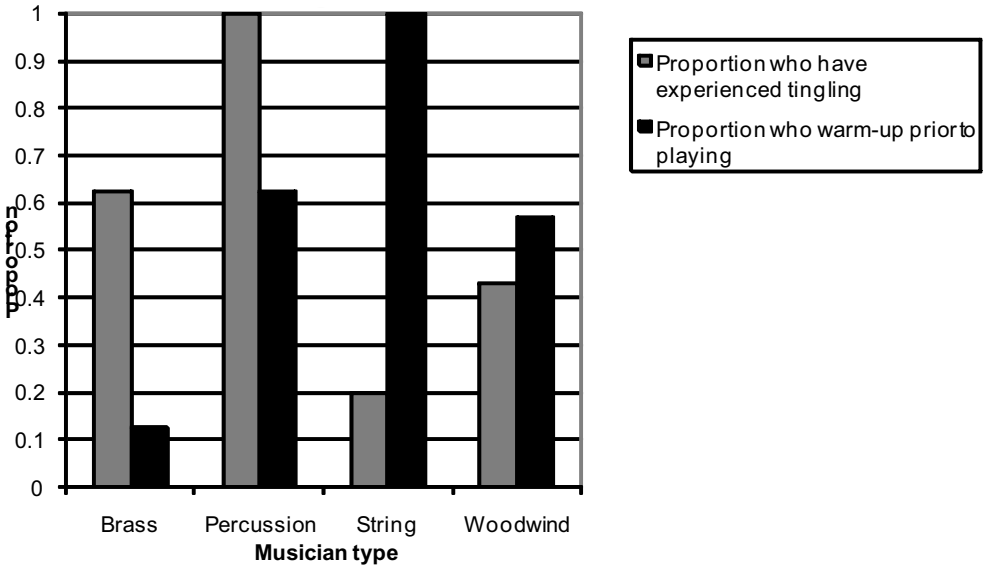

Figure 8. The correlation between tingling and warm-up for each musician group. Data represent the survey results for 8 brass musicians, 8 percussion musicians, 5 string musicians, and 8 woodwind musicians.

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Gas Exchange, Chlorophyll and Growth Responses of *Betula Platyphylla* Seedlings to Elevated CO_2 and Nitrogen

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Abstract

Effects of elevated [CO_2] and nitrogen nutrition on leaf gas exchange, chlorophyll content and growth in *Betula platyphylla* seedlings were studied. The seedlings were grown in the ambient [CO_2] (AC, 350μmol mol^{-1}) and elevated [CO_2] (EC, 700μmol mol^{-1}) growth chambers, with three levels of nitrogen: LN (0mmol L^{-1} N), MN (1.5mmol L^{-1} N) and HN (3mmol L^{-1} N). HN increased photosynthesis (P_{max}), photochemical efficiency of PSII (F_v/F_m) and intercellular [CO_2] (C_i) by 120%, 8% and 11% than the LN. EC significantly increased P_{max} and C_i by 37% and 57% compared to the AC. The interaction of EC and LN was significant increased by 85% than the AC+MN, and could be attributed to both inhibition of photosynthetic light reactions and carboxylation activity of Rubisco. In AC and EC, total biomass were 3.2 and 5.1 times greater in HN compared to LN. Root biomass increased significantly in HN level under both AC and EC. Chlorophyll (Chl) *a* and Chl *b* increased with increasing N availability.

Keywords: *Betula platyphylla* Suk., C_i, Elevated [CO_2], F_v/F_m, Nitrogen

1. Introduction

Elevated [CO_2] usually enhances photosynthesis (Teskey, 1997, pp.375; Matamala and Drake, 1999, pp.93; Elizabeth et al., 2007, pp.258), plant growth and carbon accumulation (King et al., 1996, pp.635). However, the magnitude of the response is generally affected by other environmental factors. Nitrogen availability often limits plant growth more than the availability of other nutrients (Crawford and Glass, 1998, pp.389). Most studies have shown that the degree of photosynthetic stimulation in response to elevated [CO_2] was larger in seedlings receiving high-N rates than in seedlings receiving low-N rates (Murray et al., 2000, pp.421). Murray et al. (2000, pp.421) reported that chlorophyll concentration increased with increasing N supply, however others have found that chlorophyll concentration was unaffected by growth [CO_2] (Carswell et al. 2000, pp.977). Most studies have shown that elevated [CO_2] increased biomass accumulation in seedlings supplied with high levels of available N, but it had little or no effect on seedlings growing in soil with low N availability (Murray et al., 2000, pp.421; Maillard et al., 2001, pp.163). To understand the response of plants to elevated [CO_2] it is important to consider nutrient acquisition as well.

White birch (*Betula platyphylla* Suk.) is a pioneer boreal tree species of northeastern Asia, plays an important role in the boreal forest region. However, its ecophysiological characteristics have been little studied. The purpose of this study

was to investigate the response of white birch to elevated [CO_2] and different nitrogen regimes, as well as their potential interactions, focusing on how these factors influence gas exchange, chlorophyll content and biomass accumulation. We hypothesized that elevated [CO_2] would enhance nitrogen absorption and chlorophyll concentration at all levels of nitrogen availability.

2. Materials and methods

Seeds of white birch were obtained from a local experimental forest near Northeast Forestry University of China (NEFUC) (45°N, 127°E) and were grown in a greenhouse of the key laboratory of forest plant ecology of NEFUC. Forty-five days after germination, one seedling was transferred to one pot (16cm diameter, 16cm height) containing black soil and sand (2:1) obtained from an arboretum close to the laboratory. Three different nitrogen treatments, with optimum application rate of 1.5 and lower of 0 and higher of 3 mmol L^{-1} N liquid fertilizer respectively, were maintained. NH_4NO_3 was used as the nitrogen source. Other nutrients were supplied at the same time in equal amounts to every pot using Hoagland's solution (Hoagland and Arnon, 1950, pp.32). Five pots of each nitrogen treatment were transferred to ambient [CO_2] chamber (AC, 350ppm) and elevated [CO_2] chamber (EC, 700ppm). The plants were grown in climatic chambers (Conviron E8, Canada) under a temperature and light regime that simulated local natural conditions (Figure 1). The experiment lasted for three months (July–September, 2006).

Net photosynthesis (P_{max}), dark respiration rate (R_d) and chlorophyll fluorescence were measured on the uppermost fully expanded leaf of the main branch, using a Li-6400 infra-red gas analyser (Li-Cor, Lincoln NE, USA). P_{max} was measured at 1400 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) and R_d was measured after the leaves had acclimated to the dark for about 5 minutes. Chlorophyll fluorescence of the leaves was determined using a fluorometer (Li-6400-40, Li-Cor, Lincoln NE, USA). after adapted in darkness for at least 20 minutes to allow relaxation of fluorescence quenching associated with thylakoid membrane energization (Jung, 1998, pp.71). The ratio of variable to maximum fluorescence (F_v/F_m) derived from the measurement was used as a measure of the maximum photochemical efficiency of PSII (Butler, 1978, pp.348).

The chlorophyll content of leaves was determined by the dimethyl sulphoxide (DMSO) method (Küster, 2004, pp.114) Plants were divided into leaf, stem, branch and root. Root was divided into primary (<1mm), secondary (1mm-2mm) and tap (>2mm) root (King, 1996, pp.637). All tissues were dried in an oven at 70 °C to constant weight.

Five replicates of each treatment were evaluated by two-way analysis of variance (ANOVA) for P_{max}, R_d, F_v/F_m, C_i, Chl and biomass. When the interaction between nutrient and [CO_2] treatments was significant for a given parameter, a LSD test was conducted at the 0.05 significance level. The comparisons of AC and EC treatments were mean values for three nitrogen levels, respectively.

3. Results

3.1 Photosynthetic parameters

Under ambient [CO_2], increasing N availability increased P_{max} (Figure 2, Table 1). Compared with the LN treatment, the HN treatment increased P_{max} by 120%. The HN treatment also had higher F_v/F_m than either the LN or medium N (MN) treatments, increased by 8% relative to LN. C_i was 11 % and 15% higher than in the LN and MN treatment, which were 302 and 291μmol mol^{-1}, respectively. Thus, under ambient [CO_2], higher nitrogen availability promoted photosynthetic capacity of birch seedlings in both the light and dark reactions. There were no statistically significant differences in the rates of respiration among the different nitrogen levels (Figure 2D), indicating that leaf-level carbon use efficiency increased. Elevated [CO_2] increased P_{max}, F_v/F_m and C_i by 37%, 2% and 57%, respectively. There were some interactions between [CO_2] and nitrogen availability (Figure 2). The LN treatment caused a large increase in C_i in elevated [CO_2] (P<0.01), but not in ambient [CO_2]. The value of C_i in the EC+LN treatment was 85% higher than in the AC+MN treatment.

3.2 Chlorophyll content

Chlorophyll content increased with increasing nitrogen level (Table 2). The pattern was the same as the change in P_{max}. In AC treatment, HN treatment increased the Chl *a* and Chl *b* by 25% and 38% than the MN treatment (P>0.05). And LN treatment reduced the Chl *a* and Chl *b* by 66% and 61% than the MN treatment (P<0.05). There was no significant difference on the Chl *a*+*b* among the different nitrogen treatments. EC treatment had no significant effect on chlorophyll content. Neither elevated CO_2 nor nitrogen treatment had any significant effect on the Chl *b* content. However, the LN treatment decreased Chl *a*+*b* by 72% although the decrease was significant only under the EC treatment.

3.3 Biomass

Nitrogen treatment had significant effects on the total and component biomass of the seedlings (P<0.0001)(Table 1 and Figure 3). The [CO_2] treatment affected total biomass (P=0.068), total root (P=0.008) and secondary (P=0.003) root biomass. There were no significant differences in stem, leaf or primary root biomass between the two [CO_2] treatments,

indicating that the main growth response to [CO_2] was in secondary roots. In addition, there were no [CO_2] and N interactions for any of the biomass parameters, except for stem.

In ambient [CO_2], compared to the MN treatment, HN treatment caused an increase in total biomass and root, stem, leaf and primary root biomass of 80%, 21%, 150%, 176% and 58%, respectively but a decrease in secondary root biomass by 4%. The LN treatment decreased the response by 181%, 163%, 186% 300%, 153% and 176% in total, root, stem, leaf, secondary and primary root biomass, respectively compared to the MN treatment.

Elevated [CO_2] treatment caused an increase by 16%, 11%, 18%, 52%, 22% increase in total, root, stem, leaf and secondary root biomass, respectively, but an reduce by 2% in primary root biomass. There was significant interaction between [CO_2] and nitrogen availability on stem (P<0.0001) (Table 1 and Figure 3). The EC+HN treatment increased stem biomass by 103% and the EC+LN treatment reduced that by 47% relative to AC+MN treatment.

4. Discussion

Our study suggests that nitrogen availability had more important effects on the growth of white birch seedlings than elevated [CO_2]. The increase in biomass with higher N availability corresponded with increased P_{max} and a lack of increase in R_d. Increased soil nitrogen concentration caused a great enhancement in photosynthesis in the birch seedlings, suggesting this was the main cause of the growth response. Increased N availability also enhanced F_v/F_m. F_v/F_m reflects the potential quantum efficiency of PSII and is used as an indicator of plant photosynthetic performance15. Increased nitrogen supply to plants has been reported to cause an increase in F_v/F_m in many plants (Kaakinen et al., 2004, pp.712). In ambient [CO_2], HN also increased intercellular CO_2 concentration, indicating that stomatal conductance was higher. The HN treatment also increased P_{max}, thus, in ambient [CO_2] the HN treatment promoted both the light and carbon reactions synchronously, greatly enhancing the photosynthetic capacity of the birch seedlings. Nitrogen is often the most limiting mineral element in plants and the crucial component of chlorophyll (Li, 2000, pp.190) so it is not surprising that chlorophyll concentration was strongly affected by nitrogen availably. These results are agreement with the finding of Guo et al. (2005, pp.589) who reported that total chlorophyll content was lower in larch seedlings receiving low N fertilization rates than in seedlings receiving high-N rates. There was a good correlation between chlorophyll content, F_v/F_m and photosynthetic capacity in the birch leaves. In contrast, dark respiration did not significantly differ among the six treatments, suggesting that higher N fertilization enhanced carbon use efficiency.

In the present study, EC+HN and EC+MN treatment promoted total biomass accumulation of birch seedlings to a greater extent than the EC-LN (P=0.003 and P=0.001) (Figure 3). This is consistent with Uprety and Mahalaxmi(2000, pp.272) who reported that nitrogen fertilizer significantly increased the dry weights of biomass including leaves (41%), stems (15%) and roots (11%) in elevated [CO_2] conditions. EC increased leaf, stem and root growth over AC, and the pattern were the same in the LN, MN and HN treatments.

Elevated [CO_2] increased P_{max} in MN and HN treatment, but not in LN treatment. Elevated [CO_2] significantly increased C_i in LN and MN treatments (Table 1, Figure 2). The higher C_i and lack of change in P_{max} in the EC+LN treatment indicated that N availability was more limiting to photosynthetic capacity than the [CO_2]. Elevated [CO_2] reduced the Chl a+b and Chl a. This is consistent with Luomala et al. (2003, pp.657) who reported that elevated [CO_2] reduced chlorophyll content. Uprety and Mahalaxmi (2000, pp.274) attributed such a reduction to the reduction in Rubisco.

Typically, elevated [CO_2] enhances the rate of photosynthesis by promoting carboxylation of Rubisco. Ribulose-1,5-bisphosphate (RuBP) is the principal substrate in the Calvin cycle, catalyzed by the enzyme Rubisco. The concentrations of CO_2 and O_2 strongly affect the catalyzing reaction. If the CO_2 concentration increases, more combinative positions of Rubisco are taken by CO_2 and the rate of carboxylation (carbon-reduction of photosynthesis, PCR) increases, therefore, the rate of photosynthesis increases. Likewise, the ratio of [O_2]/[CO_2] inside chloroplasts declines, passivating enzymes of glycolic acid cycle. Thus photorespiration (carbon-oxidation of photosynthesis, PCO) is restricted and net rate of photosynthesis increases. However, the results from this experiment suggest nitrogen has a bigger limiting effect on seedlings than carbon, in other words, even under elevated [CO_2], the nitrogen level will be also more pivotal. i.e., elevated [CO_2] promoted absorption of high nitrogen. This conclusion is consistent with the conclusions of Johnson et al. (2000, pp.117) and Demmers-Derks et al. (1998, pp.829). They found a similar lack of growth response to elevated [CO_2] at low nutrition supply (It was a CO_2 and temperature study) Also see Lewis et al. (2003, pp.359), who did not find much response to CO_2 and concluded that N was an important controlling factor.

In EC+LN condition F_v/F_m was much lower but C_i was much higher comparing with that in AC+MN. The decline of F_v/F_m is a remarkable characteristic of photosynthetic photoinhibition, and generally seemed as a criterion to judge if photoinhibition or not (Guo et al., 2005, pp.592). A similar decrease of F_v/F_m under nitrogen-poor had also been found in larch (Guo et al., 2005, pp.589). A possible explanation for the high C_i is that when the C/N ratio is imbalanced, photosynthetic substrates are reduced and yield less NADPH and ATP, suppressing the light reactions. At the same time,

low nitrogen supply reduces Rubisco activity, the rate of carboxylation declines and assimilation of CO_2 is reduced (Guo et al., 2005, pp.592). Thus, even with enough CO_2 the EC+LN treatment has less photosynthetic capacity and biomass than EC+MN or EC+HN. Other studies have reported that photosynthetic acclimation, the shift to a decreased carboxylation capacity in elevated [CO_2], is more marked in nutrient-limited than in well-fertilized plants, and that elevated [CO_2] leads to a larger decrease of Rubisco in nitrogen-limited plants than in well-fertilized plants (Guo et al., 2005, pp.562). Elevated [CO_2] promoted growth of seedlings to a certain extent in the EC+LN treatment, but N availability was insufficient to allow full utilization of the increased [CO_2].

The investigation indicates that, as atmospheric [CO_2] increases, gas exchange and growth of white birch is responsive to nitrogen availability by a balanced C/N ratio. In the future, nitrogen nutrition needs to be maintained for gaining greater productivity of plants.

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Table 1. *P*-values of two-way ANOVA for the effects of [CO_2], nitrogen availability and their interaction on maximal net photosynthetic rate (P_{max}), respiration (R_d), intercellular [CO_2] (C_i), photochemical efficiency of PSII (F_v/F_m), Chlorophyll (Chl) *a*+*b*, Chl *a*, Chl *b* and root, stem, leaf and total biomass of white birch seedlings. The seedlings were grown under two [CO_2] (350ppm and 700ppm) and three levels of nitrogen treatments (low, medium and high) for about three months.

Source of variance	CO_2	Nitrogen	CO_2×Nitrogen
P_{max}	0.033	<0.0001	0.073
R_d	0.301	0.861	0.922
C_i	<0.0001	0.045	0.006
F_v/F_m	0.734	<0.0001	0.055
Chl *a*+*b*	0.147	0.350	0.003
Chl *a*	0.089	0.005	0.577
Chl *b*	0.281	0.013	0.702
Total biomass	0.068	<0.0001	0.472
Root	0.008	<0.0001	0.401
Stem	0.613	<0.0001	0.004
Leaf	0.228	<0.0001	0.852
Secondary root	0.003	<0.0001	0.871
Primary root	0.151	<0.0001	0.429

Table 2. Effects of [CO_2] and nitrogen treatments on chlorophyll contents (Chl *a*, Chl *b*, Chl *a*+*b*) in white birch seedlings. Data represent the mean± SE of three replicates. See Figure 1 for other explanations.

CO_2 treatment	Nitrogen treatment (mmol L^{-1} N)	Chl *a* (mg g^{-1})	Chl *b* (mg g^{-1})	Chl *a*+*b* (mg g^{-1})
Ambient [CO_2]	0 (LN)	0.76±0.03b	0.23±0.01b	1.00±0.04a
	1.5(MN)	1.26±0.05a	0.37±0.02ab	1.63±0.07a
	3(HN)	1.58±0.37a	0.51±0.15a	2.09±0.52a
Elevated [CO_2]	0 (LN)	0.72±0.09b	0.24±0.04a	0.95±0.12b
	1.5(MN)	0.89±0.24a	0.29±0.07a	1.18±0.31a
	3(HN)	1.25±0.07a	0.41±0.02a	1.67±0.07a

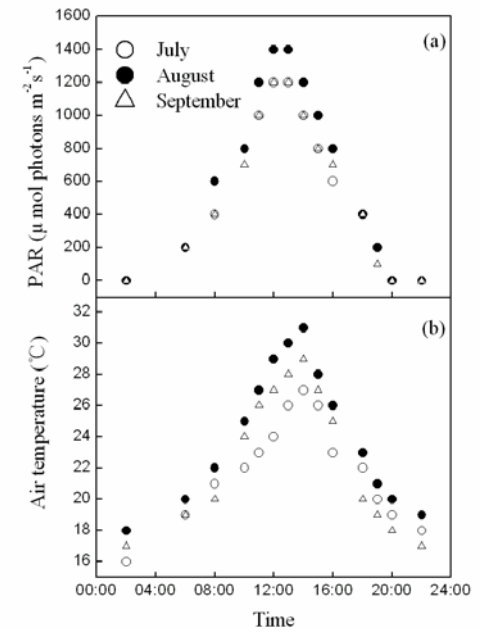

Figure 1. Diurnal pattern of (a) photosynthetically active radiation (PAR) and (b) air temperature during the experiment. The seedlings were grown under two [CO_2] (350ppm versus 700ppm) and three levels of nitrogen amendment, low (LN), medium (MN) and high (HN) for about three months.

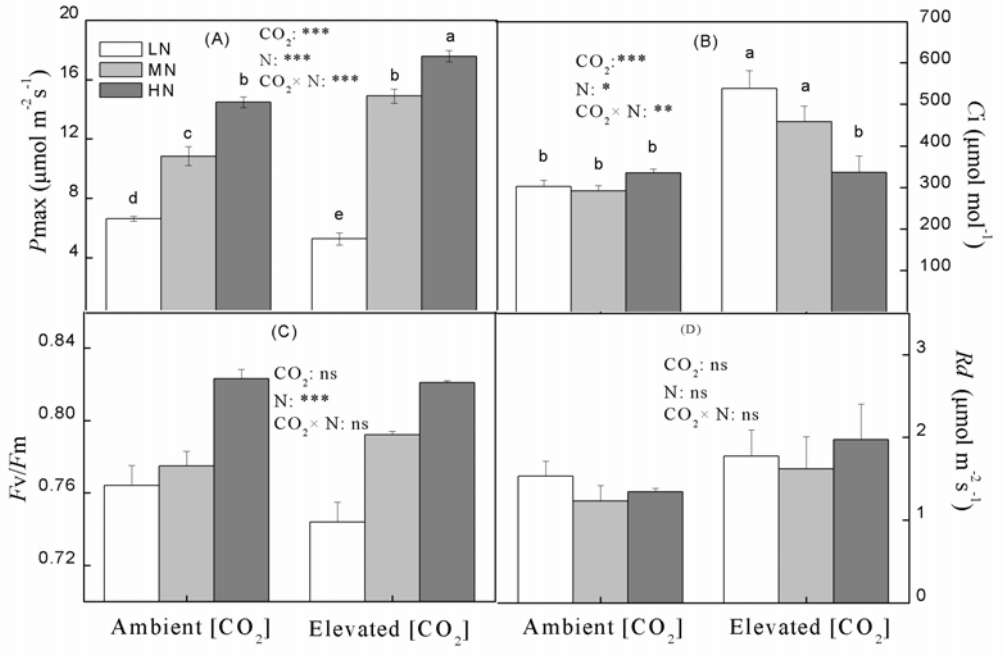

Figure 2. Effects of [CO_2] and nitrogen level on (A): maximal net photosynthetic rate (P_{max}), (B): intercellular CO_2 concentration (C_i), (C): maximum PSⅡ efficiency (F_v/F_m) and (D): respiration rate (R_d) in current-year-old white birch seedlings. The seedlings were grown under two [CO_2] (350ppm versus 700ppm) and three levels of nitrogen amendment, low (LN), medium (MN) and high (HN) for about three months. (mean ± SE, n = 5). Significance values (*: P<0.05; **: P<0.01; ***: P<0.001 and ns: P>0.05) are based on two-way ANOVA. LSD test was conducted where there was a significant interaction between [CO_2] and nitrogen treatment. Means sharing the same letter are not significantly different from each other.

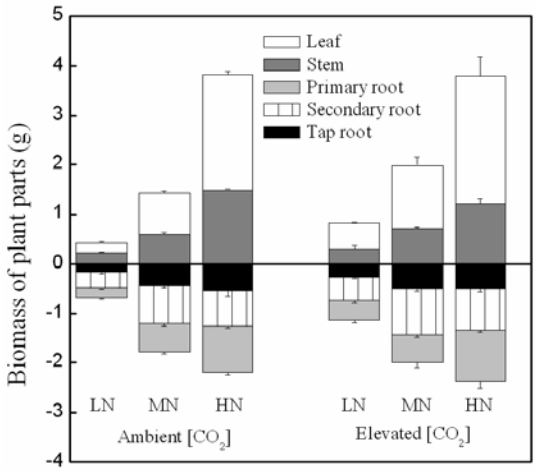

Figure 3. Aboveground (upper part of chart) and belowground (lower part of chart) biomass at two [CO_2] (350ppm and 700ppm) and three nitrogen treatments (low, medium and high). (Mean ± SE, n = 5). See Figure 1 for other explanations.

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Effects of Sucrose on Germination and Seedling Development of *Brassica Napus*

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Abstract

Sucrose is the major carbon form translocated in higher plants, and also works as an important signaling molecule that regulates genes involved in photosynthesis, metabolism, and developmental processes. Many studies focused on glucose, normal decomposed product of sucrose, demonstrated that glucose resulted in a delay of germination and an inhibition of seedling development. In this study, we determined the effects of sucrose on germination kinetics and seedlings development. Our results indicated that exogenous sucrose didn't have a similar role to glucose, showed 167mM sucrose delayed the rate of seed germination in wild-ecotype seeds by extending peak time of germination, and had multiple roles to seedling development. Overhigh concentration of sucrose (333mM) restrained germination and seedling development seriously. Further experiments of transcript profiles of genes involved in energy and carbohydrate metabolism indicated that seedlings absorbed and utilized the exogenous sucrose as a priority while reduced the need for internal source.

Keywords: *Brassica napus*, Sucrose, Germination, Seedling

1. Introduction

Sucrose is an important carbohydrate in most plants. It has multiple functions such as regulating photosynthesis and respiration, serving as storage compound and helping to maintain the osmotic pressure in the cytosol (Rita Teresa Teixeira, 2005).In oilseeds, sucrose is major carbohydrate transport form upon seeds germination and seedling development.

During germination and seedling development, storage triacylglycerols are cleaved by lipases from their glycerol backbone in the oil body, and convert to carbohydrates major as sucrose for transport to the root and shoot axes (Eastmond, 2006). Sucrose is used as a substrate for biosynthesis and is respired for energy. After being transported to the glyoxysome, sucrose is degraded by beta-oxidation to acetyl-CoA. The glyoxylate cycle ultimately catalyzes the condensation of two of these acetyl-CoA molecules to form succinate, which is then transported to the mitochondrion and metabolized by a partial TCA cycle (Kimberly L. Falk, 1998).

Sucrose also functions as an important signaling molecule in the regulation of germination and seedling development although sucrose is readily converted into fructose and glucose. Combined treatments of glucose and fructose were less

effective than sucrose treatments (Francisco Arenas-Huertero, 2000), suggesting that sucrose functions as a signaling molecule.

The sucrose-mediated translational control mechanism is an important sucrose-dependent regulation of mRNA translation. Sucrose acts as a signaling molecule in the control of translation of the S1 class basic leucine zipper transcription factor bZIP (Hummel M, 2009). The main bZIP open reading frames (ORFs) are preceded by upstream open reading frames (uORFs), which have general function of Sucrose-induced repression of translation (SIRT) (Anika Wiese, 2004). Arabidopsis homologues of ATB2/AtbZIP11, which harbour the conserved uORF, also show SIRT(Wiese A, 2005). AtbZIP11 is translational repressed in response to sucrose. Target genes of such transcription factors will then be regulated in sucrose-dependent way, resulting in sucrose-regulated changes in metabolism (Rahmani F, 2009).

Previous study always focused on glucose, which illustrated that glucose negatively affected seed germination and early seedling development. Sucrose is an important carbon source for development in plants and wildly used in plant culture. So we evaluated the role of sucrose on germination and seedling development of *Brassica napus*. Our findings suggested that extraneous sucrose influenced activity of seedling metabolism dramatically, both as metabolite and singalling molecular in *Brassica napus*, and regulated expression of the genes involved in energy and carbohydrate metabolisms.

2. Materials and methods

2.1 Growth of Plant Material and measurement of developmental conditions

Brassica napus seeds were surface-sterilized and water-imbibed in the dark for 2 d at 4°C. Seeds were transferred to 1×Murashige and Skoog medium, sucrose was also added where indicated. The plant material was incubated in the dark at 4°C for 2d to break dormancy and then was transferred to light at 24°C. All cultures were grown with a photoperiod of 16 h of light and 8 h of dark. Germination kinetics was determined by measuring the time of radicle emergence from repeated experiments with duplicate plates of approximately 80 seeds, and selected the seeds germinating in the same period to measure the length of hypocotyls and roots. to be used for phenotype analyses.

2.2 RNA Extractions and Reverse transcription

The whole developing seedlings were sampled at 2, 4 and 6 days after germination (DAG), respectively, and stored at -70°C for RNA isolation. Total RNA was extracted by Trizol (invitrogen) reagent. The RNA was quantitated by A260 measurements and by ethidium bromide staining intensity and then stored as an ethanol precipitate at -20°C until use. cDNA was synthesized using 2μg of RNA from each treatment by M-MLV Reverse Transcriptase (Takara, Japan) according to the manufacture's protocol.

2.3 Semi-quantitative reverse transcription (RT)-PCR analysis

For RT-PCR assays, the cDNAs of different treatments were used as template. The primers sequences are shown in Table 1. The PCR were performed as following procedure: Denatured at 95°C for 4 min followed by 35 cycles (95°C for 35s, 56°C for 40s and 72°C for 1 min). The same cDNAs were also used to amplify the Actin as an internal control. 10μl products of each reaction were electrophoresed on 1% agarose gel stained with ethidium bromide.

3. Results

3.1 Exogenous sucrose delay the seed germination of Brassica napus

As degraded product of sucrose, glucose caused ABA accumulation, which resulted in a delay of germination in wide levels of concentration (John Price, 2003). As our results showed, exogenous sucrose also caused a delay of germination like glucose (Figure1). We compared germination kinetics with intermediate (167mM), high (333mM) concentration of sucrose. The difference from glucose was that seeds treated with intermediate concentration of sucrose (167mM sucrose) and WT seeds reached the peak time of germination at the same time, while seeds treated with 167mM sucrose led to the extension of active phase of germination. What was similar to the seeds treated with glucose was that high concentration of glucose inhibited the seeds from germinating seriously and resulted in a delay of germination peak time (figure2).

3.2 Exogenous sucrose has multiple roles on seedling development

It has been suggested that availability of abundant glucose can exert a profound influence, resulting in seedling developmental arrest(Jang JC, 1997).Similar to glucose, High levels of exogenous sucrose inhibited seedlings development seriously, and this effect could not be mimicked by an osmotic effect (John Price, 2003). Our results showed that in *Brassica napus*, the development of hypocotyls was inhibited (figure 3) while roots were promoted (figure 4) in the medium containing 167mM sucrose, which was different from the pattern in *Arabidopsis.*

3.3 Exogenous sucrose regulates expression of the key genes involved in metabolism

Exogenous sucrose as an environmental factor greatly impacted plant gene expression and concentrations of cellular

metabolites (Solfanelli C, 2006; Hummel M, 2009). In this study, expression profiles of genes involved in energy metabolism in *Brassica napus* were analyzed (Figure 5). Hexokinase (HXK) and pyruvate dehydrogenase (PDH) are rate limited enzymes in glycolysis, HXK also plays a central role as a conserved glucose sensor in sugar signaling pathway (John Price, 2003). Citrate synthase (CS) is the first enzyme of the tricarboxylic-acid cycle. ADP-glucose pyrophosphorylase (ADG) is a key-enzyme of starch synthesis in plant. Glyoxysomal Isocitrate lyase (GIL) is unique to the glyoxylate cycle. As to the control treated without sucrose, each gene had the similar transcription profile, and attended to the top point at the 4 DAG, then decreased. Surprisingly, exogenous sucrose affected the expression of all genes strongly. Collectively, exogenous sucrose restrained expression of all these genes. Transcripts of *BnHXK* and *BnPDH* began to accumulate at the 2 DAG. Conversely, *BnCS*, *BnADG* and *BnGIL* gradually decreased. It's suggested that exogenous sucrose influenced the energy and carbohydrate metabolism of the seedling dramatically.

4. Discussion

Our results indicated that germination and seedling development of *Brassica napus* was regulated by sucrose, and sucrose has more complicated roles compared with glucose. For the seedlings of *Brassica napus*, exogenous 167mM sucrose only inhibited the hypocotyls growth but promoted the roots, while exogenous 167mM glucose caused ABA accumulation and inhibited seedling development in Arabidopsis (John Price, 2003). Our results implied that Sucrose promoted the roots growth probably because the sucrose was absorbed and used as carbon source by the roots, which improved growth of roots by counteracting negative regulating of sucrose signaling. However, the hypocotyls couldn't absorb exogenous sucrose, and the growth was inhibited by the sugar signaling. As for this process, whether the ABA work as an important signaling molecule is not clear.

Transcript profiles also indicated that sucrose play important roles in seedling developments. Compared to the controls, the expression of genes involved in metabolism in seedling treated with sucrose had been inhibited obviously and had complicated alterations. These results suggested that the seedlings absorbed and utilized the exogenous sucrose as a priority while reduced the need for internal source. The decreased expressions of *BnCS, BnADG* and *BnGIL* indicate the depression of the gluconeogenesis. While the expression of *BnPDH* and *BnHXK* related with catabolism which take sugar as their substrate (mainly glucose) increased gradually. Glucose, the decomposed products of sucrose, might play an important role in this regulation. It also has been reported that glucose affect the expression of a diverse array of genes involved in carbohydrate metabolism (Wenyan Xiao, 2000; John Price, 2003),

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Table 1. Oligonucleotide primers used in RT-PCR analysis

Genes	Accession numbers	Primer	Nucleotide sequence
BnGIL	Y13356	F	5'- ATCCATACGATACCGTTCCTAA
		R	5'- TCATCTGCTGATCCGTCATACC
BnHXK	AF454961	F	5'- CTCGGTGGCAAGCAAGAC
		R	5'- GCATAGCAGACATGGTAGGA
BnPDH	ES902002	F	5'- TTACGGGGGCTCCTACAA
		R	5'- ACACTCCTCCACTATCAAAACC
BnCS	ES900694	F	5'- GTATCAGGTCCCAGTCTCGG
		R	5'- CAACACCACTAGAGGCAAGA
BnADG	AJ271162	F	CGTCCGCAAAACTCTTCAAC
		R	TCTATCTCCTGCTTTCTCGC
Actin	AY570244	F	5'-ATGGCCGATGGTGAGGACATTC
		R	5'-GGTGCGACCACCTTGATCTTC

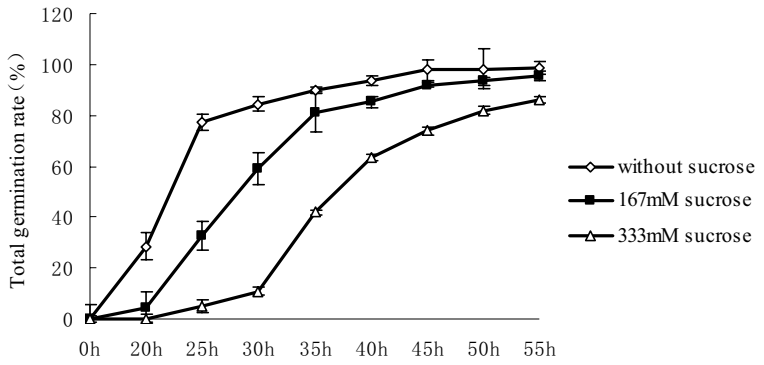

Figure 1. Exogenous sucrose resulted in a delay of germination as glucose. *Brassica napus* seeds were surface-sterilized and water-imbibed in the dark for 2 d at 4°C. Seeds were transferred to Murashige and Skoog plates without sucrose or with sucrose (concentration indicated in the figure). The plant material was incubated in the dark at 4°C for 2 d to break dormancy and was then transferred to light at 24°C. Germination kinetics was determined by measuring the time of radicle emergence after transfer to constant light and 24°C.

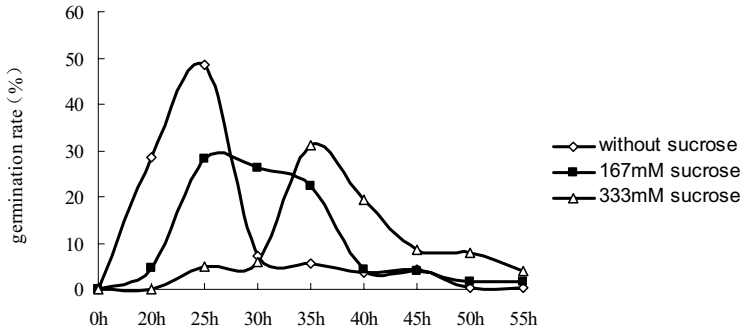

Figure 2. Compared with control treated without sucrose, 167mM sucrose treatment caused the extension of peak time of germination, while 333mM sucrose resulted in a delay of peak time of germination as glucose.

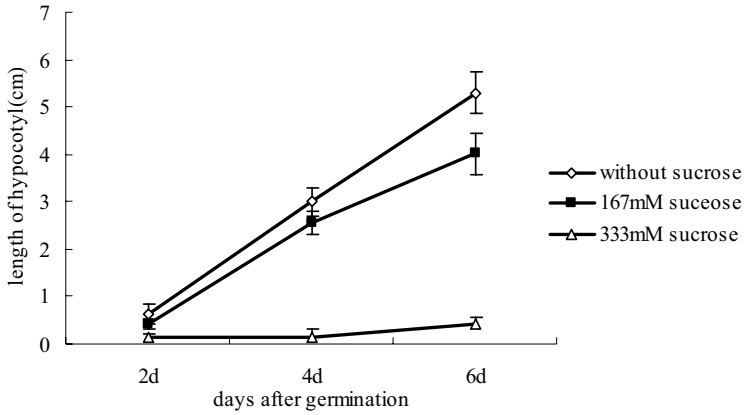

Figure 3. The development of hypocotyls was inhibited in Murashige and Skoog plates containing sucrose, especially in the medium with 333mM sucrose, and this effect could not be mimicked by an osmotic effect.

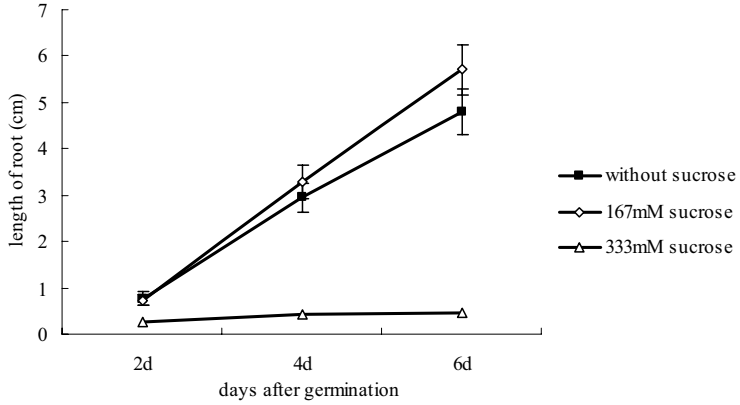

Figure 4. Contrast to hypocotyls, development of roots was promoted in Murashige and Skoog plates containing 167mM sucrose. While in the medium with 333mM sucrose, development of roots was still inhibited strongly.

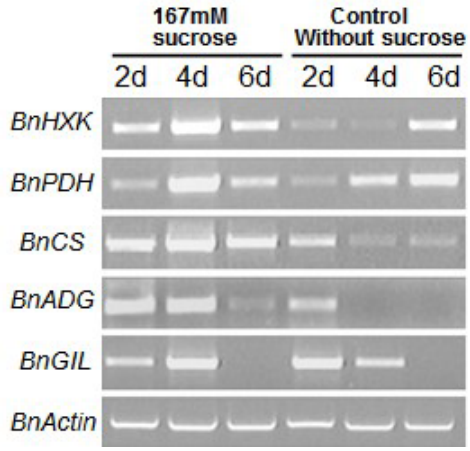

Figure 5. Exogenous sucrose regulated expression of the key genes involved in metabolism. As to the control treated without sucrose, each gene had the similar transcription profile, and at the 4 DAG attends to the top point, then decreases. Surprisingly, exogenous sucrose affected the expression of all genes strongly. Transcripts of *BnHXK* and *BnPDH* began to accumulate at the 2DAG, conversely, *BnCS*, *BnADG* and *BnGIL* gradually decreased. Generally speaking, exogenous sucrose restrained expression of all genes.

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Trace and Toxic Elements Accumulation in Food Chain Representatives at Livingston Island (Antarctica)

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Abstract

First data for concentrations and distribution of trace and toxic elements in tissues and organs of brown skua (*Catharacta lonnbergi*), Gentoo penguin (*Pygoscelis papua*), crabeater seal (*Lobodon carcinophagus*), notothenioid fish (*Notothenia coriiceps*) and in krill (*Euphausia superba*), collected on Livingston Island (South Shetlands), Antarctica are presented. Lead, cadmium, copper, zinc, manganese and cobalt were studied in liver, kidney, bone, spleen, muscle, heart and feathers. Generally, higher concentrations of toxic elements were found in liver and kidneys than in other investigated tissues. The same elements in krill and notothenioid fish were also determined. The obtained data could

serve as a starting point for broader investigation of the possible changes in the ecosystem of Antarctic Peninsula under climate and environmental changes.

Keywords: Heavy metals, Brown skua, Gentoo penguins, Crabeater seal, Notothenioid fish, Krill

1. Introduction

For the study of toxic elements concentrations in predators' fauna Antarctica is an area of special interest. It was believed that such remote environment was practically unpolluted. Recently however anthropogenic source seems to be an explanation for a significant excess of lead and zinc concentrations in the environment (Dick, 1991). Along with this, heavy metals may be high in Antarctic wildlife, which is also the case for the increasing quantity of cadmium (Honda et al., 1986; Szefer et al., 1994). Upwelling of natural cadmium rich deep water (Holm-Hansen, 1985) might be the source for elevated cadmium loads in krill (*Euphausia superba*) (Petri and Zauke, 1993). It is the main food source for most Antarctic marine predators and is present all year round in marine surface layers (Smith et al. 1995). It composes a substantial part of the diet of notothenioid fish (*Notothenia coriiceps*) (Barrera-Oro, 2003), crabeater seals (*Lobodon carcinophagus*) (Siniff, 1991) and Gentoo penguins (*Pygoscelis papua ellsworthi*) (Volkmann et al., 1980). Brown skua (*Catharacta lonnbergi*) feedings on penguin eggs, chicks, krill, fish and scavenge (Smith et al., 1995) represents as one of the top predators of an Antarctic food web. All these species are excellent subjects for examination of heavy metals presence because they are long living, feed at different distances from land and exhibit different trophic levels (Smith et al., 1995; Trivelpiece and Trivelpiece, 1990; Walsh, 1990).

Since all Antarctic species are protected under the Antarctic treaty (from 1959) there is impossible to collect animals tissues and organs for analyzes. Because of that in any case when is possible it is convenient to collect biological materials from the dead animals. This way becomes a chance to provide additional to the existing scare literature data.

Several studies report metal concentrations in the predators' tissues of various Antarctic areas. However no enough data for a set of representatives from different levels of the relatively short trophic chain from many areas are available to date. The objective of the study is to gather baseline information for trace metals lead, cadmium, copper, zinc, manganese and cobalt in Antarctic Ocean biota from Livingston Island. It may be needed to detect, measure and monitor future environmental changes and aimed at contributing on potential pollutants of ecotoxicological interest in the Antarctic ecosystem.

2. Material and Methods

The present material consisted of different Antarctic animals, mainly top predators collected during three Antarctic summer seasons (2002/3, 2003/4, 2005/6) at South Bay, Livingston Island (62º38´29´´ S and 60º24´53´´ W), South Shetlands. The island lies in the zone of oceanic Antarctic climate.

Along with the fresh dead specimens, an opportunistic approach in collecting sample material was used. Only one brown skua (*Catharacta lonnbergi*), crab eater seal (*Lobodon carcinophagus*) and Gentoo penguin (*Pygoscelis papua ellsworthi*) were collected. The notothenioid fish (*Notothenia coriiceps*) (n = 3) and krill (*Euphausia superba*) (n = 19) were collected deliberately by fishing and using a zooplanctonic net.

Tissue concentrations of trace elements (Pb, Cd, Cu, Zn, Mn and Co) were determined in liver, kidneys, spleen, bones, muscle, heart, feathers in adult individuals of brown skua, Gentoo penguin and crab eater seal. The same elements in the whole body of krill and notothenioid fish were determined.

All the collected samples were dried at 60 ºC until constant weight and wet mineralized. About 1-1.5 g of the dried material was treated with 15 ml nitric acid (9.67 M) overnight. The procedure was continued with heating in a water bath, followed by addition of 2 ml hydrogen peroxide. This treatment was repeated until full digestion. The filtrate was diluted with double distilled water up to 25 ml. All solutions were stored in plastic flasks. Duplicates of each sample were prepared independently. The elements (Mn, Zn, Cu, Pb, Cd and Co) were determined by atomic emission spectrometry (AES) with inductively coupled plasma (ICP). VARIAN VISTA-PRO instrument was used. The detection limits in the samples analyzed were: 0.004 mg/l for Zn, Cu, Cd and Co, 0.002 mg/l for Mn, and 0.03 mg/l for Pb. The analytical precision was secured by replicating (deviation between the duplicates was below 5% in all cases) and by use of blanks and stock standard solutions (1000 μg/ml Merck) for the preparation of working aqueous solutions. The mean analytical errors of the various samples (3 measurements for each solution) were higher for Pb (40.0%), Co (30.6%), Cd (23.6%), followed by Mn (9.0%), Cu (5.0%) and Zn (1.0%). Quality control was checked by standard reference materials (CRM 281 and CRM 142R). The concentrations were expressed as mg/kg dry weight.

3. Results and Discussion

3.1 Antarctic krill (Euphausia superba)

Mean concentrations of Pb (0.40 ± 0.18 mg/kg), Cd (0.60 ± 0.14 mg/kg), Cu (42.8 ± 0.33 mg/kg), Zn (50.0 ± 0.49 mg/kg), Mn (14.0 ± 0.14 mg/kg) and Co (0.40 ± 0.12 mg/kg) in krill sampled at Livingston Island (Table 1) were comparable to the values for Pb, Cd, Cu, Zn and Mn obtained from East Antarctica. Karasawa and Mishima (1981),

Honda et al. (1987), Yamamoto et al. (1996). Stoeppler and Brandt (1979) and Soszka et al. (1981), who examined some trace elements in the Antarctic krill in the areas of Scotia Sea, Bellingshausen Sea and the Weddell Sea, found comparable levels. Locarnini and Presley (1995) obtained similar values along the Antarctic Peninsula. The relatively high concentrations of Cd found in krill in the present study should be the main reason for elevated levels in its predators.

3.2 Notothenioid fish (Notothenia coriiceps)

Trace and toxic elements concentrations data in the whole body of notothenioid fish are scarce. Such data are important when tracing biomagnifications in Antarctic trophic chains. The results for *Notothenia coriiceps* from South Bay (Livingston Island) presented on table 1, were: Pb (0.76 – 1.34 mg/kg), Cd (0.26 - 0.29 mg/kg), Cu (1.6 – 1.78 mg/kg), Zn (90.8 - 96.7 mg/kg) and Mn (8.75 - 10.22 mg/kg). Few authors provided data for whole body of notothenioid fish and the available data were expressed in mg/kg wet weight, being obtained from other Antarctic regions (e.g. Honda et. al., 1987).

Demersal and pelagic fish and krill show highest element concentrations associated with seafloor sediment, the lowest with seawater particulates and organism tissues.

Livingston Island is the closest to Deception Island, where the whole ecosystem is contaminate with trace elements from local geothermal activity, which is also reflect in the pattern of element contamination in organisms (Deheyn et al., 2005). Accordingly, element concentrations were higher in organisms collected at Deception Island which suggest that toxic and trace elements in this area were incorporated into the marine food web mainly through a dietary route (Deheyn et al., 2005).

3.3 Crab eater seal (Lobodon carcinophagus)

Trace and toxic element concentrations in crab eater seal tissues and organs are present in Table 1. The lowest cadmium concentration was found in bones and muscles (0.1 ± 0.02 mg/kg dry weight and below the detection limit), the highest - in kidney (1.2 ± 0.14 mg/kg dry weight), followed by liver (0.60 ± 0.14 mg/kg dry weight). Copper concentrations were found to be from 0.30 ± 0.09 mg/kg dry weight in bones to 30.5 ± 0.24 mg/kg dry weight in kidneys. The concentration of zinc varied from 52.0 ± 0.38 mg/kg dry weight in spleen to 95.0 ± 0.7 mg/kg dry weight in liver. As in the case of Cd the concentrations of Cu and Zn were the highest in liver and kidney. Several authors have reviewed levels of metals in seals, however relatively few (Schneider et al., 1985; Steinhagen-Schneider, 1986) have reported the concentration of metals in crabeater seals organs and tissues. They obtained much higher concentration of Cd in liver and kidney in crabeater seal from Weddell Sea. De Moreno et al. (1997) reported cadmium levels in other marine mammals for muscles of southern elephant seals, where they were above the detection limit, whereas in Antarctic fur seals (Malcolm et al., 1994) they were below it.

For Brandsfield Strait there is no available data to compare with, only few data on toxic elements in crabeater seal exist to date. Lead concentrations (Table 1) varied from 0.2 ± 0.09 mg/kg dry weight in muscles to 0.96 ± 0.04 mg/kg dry weight in kidney. Honda et. al. (1987) determined very low concentrations for Weddell seal in East Antarctica (0.01 mg/kg dry weight for liver, kidney, muscle and whole body).

The highest concentrations of manganese and cobalt were found in liver (13.0 ± 0.13 mg/kg dry weight, and 0.50 ± 0.15 mg/kg dry weight respectively (Table 1).

3.4 Gentoo penguin (Pygoscelis papua ellsworthi)

The concentrations of lead in Gentoo were low (under detection limit in spleen, liver, feather and muscles). The highest concentration was found in bones (0.30 ± 0.14 mg/kg dry weight) (Table 1). The obtained results were close to the corresponding data reported by Szefer et al. (1994), Honda et al. (1986, 1987) and Karasawa and Mishima (1981).

Cadmium concentrations were highest in kidney (41.2 ± 0.67 mg/kg dry weight) and lowest in heart and bones (0.10 ± 0.02 mg/kg dry weight). A high level of cadmium in some species seems likely to be due to diet (Lock et al., 1992). Although apparently natural, levels of cadmium in some species greatly exceed those known to have toxic effects in some terrestrial birds. The highest concentrations of copper were found in spleen (24.70 ± 0.19 mg/kg dry weight) and of zinc - in kidney (232.0 ± 2.67 mg/kg dry weight).

In Adelie penguins (Smichowski, 2006) from Potter Cove (King George Island), element concentrations in different organs were observed and the ranges ascertained were as follows: Cd <0.07 in muscle and 3.4 mg/kg dry weight in kidney; Cu - 0.6 in muscle and 18 mg/kg dry weight in liver; Pb - 0.1 in muscle and 2.0 mg/kg dry weight in liver. The levels of investigated metals in Gentoo at Livingston Island were, in general terms, higher.

3.5 Brown skua (Catharacta lonnbergi)

The highest concentrations of lead were detected in feathers (2.80 ±1.13 mg/kg dry weight) followed by kidney (1.30 ± 0.52 mg/kg dry weight) (Table 1). The bioaccumulation of lead in other organs and tissues were around the detection

limit. The concentration of Cd varied between 0.2 ± 0.05 mg/kg dry weight and 70.4 ± 0.70 mg/kg dry weight mg/kg being the lowest in bone and feathers and the highest in kidney. These levels indicate that brown skua lives on cadmium- rich diet. The results follow the distribution pattern in the closely related South polar skua (*Catharacta maccormici*) (Schneider at al., 1985) – kidney > liver > muscle. Karasawa and Mishima (1981) obtained very low values in skua muscles from East Antarctica.

Highest copper concentrations were determined in liver (16.40 ± 0.13 mg/kg dry weight mg/kg), followed by kidney (14.4 ± 0.11 mg/kg dry weight mg/kg) and muscles (13.70 ± 0.11 mg/kg dry weight). Schneider at al. (1985) obtained similar results for the liver and muscles and very high concentrations in kidney.

Zinc seems to be more evenly distributed in organs and tissues than the other studied elements and varied between 44.0 ± 0.43 mg/kg dry weight in muscles and 203.0 ± 2.33 mg/kg dry weight in feather.

Manganese was higher in liver (15.0 ± 0.15 mg/kg dry weight) followed by kidney (7.1 ± 0.17 mg/kg dry weight). Cobalt was evenly distributed and its values were near and under the detection limit.

4. Trace element presence in the food chain

Marine organisms accumulate trace metals in tissues up to concentrations several order of magnitude above the environmental levels. Previously the Antarctic environment was considered to be unpolluted and the accumulations of toxic elements were expected to be at the physiologic level. However, it has been shown that the quantities of some trace elements may be higher in many Antarctic species (Honda et al., 1986; Szefer et al., 1994). Due to specific detoxification mechanisms one should expect different degree of biomagnifications of particular trace elements.

Global environmental pollution with Pb has been shown to influence the ecological system of Antarctica at least to some degree (Sun and Xie, 2001). Anthropogenic sources seems a likely explanation for significant excess of lead and zinc relative to estimated aerosol contributions (Dick, 1991; Bargagli, 2000; Sanchez-Hernandez, 2000) and are generated after long- range transport (Prendez and Carrasco, 2003). Khale and Zauke (2003) mentioned that lead concentrations in Antarctic crustaceans were generally low or below the limits of detection, but elevated levels were measured in some coastal or scavenging species. The results obtained support this and the highest lead concentration was found in brown skua feather.

Cadmium concentrations in all tissues and all the species analyzed were similar, being the highest in predators' kidney followed by liver, spleen and muscle. They fall within the ranges obtained for other marine birds and mammal species (Norheim, 1987; Eisler, 1981). The exact source of metals in the marine ecosystem is unknown, but it is possible that there is a natural rather that anthropogenic origin for cadmium, such as upwelling of cadmium- enriched waters by ocean currents. Along with this, local volcanism, typical for South Shetlands, undoubtedly increases the bioavailability of metals in the Antarctic environment (Honda et al., 1987; Sanchez-Hernandez, 2000). In most Antarctic coastal ecosystems concentrations of Cd in the waters and biota may be higher than in waters and related species of organisms from polluted coastal areas (Bargagli, 2000). The studied predators depend mainly on Antarctic krill, krill feeding prey and cephalopods, and this is probably the immediate source for the ascertained bioaccumulation of this element in the studied representatives of the Antarctic food chain (Nygard et al., 2001). Comparisons of the Cd levels of the cephalopods showed that those from sub Antarctic area contained very high cadmium concentrations compared to those from lower latitudes. High levels of Cd in cephalopods from the sub-Antarctic zone correspond closely to the reported high Cd accumulation in the tissues of top vertebrate predators from the same area. Comparisons showed that top vertebrate predators are often subjected to Cd doses far in excess of those recommended for humans (Bustamante and al., 1998). The high levels of cadmium in the studied top predators may be explained by its concentration in the main food sources and the "polar cadmium anomaly" (Petri and Zauke, 1993; Bargagli et al., 1996).

Copper and zinc are essential elements. Their concentration is expected to be metabolically regulated in vertebrates, and copper is important for tissue and feathers formation (Underwood, 1977). Therefore, these elements are non-informative for monitoring of anthropogenic impact in Antarctic.

Manganese and cobalt were poorly studied in Antarctic predators. Szefer et al. (1994), Honda et al. (1986), Yamamoto et al. (1996) have published some data on Adelie penguins and krill. Both metals are essential microelements, their toxicity is low and elevated concentration in the environment is inducing by industry. The physiologic levels of these elements in Antarctic fauna are not specified. Thus, further baseline data have to be gathered.

Most of the data in this study fall within the ranges of past investigations undertaken on much larger sample sizes. They enlarge the information about the bioaccumulation in organs and tissue distribution of investigated metals in Antarctic predators. Nova days, all the Antarctic fauna is under strong protection according to the decisions of the CCAMLR 2004 – (Commission for the Conservation of Antarctic Marine Living Resources). Therefore it is necessary to build the future monitoring investigations on noninvasive approaches, including sampling of dead bodies and metabolic nonactive tissues – e.g. molting feathers and skin. The present study supports the view, that current toxicological investigations in Antarctica could be based on noninvasive approaches.

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Table 1. Levels of lead, cadmium, copper, zinc, manganese and cobalt (mg/kg dry weight ± SD) in different organs and tissues of different Antarctic predators

Tissue	Pb	Cd	Cu	Zn	Mn	Co
brown skua (*Catharacta lonnbergi*)						
liver	<0.4	9.7±0.36	16.4±0.13	84.0±0.62	15.0±0.15	0.2±0.06
kidney	1.3±0.52	70.4±0.70	14.4±0.11	154.0±1.77	7.1±0.17	0.4±0.12
bone	0.8±0.36	0.2±0.05	1.6±0.20	118.0±1.36	2.3±0.21	<0.1
spleen	0.8±0.36	7.5±0.28	3.1±0.38	100.0±0.74	4.4±0.40	<0.2
muscle	<0.5	0.4±0.09	13.7±0.11	44.0±0.43	1.4±0.13	<0.1
heart	<0.7	0.3±0.07	11.0±0.08	54.0±0.40	1.9±0.17	0.2±0.06
feathers	2.8±1.13	0.2±0.5	10.0±0.56	203.0±2.33	4.0±0.36	<0.3
gentoo penguin (*Pygoscelis papua)*						
liver	<0.5	2.32±0.27	24.7±0.19	72.0±0.53	7.1±0.17	0.20±0.06
kidney	0.1±0.5	41.2±0.67	8.1±0.45	232.0±2.67	4.9±0.44	0.2±0.06
bone	0.3±0.14	0.1±0.02	0.9±0.028	81.0±0.60	2.5±0.23	0.1±0.3
spleen	<0.95	3.5±0.41	24.7±0.19	232.0±2.67	6.3±0.15	0.1±0.03
muscle	<0.6	0.5±0.12	5.6±0.31	24.0±0.24	1.4±0.13	<0.09
heart	0.2±0.09	0.10±0.02	11.3±0.09	91.5±0.67	1.0±0.25	<0.1
feathers	<1.9	0.5±0.12	15.9±0.12	73.0±0.54	1.65±0.15	<0.3
crabeater seal (*Lobodon carcinophagus*)						
liver	0.3±0.14	0.6±0.14	17.3±0.13	95.0±0.7	13.0±0.13	0.5±0.15
kidney	0.96±0.04	1.2±0.14	30.5±0.24	80.0±0.59	5.7±0.14	0.2±0.06
bone	0.4±0.18	0.1±0.02	0.3±0.09	69.0±0.51	5.4±0.13	0.1±0.03
spleen	0.78±0.09	0.2±0.05	4.5±0.55	52.0±0.38	2.3±0.21	<0.1
muscle	0.2±0.09	<0.1	4.0±0.49	75.0±0.56	1.4±0.13	<0.1
notothenioid fish (*Notothenia coriiceps*) n = 3, min - max						
whole body	0.76-1.34	0.26-0.29	1.6-1.78	90.8-96.7	8.75-10.22	
krill (*Euphausia superba*) n = 19						
whole body	0.40±0.18	0.6±0.14	42.8±0.33	50.0±0.49	14.0±0.14	0.40±0.12

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Current Situation and Progress in Treatment of Ascites by Reinfusion

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Abstract

Ascites, an abnormal accumulation of fluid in the abdomen, is often associated with liver cirrhosis, peritoneal tuberculosis, peritoneal metastatic carcinoma and primary peritoneal tumor. It not only affects the quality of life, but also shortens the survival time. Ascites is usually treated with the conventional therapy, diuretic drugs, abdominal paracentesis, peritoneovenous shunt, volume expansion therapy, liver transplantation and etc. Treatment of ascites by reinfusion is easy to handle, cheap, effective and easily accepted by sufferers. Therefore, this paper introduces the current situation and progress in the treatment of ascites by reinfusion.

Keywords: Ultrafiltration of ascitic fluid, Ascites reinfusion

1. Introduction

Ascites is an abnormal accumulation of fluid in the abdomen. Small amounts of fluid in the abdomen (<100 mL) can lubricate the internal organs, such as stomach and intestinal tract. However, some patients develop excessive free liquid (common) or localized liquid (relatively uncommon) in the abdomen, which is usually more than 100 mL, sometimes reaches hundreds or even ten thousands of millilitres (Xie, Pengyan, 2006, p. 2-3). The reasons that cause ascites are complicated, but liver cirrhosis, tumor and tuberculosis are responsible for about 50% of all instances of ascities (Xu, Dayi, 2007). Laboratory analysis of fluid extracted by abdominal paracentesis is a preferred diagnosis technology, which can determine whether the fluid is an exudate or a transudate, benign or malign. With the development of biochemical technology, the biochemical indexes and radiography of fluid play important roles in diagnosis of ascites, and help identify the cause of the accumulation. However, ascites of different natures have many overlapped indexes. Therefore, ascites can not be accurately characterized by single index, and combined detection of several indexes may increase the accuracy of diagnosis (Ni, Runzhou et al, 2007, p. 110-113).

Ascites is usually treated with the conventional therapy, diuretic drugs, abdominal paracentesis, peritoneovenous shunt, volume expansion therapy, liver transplantation and etc. However, different from paracentesis in which fluid is directly drained from a body cavity, and also different from albumin injection in which albumin functions to maintain the osmotic pressure in a normal range and keep fluid inside the blood vessels, treatment of ascites by reinfusion can help eliminate excess fluid and expand volume at the same time, meanwhile, it is easy to handle, cheap, effective and easily accepted by sufferers. Ascites reinfusion includes intravenous reinfusion of ascites, abdominal reinfusion of concentrated ascites and subcutaneous reinfusion of concentrated ascites.

2. Intravenous reinfusion of ascites

2.1 Peritoneovenous shunt (PV - shunt)

Take Leveen peritoneovenous shunt as an example, the surgeon attaches a pressure-sensitive one-way valve to prevent backflow, and inserts a silicone tube that connects the peritoneal cavity to the jugular vein. PV-shunt may be performed to achieve the continuous emptying of ascitic fluid into the venous system, increase the renal blood flow and urinary output, inhibit renin and angiotonin, and relieve patients' pain, to whom urine-producing drugs are ineffective. However, it usually causes complications, such as DIC, peritonitis, hematosepsis, cardiac insufficiency and etc. (Wang, Jinke, 2000, p. 1277-1282). The severest defect thereof is that the silicone tube is easy to be blocked. To overcome this shortcoming, Zerros et al developed a Denver-type shunting tube that is effective to 48 instances of intractable ascites (Wang, Kangying et al, 2000, p. 113). However, this tube could still be blocked in one year, and caused many complications.

2.2 Direct intravenous reinfusion of ascites

A direct intravenous reinfusion process refers to a procedure in which ascitic fluid extracted by abdominal paracentesis is directly reinfused into patient's venous system. It consists of single intravenous reinfusion process and intravenous reinfusion-diuretics combination process. This method can promote blood circulation, increase urinary output and U_{Na} excretion, decrease ascitic fluid, and avoid loss of protein and electrolytes (Bai, Wenyuan et al, 2002, p. 159). After direct intravenous reinfusion of ascites, patient's body reveals the increase of creatinine removal rate, free water removal rate and albumin level, and decrease of plasma aldosterone. However, it may cause early adverse reactions. Li, Yuling et al (1999, p. 91) reported that reinfusion of intravenous medicine-contained ascites, followed by intravenous injection of 20 mg furosemide, had good effects on 30 patients. Xu, Linshou et al (2001, p. 507-508) reported that direct intravenous reinfusion, followed by intake of Chinese herbs containing hard lump removers and spleen invigorator, not only had good effects on treatment, but also prevented new fluid accumulations, and its 3-month non-recurrent rate is up to 76.6%.

2.3 Intravenous reinfusion of concentrated ascites

Part of water and micromolecular compounds are removed from ascitic fluid by ultrafiltration or dialysis, then the obtained concentrated ascites containing protein and other useful substances is infused back into the patient. Its action mechanisms are as following: (1) the remarkable decrease of ascitic fluid causes the decrease of intra-abdominal pressure, improvement of renal blood perfusion, as well as increase of glomerular filtration rate and excretion of water and Na^+; (2) the protein in reinfused fluid can increase osmotic pressure of plasma colloid; (3) the increase of effective blood volume and renal blood flow stops the activity of renin-angiotonin-aldosterone system, and inhibits the excretion of aldosterone and antidiuretic hormone. Although avoiding loss of protein, intravenous reinfusion of concentrated ascites may sometimes cause hematosepsis, excessive volume load, disseminated intravascular coagulation and other complications. At first, ascites is drained from a body cavity, and then reinfused into the abdomen after extracorporeal concentration, however, in which the open sterile chamber is susceptible to bacteria contamination, and it is rather time-consuming to perform hemodialysis and reinfusion separately. Therefore, many new methods for ascites concentration have been reported recently. Xu, Linshou et al (2001, p. 507-508) studied the treatment of abdominal ascites by concentration and reinfusion with artificial kidney. He performed abdominal paracentesis with fistula needle at both sides of lower abdomen, drained the ascitic fluid into an artificial kidney to form a closed cyclic dialysis and ultrafiltration system of ascites, and reinfused the concentrated ascitic fluid into vein. This method can reinfuse the ascitic fluid into abdominal cavity and vein, and avoid the bacteria contamination from extracorporeal sterile chamber.

3. Abdominal reinfusion of concentrated ascites

Abdominal reinfusion of concentrated ascites has been a new method for treatment of different intractable ascites in recent years home and abroad. It is a simple, safe and reliable procedure in which the ascitic fluid is drained from abdominal cavity, and infused into abdominal cavity again after ultrafiltration concentration. This method avoids the loss of endogeneous protein, and increases the activity of opsonin in the remained fluid. Compared with intravenous reinfusion, this method reveals a comparative curative effect, but a higher safety, avoids the adverse effects and complications that intravenous reinfusion could cause, and broadens the treatment possibilities of intractable ascites, such as ascites caused by cancer, hemorrhagic disease, infection, cardiogenic disease and nephrogenic disease.

Hu, Darong et al (2001, p. 89) reported that 311 patients with intractable ascites had been treated by the ultrafiltration concentration and abdominal reinfusion for 921 times, and the total effective rate was 69.7%. Liu, Limin et al (2004, p. 63) treated 8 patients by abdominal reinfusion of concentrated ascites, and compared with other 6 patients treated by intravenous reinfusion of ascites. The result revealed that abdominal reinfusion of concentrated ascites was more effective in preventing patients from infection, and caused fewer complications than other methods. Zhang, Jinlong et al (2003, p. 294-295) treated 42 patients by abdominal reinfusion of concentrated ascites, followed by injection of 20240 mg dopamine, 402120 mg furosemidum, as well as 1 g ceftriaxone sodium or 0.75 g cefuroxime into abdominal cavity. This method not only solved the issues of massive ascites and deficiency of effective circulating blood volume, but also prevented patients from infection and complications. Its total effective rate was up to 95%.

As for the treatment of intractable ascites, abdominal reinfusion of concentrated ascites is a much safer, more effective, more time-saving and cheaper method, which solves some hard issues, and improves the patients' life quality. However, abdominal reinfusion of concentrated ascites still has some defects, such as fluid clotting, tube blockage, infection, as well as acute pneumonedema and bleeding of venae oesophageae resulted from rapid or massive reinfusion of concentrated ascites (Xu, Chengrun et al, 2005, p.176).

4. Subcutaneous reinfusion of concentrated ascites

Li, Haijian et al (2000, p. 388-389) reported that 17 patients had been treated by subcutaneous reinfusion of concentrated ascites. This treatment has a little effects on 8 patients, and obvious effects on 9 patients. Its curative effect is similar to that of intravenous reinfusion.No obvious complications were observed.

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A Study on Laboratory Rearing of Lady Bird Beetle (*Coccinella Septempunctata*) to Observe Its Fecundity and Longevity on Natural and Artificial Diets

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Abstract

Development and reproductive potential of the *Coccinella septempunctata* L. (CSL) was conducted under laboratory conditions to determine its fecundity and longevity on natural and artificial diets. Tests were made on laboratory rearing materials and all development stages were carefully recorded. Success ratios in these experiments were a matter of logistic and synchronization of these life cycles, the plant phytophagous and entomophagous insects. A positive correlation was obtained between food consumption and egg production, so fecundity of CSL is affected by the type of food. CSL consumed highly significant (*Rhopalosiphum maidis*) 32.2 aphids per day as compared to other treatments. Oviposition response was the maximum on these beetles which consumed 1st and 2nd instars of *Macrosiphon roseae*. CSL reared under artificial diet showed more longevity 41.6 days on plain water. Greater emphasis was pertinent on evaluation of predator specificity, understanding of colonization in new environments and assessment of community-level interactions to maximize the use of Coccinellids in Biological Control (BC).

Keywords: *Coccinella septempunctata*, Mass rearing, Fecundity, Longevity, Biological control

1. Introduction

Different types of pests are found in nature affecting life and yield of natural and cultivated crops of agro-forestry.

They hamper and destroy standing and stored foods reserves in every part of world (Kring, 1998). Scientists are trying to develop and apply different techniques to control and reduce loss of these pests on agriculture, forest and garden products. Chemical Control Method (CCM) is frequently applied as it is easy and prompt way to directly kill or repel the pests from crops and fruit tree (Katsarou, 2005). But it is reported in many experiments that CCM has not only hazardous effects on human life by increasing pollution but also it has indirect impact by disturbing ecosystems. Recently, it is reported that Biological Control Method (BCM) is better technique to control the pest of different types (Habeck, 1990).

In many studies it is known that *Coccinella septempunctata* L. (CSL) commonly called Lady bird beetle is a capable predator and can be used for the biological control of *T. tabaci* and *T. vaporariorum* in a greenhouse (Solomon, 1949). In an extensive survey of District Poonch of Azad Jammu and Kashmir, it was demonstrated there are 13 species *Coccinella septempunctata* which belong to subfamily Coccinellinae (Inya, 2005). It is demonstrated in previous studies that CSL responds to olfactory cues from their aphid prey and can even distinguish between aphid species on volatiles (Sengonca, 1994). CSL is considered to be an important bio-control agent for soft-bodied insects such as aphids, white flies, jassids and small lepidopterous larvae which were among the first to be used in this fashion (Victor, 1997). Deligeorgidis has also reported in his findings that CSL can prove to be a good bio-control source for thrips and whiteflies in green house crops also (Deligeorgidis, 2005).

The CSL larva gains size from 1 to 4-7mm in 10-30 days time span depending on food availability. It has two generations before onset of winter and hibernation activity (Katsarou, 2005). inhabits a wide variety of environments and mass rearing method of aphid predators has been considered as one of the most important conditions for the realization of their control potential (Mackauer, 1976). The life cycle of CSL is ranging from 19-24 days depending on prey species they feed. The insect predator may commit to reproduction to varying degree depending of the nature of the prey they consume (Evans, 2004). To be successful, the CSL searching strategy pre-supposes a high capacity to meet variation in food, plus a capacity for learning as suggested by Vet Lem (1992). Hodek discussed the potential advantages for some ladybird species with mixed feeding i.e. a diet that contains aphids as well as pollen (Hodek, 1996). A valuable contribution on habituation of CSL to one aphid species proved to diminish the capacity to use another species as optimal food (Rana, 2002). It is known that fecundity of CSL is also affected by the quality of food eaten, so there is a positive correlation between food consumption and egg production (Ibrahim, 1955). But no work is conducted on quantitative assessments of the efficacy of Coccinellids have not been done for most species in most agricultural crops. In this study, it was objected to find the relation of CSL with different types of food and how it affects the longevity and fecundity. Furthermore, it was also an aim to explore the food and environment correlation for population density and life span of CSL and its importance as BCM as environment friendly technique.

2. Materials and methods

The present study was conducted in Entomological laboratory (28°C, 65% RH and 14 D: 10L) at University College of Agriculture, Rawalakot, Azad Kashmir, Pakistan during the year 2003. The materials used and methods applied in the study are given below.

2.1 Materials

During the experiment following materials were used:

1. Rearing Cages
2. Disposable Petri dishes
3. Beakers
4. Camel hairbrush (0.00)
5. Plain water
6. Sugar syrup
7. Honey syrup
8. Cotton bolls
9. Filter papers
10. Thermometer

Initially field collected adults of CSL were reared in laboratory and later on all developmental stages were reared and kept under study. Three aphid prey species (*Bravicoryne brassicae*, *Macrosiphon roseae* and *Rhopalosiphum maidis*) were also reared within cages for feeding of CSL. They were maintained all the time on Cabbage seedlings, Rose plants and Maize plants. All the four instars were tested for feeding.

2.2 Methods

Adults of CSL were collected from the agriculture fields and reared in Entomology laboratory. The field-collected material was sorted out in the laboratory and pairs were selected for oviposition. The selected pairs were kept in separate petri dishes to get the batches of eggs for single cohort offspring to minimize the variation in the experiments. Then beetles were reared on aphids. The experiment was carried out in the laboratory under homogeneous condition. The experiment was laid out according to Complete Randomized Design (CRD) with

three replications and eight treatments. The treatments means were analyzed using analysis of variance (ANOVA).

Natural diet

T1	*Brevicoryne brassicae*
T2	1st and 2nd instars of *Macrosiphon roseae*
T3	Alete of *Macrosiphon roseae*
T4	*Rhopalosiphum maidis*
T5	*Rhopalosiphum maidis* on filter paper surface

Artificial diet

T1	Honey syrup
T2	Sugar syrup
T3	Plain Water

The variables under study were fecundity and longevity. The fecundity of CSL was checked and data were recorded daily. In this experiment a copulating pairs were selected and put in petri dishes for mating purpose and then to get the batches of eggs. During experiment following observations were recorded.

Number of aphids consumed by each predator pair per day/Total number of eggs laid by each predator pair during the experiment

To determine the longevity of the predator, the adults were fed with honey syrup, sugar syrup and plain water alone. Three replications of each were tested. The number alive or dead was recorded daily.

Longevity on Honey Syrup

The Honey syrup was given to CSL with the help of cotton boll every day to observe the longevity and data was recorded daily.

Longevity on Sugar Syrup

Daily Sugar syrup was given to CSL in cotton boll to check the longevity on Sugar syrup, and data was recorded.

Longevity on Water

The boiling water in cotton boll was also given to CSL to observe the longevity and data was recorded daily.

3. Results and discussion

Keeping in view the role of this predator, the present study was initiated by considering two paramount features of this particular predator. The population of the predator was at higher level during the month of March – April. Meanwhile, the population of aphids is also at higher level during this period. Under field conditions, the activity of both predator and the prey is quiet visible. The data obtained from comparative feeding behavior of CSL was collected and formulated in the tabular form. The prey-predator interaction of each day was calculated and their data are presented in Table 1. This correlation of prey-predator is analyzed statistically using ANOVA and DMR test represented by Table 1.1 and 1.2, respectively.

The Table 1.1 indicates a highly significant consumption by CSL on treatment T4 (*Rhopalosiphum maidis*) with (32.21 aphids/day) compared to very low consumption by predators on treatment T1 (*Brevicoryne brassicae*), T2 (*Brevicoryne brassicae*) and treatment T3 (Alete of *Macrosiphon roseae*) with (18.39 and 10.57) aphids/day, respectively as indicated in Fig.1. It is because of the *Brevicoryne brassicae* covered with powdery material which was not palatable for CSL which supports previous findings of Jonathan (Jonathan, 2005). However, in present study prey consumption by CSL on treatment T1 (*Brevicoryne brassicae*) was in agreement to that of peach aphids (*Phyllaphus fagi*) which cover their bodies and surround their colony with a white flaky material. This may inconvenience to predators by making individuals prey selection as difficult for feeding. It was found during the study that treatment T3 (Alete of *Macrosiphon roseae*) has taken less number of aphids 10.57 aphids/day by predators because it was fully wing developed and skin of the aphid was very hard, so the Coccinellids were reluctant to eat. Whereas, the consumption recorded on treatment T5 (*Rhopalosiphum maidis* on filter paper) and treatment T2 (1st and 2nd instars of *Macrosiphon roseae*) was (24.50 and 18.39) aphids, respectively. The consumption by CSL on treatment T5 was also higher as compared to other treatments like T1, T2 and T3. It was due to the rough surface of filter paper in petri dish or due to roughness the predator moved quickly and prey-capturing speed was fast. But it is seen that CSL eagerly feed on *Rhopalosiphum maidis* that might be its favourite diet and captures it more conveniently (Smith, 1965; 1966).

The Table 2 shows significant correlation between egg laying of CSL and food consuming the treatment T2 (1st

and 2nd instars of *Macrosiphon roseae*), (17.33 eggs) as compared to other treatments T1, T3, T4 and T5 (Fig.2). It may be due to temperature factor (Katsarou 2005). The temperature also affects the oviposition rate and fecundity as shown in Fig. 2 (Singh and Sing, 1994). The results obtained in Table 2.1 were similar with that maximum oviposition rate was attained at 32˚C and fecundity was highest at 30˚C but fecundity was low at 26˚C and 34˚C (Fig. 2). The present study was conducted at 26˚C in the Entomological laboratory. So, the temperature may also affect the fecundity (Hemptinne, 2001). Another factor which influences the fecundity was over wintering of predator (Katsarou, 2005). Lack of food and relatively low temperature is unsuitable for high level of activity. Most species start over winter in September and October. Fecundity is also affected by the quantity of food eaten, so there is positive correlation between food consumption and egg production (Ibrahim, 1955). Similarly, it was reported that *Menochilus sexmaculatus* did not reproduce when aphid's number was less or low than 29 aphids.

The results obtained about fecundity are shown in Table 2.1 which are in contrast with previous studies (Rajamohan, 1973; Rajamohan, 1974). In their research it was reported that *Menochilus sexmaculatus* had the highest fecundity when reared on *Ahpis craccivora* (1107 eggs), followed by *Aphis gossypii* (718 eggs), *Rhopalosiphum maidis* (522 eggs) and *Aphis umbrella* (276 eggs), respectively. The results of this study prove that difference in egg lying of CSL is due to temperature fluctuation (Katsarou, 2005). Other factors such as feeding behavior and climatic or lab environment variations may also affect the life cycle of CSL; more detailed and comprehensive studies are suggested to unravel this plethora.

The predator in Table 3.1 showed significantly higher longevity on treatment T3 (Plain water), (41.67 days) as compared to very low longevity recorded on treatment T1 (honey), (27.67 days) as depicted Fig.3, because in natural water is only major source for survival of insects which is present ever where, so the beetles could prolong its life on water when there is no other food available. Whereas the longevity recorded on treatment T2 (sugar syrup) was 36.00 days. However, the present study with regard to longevity on water and sugar syrup was in agreement to those of (Samalo, 1976) that *M. sexmaculatus* adult could live for 28 days on sugar syrup and water alone. The present study was carried out at 28˚C in the Entomological laboratory. So, the difference in finding in days of longevity was due to temperature.

Findings of this experiment predict that there is a profound reliability among various parameters such as food type and temperature; which affect the relationship among predators (CLS) or preys i.e. aphids and other insects (Zarpas, 2005). The life form study of CSL and aphids in different climates can lead us to discover the ways and means to overcome the damages caused by different insects to of various crops that is congruently favouring the biocontrol mechanism (Bierne,1962; Greathead). So, this technique can be used as bio-control technique for avoiding loss in yield of various cash crops and this is also studied in past years (Yamamura & Yano, 1988). It is better to use organisms of kind to suppress the control the other harmful or crop destroying pests or insects which will be environment friendly without interrupting natural chain and setup of ecosystems (Gautam, 1994). This will be not only a boosting step for our economy by enhancing per hectare yield but also cost effect and affordable method for layman and farmers at broad level.

4. Concluding perspectives

It is recommended by analyzing the experimental results that CSL growth rate population size is affected by type of food and temperature. Further more, fecundity and longevity parameters have coincidence with egg lying capacity of CSL and is directly affected by type diet available. When CSL consumed prey *Rhopalosiphum maidis* then it was predicted that there was a significant increment in its population. CSL demonstrated more yield on artificial diet than on plain water. Finally, it is conclude that knowing the all behavioral style of CSL can be helpful in enhancing its population in lab and it can be applied in fields for pest control as technique of BCM.

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Table 1. Number of aphids consumed by CSL/day

Treatments	**R1**	**R2**	**R3**
T1	6.08	8.00	7.83
T2	22.16	16.5	16.5
T3	12.14	10.28	9.28
T4	32.00	32.8	31.83
T5	23.00	24.75	25.75

Table 1.1 ANOVA for Number of aphids consumed by CSL/day

S.O.V	**D.F**	**S.S**	**M.S**	**F-value**	**Prob.**
Between	4	1236.710	309.177	95.896**	0.032
Within	10	32.241	3.224		
Total	14	1268.91			

If Prob. < 0.05, it is significant and if Prob. < 0.0001, it is highly significant; "DMR" test is presented below in Table 1.2 for comparison of their means.

Table 1.2 Means of aphid consumption of CSL/day

Original order			**Ranked order**		
T1	7.303	E	T4	32.21	A
T2	18.39	C	T5	24.50	B
T3	10.57	D	T2	18.39	C
T4	32.21	A	T3	10.57	D
T5	24.50	B	T1	7.303	E

T1 *Brevicoryne brassicae*

T2 1st and 2nd instars of *Macrosiphon roseae*

T3 Alete of *Macrosiphon roseae*

T4 *Rhopalosiphum maidis*

T5 *Rhopalosiphum maidis* on filter paper surface

Table 2. Egg laying capacity of CSL on different treatments

Treatments	R1	R2	R3
T1	-	-	-
T2	14	5	33
T3	-	-	-
T4	-	-	-
T5	-	-	-

T1 *Brevicoryne brassicae*

T2 1st and 2nd instars of *Macrosiphon roseae*

T3 Alete of *Macrosiphon roseae*

T4 *Rhopalosiphum maidis*

T5 *Rhopalosiphum maidis* on filter paper surface

Table 2.1 ANOVA for oviposition response of CSL

S.O.V	D.F	S.S	M.S	F-value	Prob.
Between	4	721.067	180.267	4.411*	0.0260
Within	10	408.667	40.867		
Total	14	1129.733			

If Prob. < 0.05, it is significant and if Prob. < 0.0001, it is highly significant and "DMR" test was conducted and presented below in Table 2.2 for comparison of their means.

Table: 2.2 Means of oviposition response of CSL

Original Order			Ranked Order		
T1	0.0000	B	T2	17.33	A
T2 A		17.33	T1	0.0000	B
T3	0.0000	B	T3	0.0000	B
T4	0.0000	B	T4	0.0000	B
T5	0.0000	B	T5	0.0000	B

T1 *Brevicoryne brassicae*

T2 1st and 2nd instars of *Macrosiphon roseae*

T3 Alete of *Macrosiphon roseae*

T4 *Rhopalosiphum maidis*

T5 *Rhopalosiphum maidis* on filter paper surface

Table 3. Longevity of adult CS L on different treatments

Treatments	**R1**	**R2**	**R3**
T1	34	25	24
T2	33	35	40
T3	39	40	46

T1 Honey syrup

T2 Sugar syrup

T3 Plain water

Table 3.1 ANOVA for Longevity of adult CSL

S.O.V	**D.F**	**S.S**	**M.S**	**F-Value**	**Prob.**
Between	2	297.556	148.778	7.740*	0.0218
Within	6	115.333	19.222		
Total	8	412.889			

If Prob. < 0.05, it is significant and Prob. < 0.0001, it is highly significant and "DMR" test was used to compare means as in presented below in Table 2.2.

Table 3.2 Means of longevity of adult CSL

Original order			**Ranked order**		
T1	27.67	B	T3	41.67	A
T2	36.00	AB	T2	36.00	AB
T3	41.67	A	T1	27.67	B

T1 Honey syrup

T2 Sugar syrup

T3 Plain water

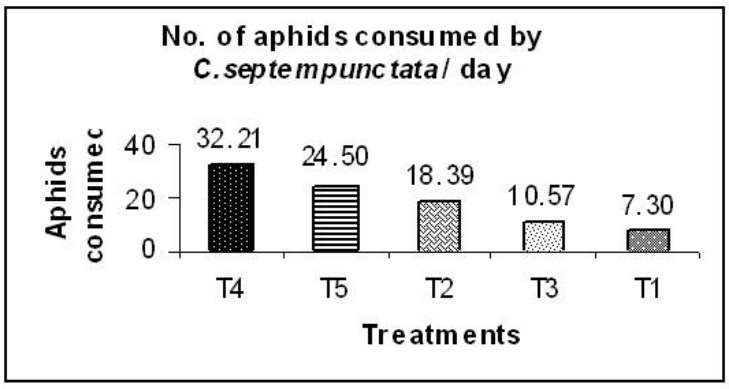

Figure 1. Showing number of aphids consumed by adult CSL/day

T1 *Brevicoryne brassicae*

T2 1st and 2nd instars of *Macrosiphon roseae*

T3 Alete of *Macrosiphon roseae*

T4 *Rhopalosiphum maidis*

T5 *Rhopalosiphum maidis* on filter paper surface

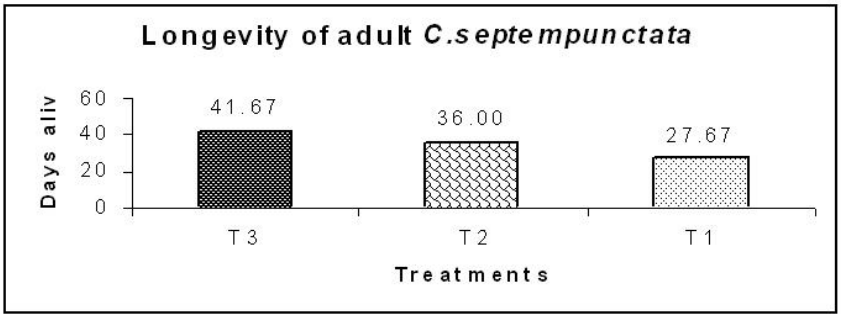

Figure 2. Showing oviposition response of adult CSL on different treatments

T1 *Brevicoryne brassicae*

T2 1st and 2nd instars of *Macrosiphon roseae*

T3 Alete of *Macrosiphon roseae*

T4 *Rhopalosiphum maidis*

T5 *Rhopalosiphum maidis* on filter paper surface

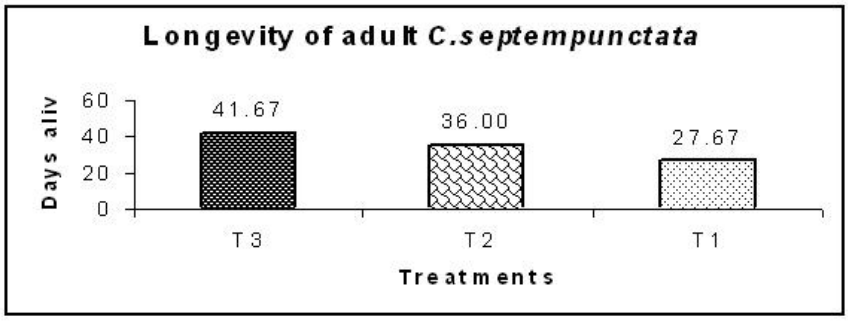

Figure 3. Showing longevity of adult CSL on different treatments

T1 Honey syrup

T2 Sugar syrup

T3 Plain water

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Healthy Culture of Aquatic Animals and Development of Green Fishery Medicine

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Abstract

Since reforming and opening to the outside world, Chinese aquiculture has developed very quickly. The total output of aquiculture has been ranking first in the world over ten years, but it still has many problems. In this article, many problems are listed about Chinese aquiculture, which has seriously influenced the quality of aquatic products and destroyed the whole aquiculture ecological environment, so it is imperative under the situation to implement healthy culture. At the same time, the actuality of Chinese healthy culture is analyzed, and healthy culture has been developed in freshwater and sea water. Healthy culture comes down to many aspects, and it is a very important part to reasonably use fishery medicines. The using of fishery medicines should start from many aspects including medicine materials, cause of disease, environment, aquatic animals, and human health, and only to use medicines intentionally and effectively can achieve the effect of preventing and treating diseases. The green fishery medicine is one most effective development direction at present to use medicines reasonably. Green fishery medicines include fishery vaccine, Chinese herbal medicine preparation, animalcule preparation, and biologic fishery medicines.

Keywords: Aquatic animals, Healthy culture, Green fishery medicine

1. Necessity to implement healthy culture

The total output of Chinese aquiculture has been ranking first in the world over ten years. The total output of 2000 had exceeded 40 million tons, and this number had achieved 48.9 million tons in 2008. But most Chinese aquiculture modes still are traditional aquiculture modes. With the further development of the aquiculture, the disadvantages of this mode have been represented increasingly, and it can not accord with the development requirement of aquiculture in China any more. Though the traditional aquiculture mode can increase the total output of aquiculture by increasing the aquiculture area, but the aquiculture benefit has descended obviously and the quality of aquatic products decreases significantly. The discharge of aquiculture nutriment and the use of chemical medicines will pollute water and deteriorate the environment. The diseases of main aquiculture breeds are serious and prevail explosively. In the seawater aquiculture, the artificial damage to the mudflats and aquiculture sea area have induced large-area red tides, serious deterioration of seacoast ecological environment, and decreasing aquatic biologic diversity (Shang, 2001, P.43).

As the existences of above disadvantages, though the total output of Chinese aquiculture has been ranking first in the world over ten years, the quality is not satisfactory, and the exports are often refused because of the over-standard animalcule and forbidden antibiotics (Zhao, 2002, P.66-70). The selective examination result of domestic market showed that the qualified rates of the products including shrimp, scallops frozen, and fish could not achieve 50%, and

the phenomenon of adding excessive additives artificially still exists universally. People still can memorize that the hepatitis A induced by unclean blood clam prevailed in Shanghai in the late of 1980s. In 2000, European Union passed the decision to allow China export aquatic products to European Union, but there were only 159 eligible enterprises in 5000 enterprises which can manufacture aquatic products. The safety of aquatic products has been the most serious problem facing by the aquiculture at present, and in this way, China only can be the "big country of aquiculture", but not the "strong country of aquiculture".

Therefore, people gradually realize the importance of problem, and begin to explore new aquiculture mode, and study new aquiculture technology and method to reduce the pressure of the aquiculture environment and maintain the sustainable development of the aquiculture industry. So the concept of "healthy culture" is proposed and implemented (Cai, 2001, P.54-55 & Zhao, 2002, P.63 & Yao, 2002, P.10-13 & Wang, 2002, P.5 & 36 & Han, 2002, P.25-27 & Zhu, 2003, P.34-35 & Dong, 2003, P.24-26).

"Healthy culture" is a new culture concept which was proposed in recent years, and comparing with traditional aquiculture technology and management, it contains more extensive contents, and it not only requires healthy aquiculture culture products to ensure the safety of human foods, but the aquiculture ecological environment should accord with the ecological requirements of aquiculture breeds, and the aquiculture breeds should keep relatively stable breed characteristics. The introduction of this concept is mainly because that the random of Chinese aquiculture technology and management has induced spreading diseases, degenerated breeds, decreasing product quality, and even influenced the healthy safety of foods.

2. Actuality of Chinese healthy culture

Fortunately, healthy culture in China has been carrying out (Zhang, 2000, P.19-20 & Bai, 2002, P.3-5). The Freshwater Fisheries Research Center of Chinese Academy of Fisheries Science has studied the pond dynamics and animalcule biology for a long time, and many aspects such as the beneficial organisms including photosynthetic bacteria and the interior water quality control and disease prevention in the aquiculture system have been developed quite well. The Freshwater Fisheries Research Center of Chinese Academy of Fisheries Science and Nanjing Institute of Geography and Limnology extensively studied the influences of different aquiculture modes to the water environment, and the sustainable development technology and relative mode. Professor Li Sifa of the Key Lab of Chinese Ministry of Agriculture and Shanghai Fisheries University has engaged in the fish seed inheritance improvement, healthy parent strain, and seed selection all along, and has acquired abundant results, for example, the immaterial asset of "Pujiang No. 1" selected by him has achieved 1 billion Yuan. The fishery vaccine study in the Pearl River Fisheries Research Institute of Chinese Academy of Fisheries Science from early indigenous vaccine of grass carp to present subunit vaccine and DNA vaccine all show that the good effect of vaccine in the disease prevention of aquatic animals.

In marine-culture of China, the healthy aquiculture management has been advocated and some corresponding technologies have been developed, especially the disease prevention system has been established, the diagnosis technology has been developed, the using and the development of aquatic medicines are going to standardization, the aquiculture breeding has been emphasized universally, the research of aquiculture capacity and the development of ecological aquiculture all have obtained some initial results (Chen, 2003, P.62-64).

3. Fishery medicines should be reasonably used in healthy culture

In the practice, the healthy culture includes five aspects such as seed, culture, water quality, feed, and medicine. And the use of aquatic medicines is a very important part, as the healthy culture requires scientific and reasonable using of medicine in the aquiculture (Wang, 2003, P.1-3). Its intention is to enhance the disease prevention effect of aquiculture animals and the quality of aquatic products. Reasonable use of medicines should start from medicine, disease cause, environment, aquatic animals, and human health to intentionally and effectively use medicines and achieve the effects of preventing and treating diseases (Lin, 2002, P.63-64). Medicines have both positive function and negative function. On the one hand, medicines can prevent and treat diseases or improve the environment and strengthen the constitution of aquatic animals. And on the other hand, if medicines are used more frequently, not only the disease causes will produce drug tolerance to invalidate medicine prevention, but also the aquatic animals will be harmed or stimulated to destroy the microbial environment of aquatic animals.

The reasonable use of medicines should emphasize "giving priority to prevention, and combining prevention and treatment". In the season that diseases prevailing, the medicines which can restrain and kill causes of diseases should be offered periodically according to the prevalence rule of diseases, or the medicines which can enhance the metabolism mechanism of aquatic animals should be used to prevent the occurrence of diseases. The usual method is to mix medicines into feeds, for example, adding some bacteriophages or Chinese herbal medicines (such as isatis root, rhubarb, garlic, and coptis root), and some vitamins and mineral compositions. At present, in the aquiculture, the phenomenon of "emphasizing treatment and ignoring prevention" still exists, and once the diseases come on, medicines are always abused, so the drug tolerance of disease causes will be formed.

The reasonable use of medicines should also develop and use special aquatic medicines, such as fishery vaccine, animalcule preparation, biologic fishery medicines, and natural Chinese herbal medicines. Most fishery medicines used in aquiculture are composed by human medicines and animal medicines without pertinence, and the residuals of many fishery medicines are very serious, which will seriously threaten the aquatic biological environment and human health in a long time. For the sustainable development of aquiculture and human healthy, it is urgent to study low-poison and strong pertinence fishery medicines without residuals and pollution, especially the development of fishery vaccine and Chinese herbal medicines should be the emphasis of further work. Fishery vaccine and Chinese herbal preparation will not negatively impact the aquatic animals, they are real "green fishery medicines". Study the using and effect fishery vaccine and Chinese herbal medicines is one direction of reasonable use of medicines.

4. Green fishery medicines

The so-called "green fishery medicines" means safe and harmless fishery medicines which is the high-technology product combining with agriculture science, environment protection science, nutrition science and health science, i.e. These kinds of medicines which utilize natural medicines and beneficial biology swarms, and adopt modern advanced pharmacy technologies to prevent the diseases of aquatic animals such as fish, shrimp, and shellfish and improve the environment of aquatic animals. It will not destroy the ecological balance of aquatic animals and produce residuals of medicines. It has better prevention effects, and it can not only prevent diseases but also protect the ecological environment. It mainly includes fishery vaccine, natural Chinese herbal medicine preparation, animalcule preparation, and biologic fishery medicines.

4.1 Fishery vaccine

The fishery vaccine is the most effective measure to prevent the explosive epidemics of aquiculture. Vaccine can not only prevent bacteria diseases, it is also the unique effective measure to deal with virus diseases. Different with traditional aquatic medicines, vaccine is not kill medicine causes, but strengthen aquatic animals' resistance to some intensive infectious diseases and make them to void these infectious diseases. And most aquatic vaccines need only to be used once in the whole aquatic period.

In 1969, the Pearl River Fisheries Research Institute first developed the aquatic vaccine successfully, which can be extended and used in large area of grass carp (a inactivated vaccine of organization plasm). It can essentially treat harmful explosive epidemic diseases such as broken gill, read skin, enteritis, or bleeding, and enhance the survival rate of grass carp in pond aquiculture to over 85%.

After that, the aquatic scientists of China also developed many aquatic vaccine production technologies including inactivated cell vaccine, weak poison activated vaccine, molecule vaccine, and gene engineer vaccine.

The gene engineer vaccine is the vaccine prepared by abstractubg antigenic determinants on the cytoderm of nosogenetic bacterium with modern biologic technology. It is harmless, and has high immunity protection rate, and strong specificity, and only two gamma needs to be injected for one grouper. And the vaccine can be stored for five years in normal temperature. It is also easily to be produced and transported, and the industrialized production ability has been formed at present.

Comparing with above vaccines, the gene engineering vaccine is more safe and reliable, and it has high purity, low cost and large production scale. Lately, Zhongshan University successfully developed the gene engineering vaccine of soft-shelled turtle hydrosphere monad, and the lab immunity protection rate can achieve 100%, and it has been tested in large area (Li, 2000, P.30-32).

Recently, people also successfully developed DNA vaccine (gene vaccine or nucleic acid vaccine). It means that the eukaryotic expression plasmid DNA with coding antigen gene can be incepted by host cell through directly being inoculated into inoculums, and express corresponding antigens by transcribing and translating, and generate the immunity answer of this antigen by different approaches to stimulate organisms and achieve the effect of immunity. The preparation method of vaccine is simple and fit to produce in large scale, and it has high efficiency and stability with low costs. It can be expressed for a long time in the organism, and continually stimulate the immunity system of the organism, and compose multivalent vaccine which can generate the immunity protection function aiming at multiple antigen expressions (Yin, 2001, P.87-90 & Bai, 2001, P.57-59).

4.2 Chinese herbal medicine preparations

Natural Chinese herbal medicines have many characters such as cheap cost, low poison, little side effect and difficult generation of drug tolerance and some components can not only resist bacterium, but immunize virus, and this kind of medicines can improve the immunity status of the organism, and enhance the anti-bacteria ability. The advantages of Chinese herbal medicines to prevent fishery diseases include abundant medicine sources, cheap costs, extensive function, treating both principal and secondary aspect of disease, safety and low poison, and difficult generation of drug tolerance, and can also obviously enhance the production performance of fish, and increase the economic benefits of

aquiculture (Sha, 2003, P.60 & Li, 2003, P.29-31 & Sun, 2002, P.37-38 & Wang, 2001, P.17-18).

Natural Chinese herbal medicines have wide application foreground. For example, the gallnut and sanguisorba can prevent and treat *Edwardsiella tarda*, the coptis root and phellodendron can prevent and treat the disease of pasteurellosis, the gallnut (dousing) and Chinese tallowtree leaf (mixing) and folium eucalypti (dipping) can prevent the bacterial gill-rot disease, and the garlic, wolf's milk, polygonum hydropiper and creat can prevent and treat the bacillary enteritis. In recent years, the scientists of Pearl River Fisheries Research Institute using Chinese herbal composite preparation to treat the white-soleplate disease of turtle and the red leg disease of white-leg shrimp, the result is very well.

In cultre, China is one of the most developed country in the world, and the founder of natural Chinese herbal medicines, and the headstream and village of Chinese herbal medicines. When the natural Chinese herbal medicines extensively begin to rise in the world, China should push the research and application to a new height especially in the aquiculture industry, and use the high technology to change the traditional industry and improve the ecological environment, prove the mechanism of Chinese herbal medicines, and establish the system of R & D and standards to initiate the fishery disease prevention medicines and feed additives and aquatic science and technology with Chinese characteristics.

4.3 Microorganism preparations

To prevent and treat the diseases of aquatic animals by chemical medicines or antibiotics only is temporary measure, and the broad-spectrum antibiotic can kill or restrain sensitive bacterium and keep pathogenic bacterium with drug tolerance, it also can destroy or disturb the ecological balance of the original normal animalcule region of waters, and increase the opportunity that aquatic animals infect pathogens. The residual of antibiotic in the organism will finally harm human, and the ecological prevent and treatment is a good measure to solve problems, so the research and development of animalcule preparation begin to occur. In the ecology, it mainly study the function and characters of animalcule swarm, optimize the ecological structure of aquiculture water area, and develop the aquiculture production in good circulation, and obtain larger economic, ecological and social benefits (Gui, 2001, P.86-87 & Liu, 2002, P.17-18 & Yang, 2000, P.23-24 & Gong, 2003, P.83-84). Following animalcules have been developed at present.

(1) Photosynthetic bacterium. It is the animalcule to reproduce by light as the energy. And its mycelium contains abundant proteins, various vitamins, biotins, carotenoids, coenzyme Q and other living activated materials. In addition, it has special physiological function, i.e. it can absorb the ammonia-azote, nitrite, sulfureted hydrogen, and organic acid to eliminate the harmful materials in the waters and purify the water, and the pathogenicbacteria will not survival any more.

(2) Nitrobacteria. It belongs to self-nutritional bacterium, and includes two different metabolic swarms, i.e. nitrosomonas and nitrobacter. Both of them are aerobic bacteria which can grow in oxygenic waters, and play important function to purify waters. One important function of nitrobacteria is to oxidate poisonous ammonia to innocuous nitric acid for the growth of aquatic animals.

(3) Mixed bacterium (composite animalcule). Mixed bacterium is not the name of a kind of bacterium, but the name of a kind of animalcule preparation because this preparation is composed by multiple beneficial activated animalcules which can decompose organisms and purify waters, and the method which adopts single animalcule (such as photosynthetic bacterium and nitrobacteria) to control and purify waters has certain limitation, so multiple animalcules bacterial strains existing in natural environment are selected and cultivated to form the mixed bacterium preparation in the world (Li, 1999, P.34-35).

4.4 Biologic fishery medicines

The biologic pharmacy is the process that applies the biologic engineering technology into the domain of pharmacy, and the main method is the gene engineering, i.e. utilizing the monoclonal antibody organization cultivation technology to cut, insert, connect, and rebuild DNA for acquiring medical biologic products. The biological product takes animalcule, vermin, animal toxin, and biologic organization as the starting materials, adopts the biologic technique or the separation purification technology to prepare, and use the biology technology and the analysis technology to control the middle production and the product quality to make biologic activities including bacterin, vaccine, toxin, toxoid, blood serum, blood products, immunity preparation, cell gene, antigen, monoclonal antibody and gene engineering product, DNA rebuilt products, and IVD. At present, biological products include gene engineering medicine, biologic vaccine, and biologic diagnosis reagent.

The fishery medicine manufactured in the biologic pharmacy process is biologic fishery medicine. At present, the biologic medicines have been widely applied in many diseases such as cancer, aids, coronary heart disease, multiple sclerosis, anemia, hypogenesis, diabetes, heart failure, haemophilia, cystic fibrosis and some infrequent genetic diseases. And the application of alumen, polypeptide, enzyme, hormone, vaccine, cell growth factor, and monoclonal antibody manufactured by the recombinant DNA technology in the aquiculture have shown good foreground (Yang, 1999, P.44-45).

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Combining Ability Estimates for Yield and Fibre Quality Traits in Line X Tester Crosses of Upland Cotton, (*Gossypium hirsutum)*

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Abstract

This study was to estimate the GCA of the parents and SCA considered for the development of high yielding and better quality cultivars. Eleven genotypes and 28 F_1 hybrids obtained by crossing 4 lines and 7 testers in line X tester mating system were sown in randomized complete block design. L X T analysis revealed significant GCA and SCA effects for all the traits except earliness. Among the parents: MCU 12 for number of bolls per plant, boll weight, seed cotton yield per plant, F 1861 for seed cotton yield and number of bolls per plant, SOCC 17 for earliness, SURABHI for number of sympodia and TCH 1641 for ginning outturn and lint index with high GCA. Parent F 776 and F1861 were good combiners for fibre quality traits. The high yielding quality hybrids were deducted with significant SCA effects for seed cotton yield and fibre characteristics.

Keywords: Cotton, Fibre quality traits, General (GCA) and specific combining ability (SCA), Line X Tester analysis, Lint index

1. Introduction

Cotton (*Gossypium hirsutum* L.) is an important fibre crop and plays a vital role as a cash crop in commerce of many countries such as USA, China, India, Pakistan, Uzbekistan, Australia and Africa. Cotton crop is mainly cultivated for fibre. Development of new variety with high yield and fibre quality is the primary objective of all cotton breeders. The first step in successful breeding program is to select appropriate parents. Line x Tester analysis provides a systematic approach for detection of appropriate parents and crosses in terms of investigated traits. This method was applied to improve self and cross-pollinated plants (Kempthorne 1957). Previous studies showed that variation in seed cotton yield and its components were controlled by genes acting additively and non-additively.

Studies of Shakeel *et al.*,(2001), Ahuja and Dhayal (2007) revealed that number of bolls, boll weight and seed cotton yield were influenced by the genes acting non-additively and in contrast studies of Khan and Idris (1995). Kumaresan *et al.* (1999) indicated that both additive and non-additive gene effects were important for controlling number of bolls and seed cotton yield. However, Lukange *et al.* (2007) revealed additive gene effects for fibre strength and microanire value and non-addidive gene action for fibre length. Non-additive gene action for fibre quality traits: fibre length, fibre strength and micronaire value have been reported by Baloch *et al.* (1997), Hassan *et al.* (1999 and 2000), Ahuja and

Dhayal (2007) and Preetha and Raveendran (2008). The purposes of this research were to estimate the GCA and SCA effects for seed cotton yield, its components and fibre quality traits among 4 genotypes taken as female, and 7 taken as male *G. hirsutum* lines and to determine appropriate parents and crosses for the investigated traits.

2. Materials and Methods

2.1 Plant materials

The materials used in the present study was developed by crossing four local cultivars, viz: MCU 5, MCU 12, SURABHI, and SVPR 2 with 7 diverse genetic accessions: F 776, F 1861, SOCC 11, SOCC 17, TCH 1641, TCH 1644 and TCH 1646 all belonging to *G. hirsutum.* When the parental lines started to flower, these were crossed in line x tester fashion. Some of the buds of parents were also selfed. Maximum numbers of crosses were made to develop sufficient F_1 seed.

2.2 Field layout

The F_1 seed of 28 hybrids and parents were planted in the field during Kharif 2005-2006 crop season at Cotton Breeding Station Coimbatore, Tamil Nadu (India). Each entry was sown in three replications following randomized complete block design. Each genotype seed was sown in a 3 plot of 4.5 meter length adopting a spacing of 75 cm between rows and 30 cm between the plants in the row, so as to have 15 plants per row.

2.3 Data analysis

Data were recorded on middle five competitive plants for all the 17 characters viz., Days to first flowering, days to 50% flowering on whole plot basis, plant height (cm), number of sympodia per plant, number of bolls per plant, boll weight (g), number of seeds per boll, ginning outturn (%), lint index (g), seed index (g), seed cotton yield per plant (g), 2.5% span length (mm), fibre strength (g/tex), micronaire value (µg/inch), uniformity ratio and fibre elongation (%). Lint samples were submitted to Central Institute for Research on Cotton Technology unit at Coimbatore, Tamil Nadu (India) for analysis of fibre quality traits: 2.5% span length (mm), Micronaire value (µg/inch), fibre strength (g/tex) and fibre elongation (%).

2.4 Statistical Analysis

The mean values of the characters measured in 59 genotypes in each replication were analysed for Analysis of variance, estimation of Standard Error and Critical Difference by adopting the method suggested by Panse and Sukhatme (1964). Prior to estimation of combining ability effects, the data on 16 traits was subjected to path coefficient analysis suggested by Dewey and Lu (1959) to study direct contribution of different traits and nature of their relation to seed cotton yield. The Line x Tester analysis of combining ability was performed as suggested by Kempthorne (1957).

3. Results

Significant difference among parents and hybrids revealed presence of genetic diversity among them. From the parents versus hybrids components of variance, it was observed that significant heterosis (average) was exhibited by all the characters except days to first flowering and days to fifty per cent flowering (Table 1). Path coefficient analysis at genotypic level indicated that out of sixteen traits under study, six traits viz: boll weight, number of sympodia per plant, lint index, number of seeds per boll, uniformity ratio and micronaire value depicted direct effects in desirable directions on seed cotton yield (Table 2). The genotypic correlations of these traits with seed cotton yield were also positive for all of these characters and desirable negative for days to first flowering and micronaire value. Using the technological evolution of yarn developed from solely ring-based spinning to predominately rotor and air-jet spinning, need for intensification of fibre property profiles to suit the automated spinning systems. Nevertheless, successful rotor spinning requires high fibre strength for all yarn counts, along with fibre fineness for fine count yarns. The stable more productive air-jet spinning requires a minimum, but uniform fibre length, fibre fineness, and to a less extent strong fibre. On the differing, ring spinning requires a minimum fibre length, fibre strength, and to a lesser extent fibre fineness. Thus fibre length and fibre strength being indispensable traits though showing negative direct effect on seed cotton yield were not excluded for further analysis for combining ability. Seed cotton yield exhibited positive but non-significant association with fibre length and fibre strength. Its correlation with micronaire value, uniformity ratio and elongation percentage was non-significant and negative. Mean squares of genotypes, GCA and SCA for all the characters under study were significant except days to 50 per cent flowering indicating prevalence of genetic diversity among parents and F_1 hybrids.

The combining ability analysis gives useful information regarding selection of parents based on the performance of their hybrids and further it helps for the exploitation of heterosis. Among the parents, the best general combiner was line MCU 12 and testers F 1861 and SOCC 17 recorded significant *gca* effects for bolls per plant. Apart from this MCU 12 has recorded high *gca* for seed cotton yield, number of bolls per plant and while F 1861 exhibited high *gca* effects for number of bolls per plant and seed cotton yield. Therefore the line MCU 12 and tester F 1861 were identified as good source of favourable genes in improving yield characters. Further the study revealed that high and significant *gca*

effects for seed cotton yield result from combined effect of yield component, number of bolls which is an concordance with results of Patel et al. (1992). Significant and positive gca effects were recorded for number of sympodia the line Surabhi presented in Table 3. For fibre quality characters MCU 5 and MCU12 showed high significant *gca* for 2.5 % span length and bundle strength. Similarly among the testers F776 recorded high *gca* for both fibre length and strength. In addition it also showed high *gca* for 2.5 per cent span length (F 776) as indicated in Table 3.The specific combining ability value of any cross was helpful in predicting the performance of the better parents. Out of the 28 hybrids, only three hybrids, MCU 12 x F 1861, SVPR 2 x F 776 and MCU 5 x TCH 1644 showed significantly negative *sca* effects for earliness. Beyond the 28 hybrids MCU5 x TCH 1641 for plant height and ginning outturn, MCU 12 x TCH 1641 for number of sympodia per plant, MCU 12 x SOCC 17 and MCU12 x F 1861 for number of bolls, Surabhi x F 1861 for boll weight per plant, Surabhi x SOCC 11 for number of seeds per boll, MCU 5 x TCH 1644 and MCU 12 x SOCC 11 for lint index, MUC 5x TCH 1646 for seed index were exhibited to significant high *sca* expression, presented in Table 4. The hybrid MCU 5 x SOCC11 for ginning outturn and MCU 5 x TCH 1646 for seed index had superior per se performance, with high sca effects. The hybrids can be exploited as basic material for breeding

Specific combining ability effects of these crosses were related with general combining ability effects of their parents as they involved at least one parent with high or average GCA effects for particular traits. Similar results have been reported by Punitha et al.(1991), Khan and Idris (1995), Baloch et al.(1997), Hassan et al. (1999), Kalwar and Babar (1999), Kumaresan et al. (1999), Hassan et al. (2000), Shakeel et al. (2001) and Ahuja and Dhayal (2007). Mean values for characters of parents and F_1 hybrids are given in Table 6. The means of F_1 crosses were higher than those of parents in all the characters. In majority of cross combinations having significant SCA effects showed better mean performance (average performance over three replication of a cross for a trait) as reflected by positive association between them indicating that the cross combinations may be selected either on the basis of SCA or mean performance or in combination. Several workers found that hybrids superior with fibre quality traits were not good in seed cotton yield and vice-versa [Tuteja et al. (1995); Hassan et al. (1999); Jagtap (1994); Neelam Dheva et al. (2002)]. In the present study, of the best three cross combinations: MCU 12 x F 1861, MCU 12 x SOCC 17 and MCU 12 x SOCC 11 exhibited high heterotic effect and *per se* performances for seed cotton yield per plant. Four hybrids recorded significant and positive *sca* effects for fibre elongation percentage, the hybrids being SVPR 2 x TCH 1641, Surabhi x TCH 1644, MCU 12 x SOCC 17 and MCU 5 x F 1861. Among these, SVPR 2 x TCH 1641 and MCU 12 x SOCC 17 had superior *per se* performance, significant standard heterosis with high *sca* effects for fibre elongation. For ginning outturn MCU 5 x TCH 1641 had superior *per se* performance, significant standard heterosis with high *sca* effects. These hybrids can be exploited as basic material for breeding purposes.

4. Discussion

The 11 parents and 28 hybrids used in this study varied significantly for each yield component and fibre quality parameter evaluated (Table 1, 3). These data indicated that the highest values of yield components and fibre quality parameters do not follow same pattern in every line i.e. the parent or the cross with high yield or its component traits did not necessarily had high fibre quality parameters. GCA variances were lower than SCA variances for all the characters as indicated by their lower ratios indicating predominance of non-additive gene action (dominant or epistasis) in the inheritance for all of these traits (Sprague and Tatum 1942). Studies of Shakeel et al. (2001) and Ahuja and Dhayal (2007) revealed that number of bolls, boll weight and seed cotton yield were influenced by the genes acting non-additively and in contrast studies of Khan and Idris (1995), and Kumaresan et al. (1999) indicated that both additive and nonadditive gene effects were important for controlling number of bolls and seed cotton yield. However, Lukange et al. (2007) revealed additive gene effects for fibre strength and microanire value and non-addidive gene action for fibre length. Non-additive gene action for fibre quality traits: fibre length, fibre strength and micronaire value have been reported by Baloch et al. (1997), Hassan et al. (1999 and 2000), Ahuja and Dhayal (2007) and Preetha and Raveendran (2008). Predominance of non additive gene action for days to fifty per cent flowering, plant height, number of bolls per plant and boll weight was observed by Neelam Deva et al. (2002), number of sympodia per plant reported by Valarmathi and Jehangir, (1998), number of seed per boll, ginning outturn [Sandhu et al. (1993)], lint index, seed cotton yield [Patel et al. (1992); Ahuja and Dhayal (2007)]. These results suggested that heterosis breeding was suitable for all the characters including fibre properties. The non-additive gene actions are also important for varietal adaptability.

Amudha et al. (1997); Mandloi et al. (1998) and Modi et al. (1999) also observed superior per se performance with high SCA effects for ginning outturn. The results indicate the predominance of non- additive genetic variation in the inheritance of these characters, which was in accordance with results, was obtained by Krishna Rao (1998). The hybrid combinations, which were, have good *sca* and *per se* performance for seed cotton yield, indicated the possibility for simultaneous improvement of seed cotton yield and yield-attributed traits by exploring these hybrids.

Among the derived hybrids, five hybrids recorded significant and positive *sca* effects for uniformity ratio. The hybrid between Surabhi x TCH 1644 exhibited high *sca* for uniformity ratio. Six hybrids recorded significant and positive *sca*

effects for bundle strength, with MCU 5 x SOCC 17 recording the highest value followed by Surabhi x F 776, MCU 5 x F 1861, MCU 12 x TCH 1644, MCU 12 x TCH 1641 and Surabhi x TCH 1646. Among these, the hybrid MCU 5 x SOCC 17 exhibited (good x poor general combiner) *sca* for bundle strength and MCU 12 x TCH 1644 (moderate x good general combiner) for micronaire were the other crosses with high *per se* performance and high *sca* effects for the characters mentioned (Table.5). The four cross combinations recorded significant and positive *sca* effects for fibre elongation percentage being SVPR 2 x TCH 1641, Surabhi x TCH 1644, MCU 12 x SOCC 17 and MCU 5 x F 1861. The results indicated the predominance of non- additive genetic variation in the inheritance of these characters. The presence of parallelism between *per se* and heterosis in present study suggest the possibility of direct exploitation of these hybrids for commercial exploitation. The study indicated the possibility of developing hybrids with high seed cotton yield and quality traits through heterosis breeding.

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Preparation and Swelling Behavior of Poly(N-isopropylacrylamide-co-acrylic Acid Derivatived L-phenylalanine) Hydrogels

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Abstract

A series of novel temperature-sensitive hydrogels with chiral group, poly(NIPA-*co*-AAc-*L*-Phe), were synthesized by copolymerization of *N*-isopropylacrylamide(NIPA) and acrylic acid derivatived L-phenylalanine (AAc-*L*-Phe) in ethanol with different feed ratios. Their swelling behaviors in response to temperature and deswelling/reswelling kinetics have been studied. The primary results showed that poly (NIPA-co-AAc-*L*-Phe) hydrogels are thermo-responsive and have potential applications in the biological chemistry.

Keywords: Hydrogels, Copolymerization, N-isopropylacrylamide, Temperature-sensitive, Swelling behavior

1. Introduction

The separation of chiral materials (Lu, H. 2007) has become the urgent need to solve the issue in the biochemistry and pharmaceutical industry (Huang, L. and Dai, L.X. 2002). In recent years, with the chiral materials (Izake, E. L. 2007) such as chiral stationary phase and chiral membrane have developed, a lot of techniques have been more application in Chiral drugs preparation (Dufrasne, F. and Galanski, M. 2007). But these methods are weakly demand for large-scale industry produce which have high efficiency and low costs. Thus, it is very important that we further research chiral separation materials with high selective and handling ability.

Poly(N-isopropylacrylamide) (PNIPA) is a typical thermosensitive polymeric material and demonstrates a lower critical solution temperature (LCST) at ~32^{o}C in aqueous solution (Hirokawa, Y and Tanaka, T. 1984). Below the LCST, PNIPA is hydrophilic and swells in water. In contrast, it becomes hydrophobic and shrinks dramatically above the LCST. Due to their unique properties, PNIPA hydrogels have been widely used in many fields (Zhang, X. Z. and Zhuo, R. X. 2000). For example, hydrogels have been used to recognize and capture a target molecule (Zhang, X. Z., Zhang J. T., Zhuo, R. X. and Chu, C. C. 2002) (Zhang, Z. L. and Wang, B.2008) (Kurisawa, M., Okano, T. and Yokoyama, M. 2000). By incorporating chiral recognizable groups, such as amino acids and their derivatives into the temperature sensitive PNIPA network, we speculated that PNIPA hydrogel could be used to extract enantiomeric molecules and applied to enantiomeric separation. Based on above considerations, we wish to synthesize a new hydrogel, which can combine the

temperature sensitive properties of PNIPA with the excellent capability of enantioselective separation by introducing amino acid groups into PNIPA hydrogel.

In this study, a series of new temperature-sensitive hydrogels containing acrylic acid derivatized L-phenylalanine (poly(NIPA-co-AAc-*L*-Phe)) were synthesized by copolymeration of AAc-L-Phe with NIPA in ethanol. The temperature-responsive properties and the effect of weight ratio r (r = AAc-L-Phe / (AAc-L-Phe + NIPA)) on the swelling ratio (SR), deswelling kinetics and reswelling kinetics have been studied. The poly (NIPA-co-AAc-*L*-Phe) hydrogels have potential applications in the biological chemistry.

2. Experimental

2.1 Materials

N-isopropylacrylamide **(**NIPA**)** was purchased from Kohjin Co. Ltd., Japan and purified by recrystallization three times in the mixed solvent of benzene and n-hexane. D/L-phenylalanine (L-Phe/D-Phe) was obtained from Tianjin Kermel, China. Acryloyl chloride was purchased from Alfa Aesar, USA. All other reagents, including N, N-methylenebis (acrylamide) (MBAA), Azobisisobutyronitrile (AIBN), Ethanol, Triethylamine, N, N'-dimethylformamide (DMF), Dichloromethane, Thionyl chloride and Sodium chloride, etc. were analytical grade made in China, and used as received without further purification.

2.2 Synthesis of chiral monomer (AAc-L-Phe)

L-phenylalanine ethyl ester was synthesized based on as follow: ethanol (100ml) was put in a flask. In the ice bath, under the condition of stirring, thionyl chloride (64.5mmol) was added dropwise, the mixed solution was stirred for 1h. At room temperature (25^{o}C), L-phenylalanine (50mmol) was added and stirred for 3h. Then the mixed solution was heated to reflux for 2h. The over ethanol and thionyl chloride were made by distillation; the mixed solution was precipitated to solid. The solid was filterred and dried by vacuum drying. Sodium carbonate solution (25wt %) was added dropwise in the solid, which was the solution's pH>12. The liquid was extracted by acetic ester (20ml) three times. L-phenylalanine ethyl ester was made by rotary evaporator. Then L-phenylalanine ethyl ester (10mmol) was dissolved and stirred in DMF, then added triethylamine (10ml) in a flask. In the ice bath, under the condition of stirring, acryloyl chloride (20mmol) was added dropwise. Then the mixed solution was stirred for 4h at room temperature, the result was filtered and the filtrate was washed distilled water (40ml). The synthetic scheme of chiral monomer is summarized in Figure 1.The liquid was extracted by dichloromethane (20ml) three times. The oily liquid was made by rotary evaporator. The product recrystallized in ethanol three times, and finally the obtained crystallized product.

2.3 Synthesis of poly (NIPA-co-AAc-L-Phe) hydrogels

For the synthesis of poly(NIPA-co-AAc-L-Phe) hydrogels, different quality ratios of precursors (NIPA and AAc-L-Phe) were dissolved in 1.2mL ethanol in existence of a crosslinker MBAA(3wt.% based on total precursors), according to r 0, 0.05 and 0.1, respectively. AIBN (1wt.%) were then added as initiators. After bubbling with nitrogen gas to remove oxygen, the copolymerization was carried out at 55^{o}C for 24h and then the hydrogels obtained were first immersed in ethanol at room temperature to take out the unreacted chemicals. During this period, the ethanol was replaced with fresh ethanol every several hours. Then the hydrogels were further purified with distilled water at room temperature for at least 48h. Similarly, the distilled water was replaced every several hours to let the purified hydrogels reach equilibrium following characterization.

2.4 Measurements

Both AAc-*L*-Phe monomer and dried poly (NIPA-co-AAc-*L*-Phe) hydrogels were powdered with KBr, pressed into pellets under reduced pressure, and infrared spectrum analyses were taken on a TENSOR37-Fourier Transform Infrared Spectrometer (Bruker Corporation, Germany). ^{1}H-NMR measurements of AAc-*L*-Phe monomer were conducted at room temperature using $CDCl_3$ as solvent (Varian UNITY Plus-400 NMR, USA).

Swelling ratio (SR) of hydrogels was measured gravimetrically after wiping off the excess surface water with moistened filter paper in the temperature range from 20^{o}C to 45^{o}C. Gel samples were incubated in distilled water for at least 24h at every particular temperature. SR is defined as follows:

$$SR = W_s / W_d$$

Where W_s is the weight of water in a swollen hydrogels at the particular temperature, and W_d is the dry weight of hydrogels.

The deswelling kinetics of gels was measured gravimetrically at 45^{o}C after wiping off the excess surface water with moistened filter paper. Before this measurement, the gel samples reached equilibrium in distilled water at 25^{o}C. The weight changes of gels were recorded at the course of deswelling at regular time intervals. Water retention (WR) is defined as follows:

$$WR = 100(W_t - W_d)/W_s$$

Where W_t is the wet weight of hydrogels at regular time intervals and the other symbols are the same as defined earlier.

The reswelling kinetics of the gels was measured gravimetrically at 25°C, also after blotting the excess surface water with moistened filter paper. The weight changes of gels were recorded during this reswelling process at regular time intervals. The water uptake (WU).is defined as follows:

$$WU = 100(W_t - W_d)/Ws$$

Where Ws is the weight of water in swollen gel at 25°C and the other symbols are the same as defined above.

3. Results and discussion

3.1 Structural analysis of chiral monomer (AAc-L-Phe)

In this experiment, the IR analysis was applied to the characterization of samples' structure at and the certification of functional groups existing in molecules. The synthesis route in this experiment is shown in Figure 1, simultaneously, the infrared spectrum of samples is shown in Figure 2. The structures of chiral monomer AAc-*L*-Phe were determined by IR. There is a broad band in the range of 3600-3200cm^{-1} (~, 3312cm^{-1}) which belongs to N-H stretching vibration. This can be proved secondary amine group (RNH-R'). The typical amide I band (~, 1679cm^{-1}), consisting of C=O stretch. At the same time, it is saw by the presence of the typical band of unsymmetrical vibration (~, 1210cm^{-1}), consisting of C-O-C stretch. There is which belongs to C-H stretching vibration of the aromatic hydrocarbon (~, 3033cm^{-1}). There is a peak band which belongs to C-H of olefin(RHC=CH_2) out-of-plane vibration (~, 910cm^{-1});The typical Benzene ring skeleton I band (~, 1527,1449cm^{-1}), consisting of C=C stretching vibration and Benzene ring II band (~, 743cm^{-1}), including C-H out-of-plane morph vibration were evident by spectrum.

The structures of chiral monomer AAc-*L*-Phe were determined ^{1}H-NMR measurement ($CDCl_3$) which is shown in Figure 3. The peaks at 3.26 and 5.07ppm can be assigned to –CH=C and methyl protons from AA, respectively. And the peaks at 1.28, 4.21ppm can be assigned to -CH_2- and methyl protons of ester group. The signals of 7.73, 7.29 and between 7.40 and 7.53ppm were assigned to the protons of benzene ring. The peak at 7.15ppm was attributed to the protons of -CH_2- linked with benzene ring. The signals of 6.60ppm corresponded to the chiral proton.

3.2 Structural analysis of hydrogels

IR spectra show that the FT-IR spectra of hydrogels in Figure 4, which curve A means PNIPA hydrogels and curve B represents poly(NIPA-co-AAc-*L*-Phe) hydrogels. Even though the spectrum of each hydrogels showed some changes, it is found that the FT-IR spectra were similar. Every spectrum showed a broad band in the range of 3600–3200cm^{-1}, which belongs to N–H stretching vibration of the PNIPA. The typical amide I band (~, 1641cm^{-1}), consisting of C=O stretch of PNIPA and amide II band (~, 1549cm^{-1}), including N-H vibration were evident in curve A. The existence of chiral monomer was evident by the presence of the typical band of unsymmetrical ester group (~, 1211cm^{-1}), consisting of C-O-C stretch, although this band was weak due to the low chiral monomer content in hydrogels in curve B.

3.3 Effect of temperature on SR of poly(NIPA-co-AAc-L-Phe) hydrogels

The swelling behaviors of poly(NIPA-co-AAc-L-Phe) hydrogels with different r values were investigated at various temperatures, and the results are shown in Figure 5. We can see that compared with PNIPA hydrogel, the SRs of the hydrogels decreased with the increasing contents of chiral units (AAc-*L*-Phe) at temperatures below the LCST. This result indicates that poly(NIPA-co-AAc-*L*-Phe) hydrogels maintain the temperature-sensitive characteristics of PNIPA hydrogel, which is attributed to the temperature sensitive component PNIPA. However, incorporating the hydrophobic group (benzene ring) from L-phenylalanine ethyl ester to the hydrogel increases the hydrophobic nature and exerted negative an effect on the extensibility of the hydrogel network.

The deswelling kinetics of hydrogels after a temperature jumping from the equilibrated swollen state at 25°C to the hot water at 45°C is showed in Figure 6. The most important observation is an abrupt shrinkage with all the hydrogels which lost more than 80% of their original water contents within 80min and quickly reached their stable WRs within 100min. The deswelling speed and the WR increased with the increase of r; it might be associated with the increase of the amount of the hydrophobic units (AAc-*L*-Phe) and thus the force that forces the PNIPA chain into globule conformation was strengthened with the increase of r.

We further studied the reswelling kinetics of the dry poly(NIPA-co-AAc-*L*-Phe) hydrogels in distilled water at 25°C (Figure 7). It was found that all the hydrogels reswelled and reached to equilibrium within 12h, and the water uptake decreased with the increase of r, which is also attributed to the increasing hydrophobic nature of AAc-*L*-Phe.

4. Conclusions

Acrylic acid derivatized L-phenylalanine (AAc-*L*-Phe) was successfully synthesized and incorporated into the backbone of the PNIPA hydrogel by copolymerizing in ethanol. Both monomer and poly(NIPA-co-AAc-*L*-Phe) gels were characterized by using IR spectra and 1H-NMR. The swelling and deswelling behaviors of hydrogels responding to external stimuli were studied, and poly(NIPA-co-AAc-*L*-Phe) hydrogels showed evident temperature-sensitivity.

Compared with PNIPA hydrogel, poly(NIPA-co-AAc-*L*-Phe) hydrogel exhibits better absorption property and enantioselectivity for D/L-phenylalanine. The thermosensitive poly(NIPA-co-AAc-*L*-Phe) hydrogel have potential applications the biological chemistry.

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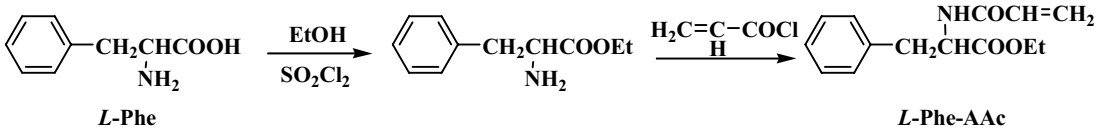

Figure 1. The synthetic scheme of AAc-*L*-Phe monomer

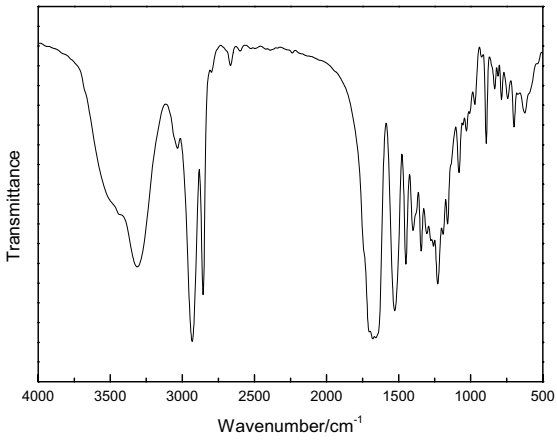

Figure 2. Infrared spectra of AAc-*L*-Phe monomer

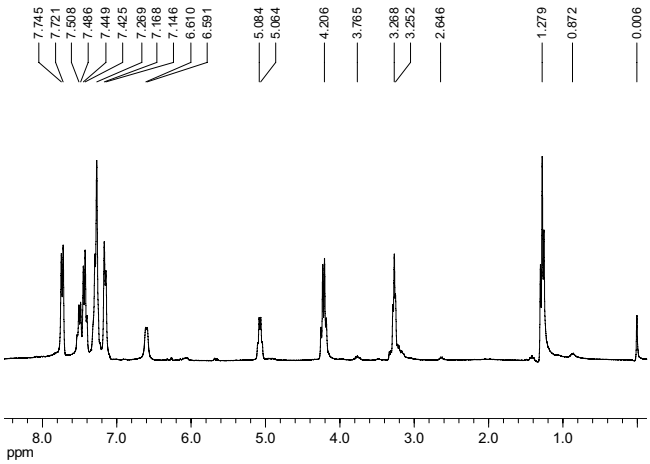

Figure 3. ^{1}H-NMR of AAc-*L*-Phe monomer

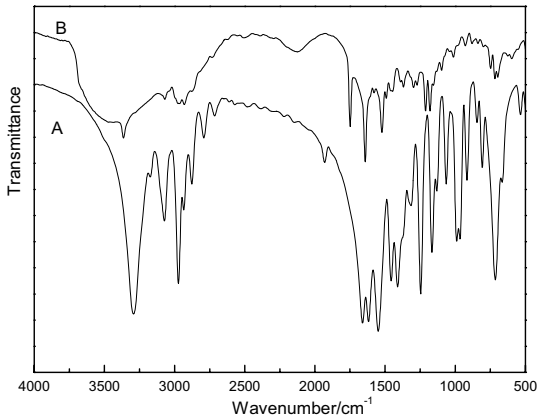

Figure 4. Infrared spectra of hydrogels

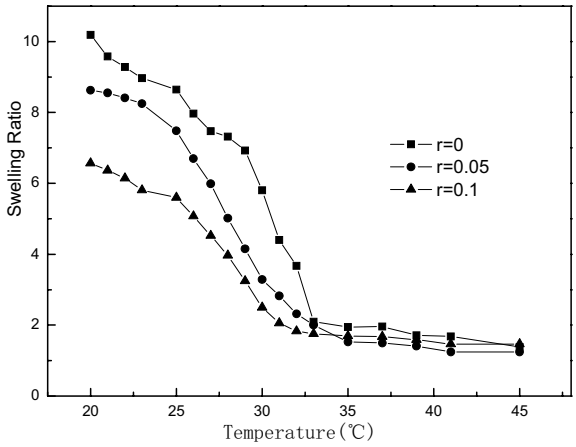

Figure 5. Temperature dependence of equilibrium SR at the temperature range from 20 to 45°C

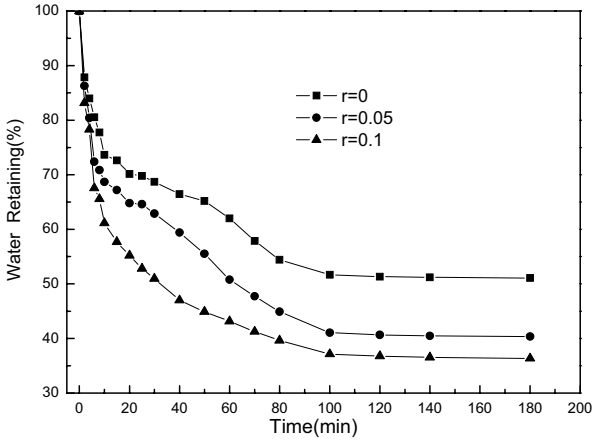

Figure 6. Deswelling kinetics of hydrogels in distilled water

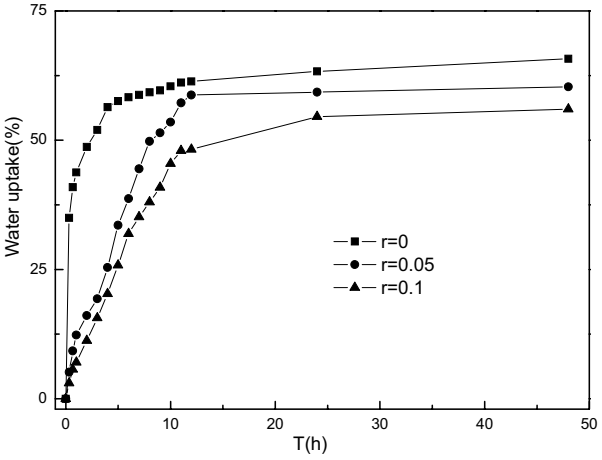

Figure 7. Reswelling kinetics of hydrogels at 25°C in distilled water

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