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Mint Extract Present Antioxidant Action on the Erythrocytes of Dairy Cows Suffering from Mastitis?

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Abstract

Bovine Mastitis is an infection of the mammary glands that represents important economic losses in milk production. As in other pathologies, medicinal plants emerged as an important alternative for treatment of this condition. In this sense, the aim of this study was to investigate the antioxidant activity of the extract of *Mentha arvensis* L. on the erythrocytes of healthy dairy cows (P1); of dairy cows with mastitis without previous treatment (P2); and of dairy cows with mastitis and previously treated with antibiotics (P3). The levels of Thiobarbituric Acid Reactive Substances (TBARS), of the protein carbonylation (PC) and reduced glutathione (GSH) were analyzed in the cows' erythrocytes before and after treatment with the extract of Mentha. This study demonstrated an occurrence of oxidative stress in the cows with mastitis. The mint extract promoted an increase in GSH levels combined with a decrease in the levels of oxidative markers, especially in cows with mastitis without previous antibiotic treatment.

Keywords: oxidative stress, bovine mastitis, *Mentha arvensis* L.

1. Introduction

Bovine mastitis is considered one of the most important diseases of dairy cattle in the world, as it is the main cause for economic loss to milk producers everywhere. This condition is characterized by the inflammation of the mammary gland – it is usually infectious, and can be classified both as clinical and as subclinical (Tomazi et al., 2015). In order to evaluate the occurrence of oxidative damage in dairy cows and its influence on milk production, we found that the so-called "oxidative stress" in periparturient dairy cows is a contributing factor to the development of breast edema, hypocalcemia, retained placenta, mastitis and decreased reproduction (Miller et al., 1993a). As a result, the authors highlight the need to supplement dairy cattle with antioxidants such as vitamin E, beta-carotene, glutathione, urate, among others. These findings emphasized that breast edema may be caused by "oxidative stress", as reactive oxygen metabolites are not well controlled or metabolized by the body in such conditions (Miller et al., 1993b).

Oxidative stress is the imbalance between the amount of reactive species (RS) and the cellular antioxidant capacity of the body (Scandalios, 2005). In the event of such imbalance, there may be oxidative damage in cells and tissues of animals, to the point of engendering the loss of biological functions important for homeostasis (Pisoschi & Pop, 2015). This may be demonstrated by the oxidation of the cell membrane lipid layer, leading to increased levels of Thiobarbituric Acid Reactive Substances (TBARS) and protein carbonyls (PC), which can be quantified by using the spectrophotometric method. The elevation of blood biomarkers indicates increased protein and lipid oxidation levels (Barbosa et al., 2010).

Therefore, the high production of RS is not harmful to the cells, as the body uses the antioxidant system, which neutralizes and prevents the action of these highly reactive species. There are two types of antioxidant mechanisms, enzymatic and non-enzymatic. The enzymatic antioxidant system consists of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR).

Other compounds form the non-enzymatic antioxidant system: reduced glutathione (GSH), uric acid, ascorbic acid (vitamin C), α-tocopherol (vitamin E) and β-carotene (Barreiros et al., 2006; Barbosa et al., 2010). Factors that disrupt the organic homeostasis, such as infections, can interfere with the biochemical processes in the body, leading to the formation of RS.

The genus *Mentha*, belonging to the Lamiaceae family, comprises about 25 species – it originates from the old world in the early days of the American continent (Watanabe et al., 2006; Garlet et al., 2007). Mints are perennial, herbaceous, fast growing plants with violet branched stems and serrated opposite leaves, blue or purple flowers arranged in terminal spikes, and fruit (achene) (Watanabe et al., 2006). Furthermore, they produce essential oils, accumulated in the glandular trichomes of leaves and inflorescences. These oils are liquid, volatile, and contain substances responsible for the refreshing aroma of these plants, very important for pharmaceutical use, cosmetics and food, as well as for its use in folk medicine (Paulus et al., 2007). Ethnoveterinary Medicine seeks to rescue traditional knowledge, the use of medicinal plants in veterinary medicine, so as to establish a connection with scientific knowledge. It also relates to Agroecology, a field of agriculture which seeks ecological productive experiences in order to develop "sustainable agriculture – one that is fairer, economically viable, and ecologically appropriate" (Wanzala et al., 2005).

The establishment of agro-ecosystems in this perspective, through the redefinition of traditional practices and the elaboration of new collective solutions, provide a sustainable alternative for ecological production. Thus, both the quantity and quality of the milk depend on solutions to livestock diseases and on using sustainable alternatives with renewable natural resources – for that matter, the use of medicinal plants show great potential (Caporal & Costabeber, 2002). Among the various types of essential oils used, those produced by the genus Mentha are in most demand – particularly *Mentha arvensis* L. (Japanese mint, vique), which has menthol as its main component (Shelepova et al., 2014). Presently, one can hardly avoid *Mentha arvensis* L. essential oil or its derivatives, for they are used in the formulation of several industrial products worldwide (Garlet et al., 2007). In this context, the present study aims to investigate the antioxidant effect of the *Mentha arvensis* L. extract in the erythrocytes of dairy cows suffering from mastitis by using tests "*in vitro*".

2. Method

2.1 Ethical Aspects

This project was submitted to the Ethics Committee for the Use of Animals (CEUA) and subsequently approved with the number 005/12.

2.2 Experimental Design

For this experiment, we used pure Holstein cows (black and white), property of Irmãos Strobel Agropecuary, from the city of Condor, located at latitude 28°12′28″ S and longitude 53°29′14″ W; 451 m of altitude, with an area of 465,64 km² and a population estimated at 6,543 inhabitants, in the northwest of the Rio Grande do Sul state. The property works with the "free stall" production system, administering a complete diet ad libitum, based on corn and concentrate. Seventy-five (75) animals were classified as to their health status in three groups: control group (P1), consisting of 25 healthy animals; group 2 (P2), consisting of 25 animals with mastitis detected by CMT (Californian Mastits Test) and SCC (Somatic Cell Count) without antibiotic treatment; and group 3 (P3), which comprised a set of 25 animals that were detected with mastitis by CMT and SCC and underwent treatment with antibiotics for at least three days preceding the time of blood collection.

In order to conduct this research, we used leaves and stems of *M. arvensis* L., collected at the Garden of Medicinal Plants of the Cruz Alta University (UNICRUZ), identified with voucher number 1102, located at the UNICRUZ Herbarium. Three hundred grams (300 g) of the plant were collected and dried in a greenhouse at 37°C for seven days; thereafter the plants were macerated and stored in 70% ethanol (v/v) in amber bottles for 30 days. Hydroalcoholic extracts (EHA) were obtained from the plant. The extracts were obtained by macerating 100 g of dry plant in 700 mL of 70% alcohol (v/v), where they remained for 30 days. After that, the extracts were filtered with a vacuum pump and each resulting mixture was stored in amber bottles. Before submitting it to use, each extract was subjected to fractional distillation rote-evaporator at reduced pressure for the extraction of alcohol. After the extraction, we proceeded to rehydration with distilled and autoclaved water, reconstituting the original concentration of the extract. The dilution concentration used in this study was 1 mg/mL.

2.3 Samples

Blood samples from the animals were collected by venipuncture of the coccygeal vein, after disinfection with 70% alcohol, using disposable needles and Vacuntainer® tubes with addition of EDTA. Blood samples were kept refrigerated at 4 °C and centrifuged at 3.000 rpm to remove the plasma and the separation of erythrocytes.

Subsequently, the erythrocytes were washed three times with buffer solution of Phosphate Buffered Saline (PBS). Then, the solutions of erythrocytes were resuspended and diluted in PBS buffer until they reached hematocrit levels of 5%. After washing the erythrocytes, we treated the samples from groups P1, P2 and P3, keeping only the supernatant in group P1, with no intervention of any product, while in groups P2 and P3 there was exposure to the extract of *Mentha arvensis* L. at a concentration of 1mg/mL for one hour. After this period, the samples were hemolysed and centrifuged, and the final product was the separation of the supernatant, which was stored at a -20 °C temperature until completion of analytical determinations (Catalgol et al., 2007).

2.4 Analytical Determinations

Lipid peroxidation was determined according to the method of formation of thiobarbituric acid reactive species (TBARS), as prescribed by Stocks and Dormandy (1971) protocols. The supernatant was added to the reaction mixture containing trichloroacetic acid (TCA) to 28% (v/v) and thiobarbituric acid (TBA) (0.1 mol/L), followed by heating at 95 °C. Readings were taken in a spectrophometer visible at 532 nm – length at which the resulting product, malondialdehyde (MDA), can be measured. The results were expressed by nmol/g Hb. Hemoglobin levels were determined through use of Labtest® material.

The analysis was carried out using the technique described by Levine et al. (1990) adapted to erythrocytes, in which the supernatant is previously diluted with Hepes and with subsequent determination of the carbonyl proteins using TCA at 10% (v/v), 2 N hydrochloric acid, 2,4-dinitrophenylhydrazine (DNPH) and 10 mM sodium dodecyl sulfate (SDS) at 3% (w/v). Readings were taken in a spectrophotometer visible at 370 nm. The results were expressed as nmol of carbonyl/mg protein. Total proteins were determined in erythrocytes diluted with Hepes, according to Labtest® protocol.

The levels of reduced glutathione (GSH) were determined according to the method described by Ellman (1959) adapted to erythrocytes, which employs potassium phosphate buffer (TFK) at 1 M in pH 7.4, and 5,5'-dithiobis acid (2-nitrobenzoic acid) (DTNB) as the color reagent. Readings were taken in a spectrophotometer visible at 412 nm. The results were expressed as μmol GSH/mL.

2.5 Statistics

Both analytical determinations, carried out in duplicate, and results were expressed by average ± SEM (Standard Error of the Mean). Data obtained from all groups studied, for the same parameter, were submitted to Analysis of Variance one way (ANOVA), followed by the Tukey-Kramer test, considering the significantly different averages with a $P < 0.05$. To do so, we employed the statistical program GraphPad Prism® 6.0.

3. Results

TBARS levels in groups P2 was increased (48.50 ± 5.55 nmol/g Hb) and P3 was reduced (1.89 ± 0.34 nmol/g Hb), before exposure to the extract of mint in comparison with the P1 (Control Group) (16.76 ± 1.99 nmol/g Hb) (Figure 1). Additionally, figure 1 shows that there was a significant reduction in TBARS levels in group P2 (13.27 ± 1.08 nmol/g Hb) analyzed after exposure to the extract of mint.

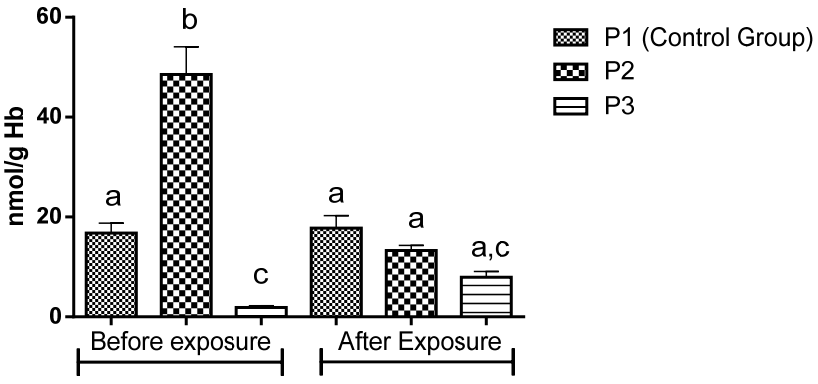

Figure 1. Levels of Thiobarbituric Acid Reactive Substances (TBARS) (nmol/g Hb) measured in the erythrocytes of dairy cows exposed to the extract of *Mentha arvensis* (1 mg/mL). Values are expressed as mean ± SEM (n = 25). Different letters represent significantly different statistics

The levels of protein carbonyl were increased P2 (121.0 ± 5.97 nmol/mg protein) and P3 (180.8 ± 19.23 nmol/mg protein) before treatment with the plant's extract in comparison with the P1 (Control Group) (51.33 ± 4.70 nmol/g Hb) (Figure 2). Furthermore, this same figure shows that there was a significant reduction in carbonyl levels in group P3 (96.73 ± 3.82 nmol/g Hb) analyzed after exposure to the extract of mint (Figure 1).

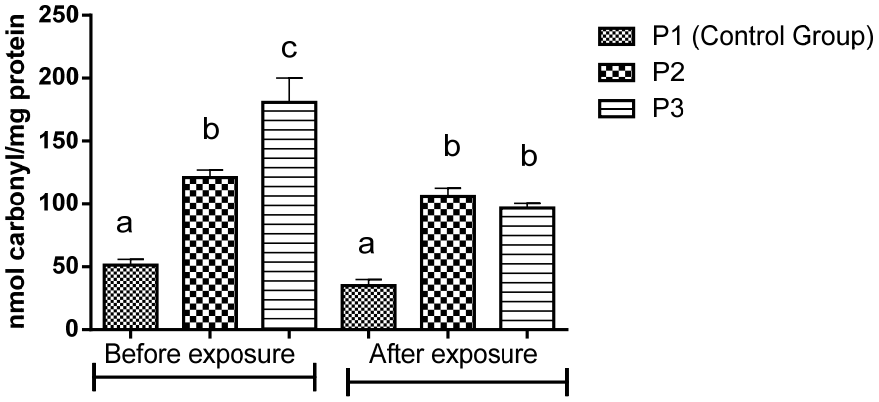

Figure 2. Levels of protein carbonyls (PC) (nmol carbonyl/mg of protein) measured in the erythrocytes of dairy cows exposed to the extract of *Mentha arvensis* (1 mg/mL). Values are expressed as mean ± SEM (n = 25). Different letters represent significantly different statistics

We determined the main marker levels of the non-enzymatic antioxidant system in cows, reduced glutathione, in order to verify if the plant could increase its protection against damage from oxidative stress. We found that GSH levels decreased in groups P2 (1.41 ± 0.05 µmol GSH/mL) and increased in group P3 (7.38 ± 0.11 µmol GSH/mL) in comparison with the P1 (Control Group) (2.83 ± 0.27 µmol GSH/mL) before exposure to the extract of mint (Figure 3). This same figure shows that there was a significant reduction in GSH levels in group P2 (3.48 ± 0.07 µmol GSH/mL) and P3 (5.95 ± 0.23 µmol GSH/mL) analyzed after exposure to the extract of mint.

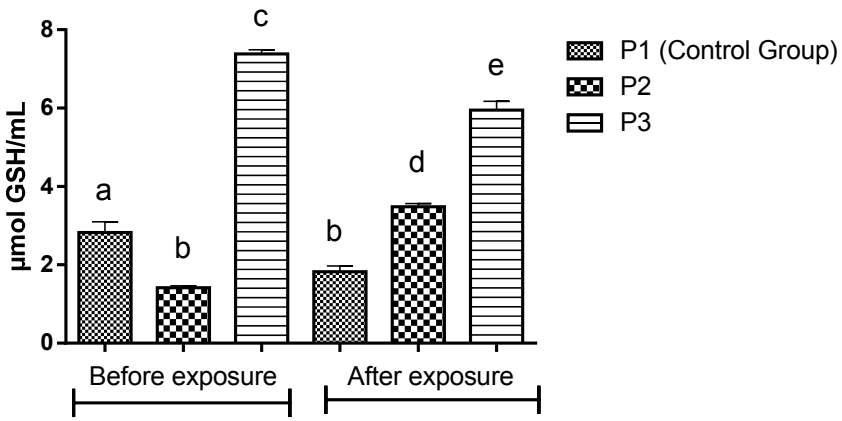

Figure 3. Levels of reduced glutathione (GSH) (µmol GSH/mL) measured in the erythrocytes of dairy cows exposed to the extract of *Mentha arvensis* (1 mg/mL). Values are expressed as mean ± SEM (n = 25). Different letters represent significantly different statistics

4. Discussion

Blood is an excellent source of oxidative stress markers "*in vivo*". In particular, erythrocytes, that are enucleate cells and don't have the ability to repair the damage caused by oxidation. Besides, the membrane of this cell is a

major target of oxidative damage, which makes a model of oxidative stress used for "*in vitro*" tests (Furman et al., 2012). Thus, oxidative damage, as well as a possible antioxidant activity demonstrated in erythrocytes may suggest cell behavior "*in vivo*".

In pathological conditions, the production of RS exceeds the antioxidant defense capacity, as they are highly reactive compounds, harmful to lipids, proteins and nucleic acids (Barreiros et al., 2006). This process is demonstrated by the occurrence of lipid peroxidation, i.e. the oxidation of the cell membrane lipid layer, which can be measured by TBARS (Barbosa et al., 2010). The results found in this study regarding TBARS (Figure 1) show a more marked occurrence of lipid peroxidation in cows with mastitis without previous antibiotic treatment (P2 group). However, after the treatment with mint extract this levels were decreased, a possible indication of the antioxidant effect of plant on cows with mastitis. In P3 group, nevertheless, the treatment with mint extract not show this effect, maybe because the used of antibiotics in this cows were already enough to reduced cellular lipid peroxidation levels, because treated the mastitis.

There may be attacks by the reactive species (RS) on the amino acids that make up proteins, which can do damage such as cleavage of connections, with or without generation of fragments and crosslinking, and cause decreased enzyme activity, difficulties in active transport through cell membranes, cytolysis, and cell death (Chondrogianni et al., 2014). Supporting the findings of this study, these changes in amino acids result in the formation of protein carbonyls, which are used as a specific marker for measuring oxidative stress (Jamel et al., 2010). The results found for the determination of levels of protein carbonyls (Figure 2), it was demonstrated that mastitis influence PC levels as expected. Nevertheless, the exposure to the extract of mint favored PCs reduction in P3 group after treatment with the plant extract.

Therefore, considering the results, it was found that the extract may serve as a preventive measure to avoid the increase of lipid and protein damage in cows with mastitis, making them less susceptible to physiological changes likely to favor secondary pathologies. Still, it's possible that mint acts helping the antibiotics to decreased cellular damage, especially PCs levels. These results corroborated with Zakaria et al. (2008), that investigated the antioxidant activity of many plants from Lamiaceae family, including the *Mentha arvensis*, and demonstrated that all the extracts investigated exhibited a rather high degree of activity (more than 40%). Jung et al. (2004), that conducted a screening for antioxidant activity of plant medicinal extracts and showed that *Mentha arvensis*, among other plants, had strong antioxidative activity in the both DPPH (2,2-diphenyl-1-picrylhydrazyl) and superoxide anion radical scavenging activities.

Glutathione, a tripeptide (γ-L-glutamyl-L-cysteinyl-glycine), exists in reduced form (GSH) and oxidized (GSSG), acting in many important biological processes such as protein synthesis, metabolism, and detoxification of xenobiotics. In particular problems in synthesis and glutathione metabolism are associated with the development of pathology (Júnior et al., 2001). This condition it was confirmed by this research, because P2 group (cows with mastitis) showed GSH levels reduced when this group was compared with P1 (control group) (Figure 3). However, after the treatment with mint extract GSH levels increased in this group showing that the plant exerts satisfactory antioxidant effect on cows suffering from mastitis with no previous treatment, as it increases the main non-enzymatic antioxidant, which participates in the detoxification of chemicals and disposal of lipid peroxidation products, having as its main capacity the neutralization of RS with great efficiency and speed (Augusti et al., 2009; Barbosa et al., 2010). In cows treated with antibiotics (P3) this level was increased before the treatment with mint, suggesting a beneficial effect on antioxidant system of medication in the mastitis. In this case, the association with the mint extract doesn't appear to be beneficial (Figure 3), because promoted decreased of GSH levels.

This study clearly demonstrated an occurrence of oxidative stress in the cows with mastitis. This factor may favor the development of other diseases, thus, it is utterly important to find alternatives for treating mastitis with no further aggravation of the animal's clinical presentation (Miller et al., 1993a). The mint extract promoted an increase in GSH levels combined with a decrease in the levels of oxidative markers, especially in cows with mastitis without previous antibiotic treatment. Therefore, in the future this plant can to be used to treat the mastitis, especially because it seems to act on oxidative damage already installed and stimulate the production of the main endogenous antioxidant.

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