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Effect of *Asperagillus awamori* on Alloxan-induced Mouse Hyperglycemia

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Abstract

Problem statement: Though alloxan-induced mouse hyperglycemia was ameliorated by feeding of 5 % *Asperagillus awamori* (*A. awamori*)-fermented burdock root diet (fermented burdock diet), it is unclear whether the anti-hyperglycemia activity is due to *A. awamori* or antioxidant activity induced by the fermentation.

Methods: A 0.05 % *A. awamori* diet was prepared. Acatalasemic mice, having a quite low catalase activity in blood, were divided three groups, and each group fed control, *A. awamori* and the fermented burdock diets for 14 weeks, separately. Then, alloxan monohydrate (200 mg/ kg of body weight) was intraperitoneally administrated to each mouse. Glucose, insulin, C-peptide contents in blood and glucose tolerance tests (GTTs) were examined.

Results: Incidence of alloxan-induced hyperglycemia in acatalasemic mice maintained with the *A. awamori* diet or the fermented burdock diet was low (20 or 25%) compared to that (75%) maintained with the control diet. Feeding the *A. awamori* diet ameliorated insulin, C-peptide in blood and GTT like as mice fed the fermented burdock diet. It indicated that *A. awamori* in these diets plays an important role for the prevention of alloxan-induced hyperglycemia.

Conclusions: It is suggested that *A. awamori* has the anti-hyperglycemia activity.

Keywords: hyperglycemia, insulin, C-peptide, alloxan, *Asperagillus awamori*

1. Introduction

Diabetes mellitus is a syndrome characterized by hyperglycemia, more than a desirable level of glucose in blood (Taylor, 1995). The morbidity causes blindness, renal failure and amputation, and diabetes is a worldwide disease and one of the major causes of death. It was reported that 171 million people suffer from diabetes at 2000 and 366 million people will do at 2030 (Wild et al., 2004). Alloxan is a diabetogenic drug for animals, and alloxan with reducing agents in the body generates reactive oxygen species to induce oxidative stress. These oxygen species selectively injure β-cells in the pancreas so as to cause hyperglycemia as like diabetes Type 1 (Szkudelski, 2001; Lenzen, 2008). Many researchers interest in finding anti-hyperglycemic compounds or products to prevent this alloxan-induced hyperglycemia (Perumal et al., 2014; Oloyede et al., 2015). As we found that acatalasemia (very low catalase activity, hereditary catalase deficiency) mice induced diabetes mellitus with smaller amount of alloxan than normal mice, we also pursued anti-hyperglycemia compounds using alloxan-induced acatalasemic mice (Takemoto et al., 2009; Kamimura et al., 2013). Burdock root, *Artium lappa*, is a popular vegetable in Japan and Korea. It contains a considerable amount of polyphenols such as chlorogenic acid, caffeoylquinic acid, hydroxycinnamoylquinic acids and related compounds and indicates antioxidant activity (Maruta, Kawabata & Niki, 1995; Lin & Harnly, 2008). Okazaki et al. (2013) prepared *A. awamori*-fermented burdock root to improve the functional property of burdock, and indicated that the intake improved intestinal environment and suppressed obesity in rats fed a high fat-diet. Fermentation of burdock root

with *A. awamori* indicated increases of 1, 1-diphenyl-2-picryhydrazyl (DPPH) scavenging activity and polyphenol contents (Doi et al., 2015), and feeding of 5% *A. awamori*-fermented burdock root diet ameliorated mouse hyperglycemia induced by alloxan (Doi et al., 2013; Takemoto et al., 2014). As feeding of 5 % raw burdock root diet did not ameliorate mouse hyperglycemia, we suggested that *A. awamori* plays an important role for preventing alloxan-induced mouse hyperglycemia. As it was suggested that the intake of antioxidants can ameliorate oxidative stresses (Yamaoka et al., 2008; Choi et al., 2009), we prepared 0.05 % *A. awamori* diet and examined the effect on alloxan-induced mouse hyperglycemia to confirm anti-hyperglycemia effect of *A. awamori*.

2. Materials and Methods

2.1 Materials

Normal mice (C3H/AnL CS^aCS^a) and acatalasemia mice (C3H/AnL CS^bCS^b) established by Feinstein, Braun, & Howard (1967) were bred. Both male mice were maintained on a laboratory diet (CE-2 diet, Clea Japan, Tokyo) and water ad libitum until the start of the experiments. Catalase activity in the mouse erythrocytes was measured according to previous method (Masuoka et al., 1996) and calculated as the difference between the hydrogen peroxide removal rate by hemolysate and the rate (0.73 μmol/s/g of hemoglobin) by hemoglobin (Takemoto et al., 2009).

Control diet (AIN-93M), *A. awamori* and *A. awamori*-fermented burdock diets were prepared by Ahjikan Co. Ltd (Hiroshima, Japan). *A. awamori* diet was prepared by mixing components (in **Table 1**) with 0.1 volume of water and then dried using air drying oven at 50 ℃. *A. awamori*-fermented burdock diet was prepared according to the procedure (Okazaki et al., 2013; Doi et al., 2015). Tablets of these diets (1.3 X 2 cm) were prepared and stored at -20 ℃ until use.

Table 1. Composition of prepared diets (%, w/w)

Component compounds	Control diet	Fermented burdock diet	*A. awamori* diet
Corn starch	47.0692	42.0692	47.0192
Milk casein	14.0	14.0	14.0
α-Corn starch	15.0	15.0	15.0
Sucrose	10.0	10.0	10.0
Soybean oil	4.0	4.0	4.0
Cellulose powders	5.0	5.0	5.0
Mineral mixture (AIN-93M)	3.5	3.5	3.5
Vitamin mixture(AIN-93VX)	1.0	1.0	1.0
L-cysteine	0.18	0.18	0.18
Choline	0.25	0.25	0.25
t-Butylhydroquinone	0. 0008	0.0008	0.0008
Fermented burdock root*	0.0	5.0	0.0
Aspergillus awamori spores	0.0	0.0	0.05

Control diet, AIN-93M, was prepared according to the method (Reeves, Nielson, & Fahey, 1993).

* indicates *A. awamori*-fermented burdock root powders.

2.2 Animal Experiments

The experimental procedure was approved by the Ethics Review Committees for Animal Experimentation of Okayama University of Science. Alloxan monohydrate (200 mg/ kg of body weight) was intraperitoneally administrated to induce hyperglycemia for acatalasemic mice (Takemoto et al, 2009).

Acatalasemic mice (n=25) and normal mice (n=17) were used at the age of 14 to 15 weeks old (body weight was between 22 and 37 g) and were housed in a group of four. Acatalasemic and normal mice were divided into three kinds of diet groups, respectively. Mice in each group fed control diet, *A. awamori* and the fermented burdock diets ad libitum for 14 weeks. Then, each group divided two groups, alloxan administration and the control groups. Alloxan monohydrate was dissolved with phosphate buffered saline (PBS), and the solution was intraperitoneally administrated (200 mg/ kg of body weight). To mice in the control group, PBS was injected. Mice in each diet group were maintained on the same diet for one more week. After five days, mice were fasted for 20 hrs, and GTTs were carried out. After 2 days from GTT, mice fasted were killed under diethyl ether anesthesia. Each mouse blood was collected in test tube containing heparin as the anticoagulant from heart. Blood was centrifuged, and the plasma was isolated. Insulin and C-peptide levels in plasma were examined.

Pancreas in each mouse was isolated, and the sections were prepared for microscopic studies.

2.3 Assay of Blood Glucose

After fasting, glucose content in the blood obtained from the tail was determined. As the blood volume for the determination of blood glucose was quite small (approximately 2 μL), the glucose contents in blood were measured with a "Glucose-Test-Ace R" apparatus (Sanwa Kagaku Kenkyusho Co., Nagoya, Japan) applying a glucose oxidase method.

2.4 GTT

After fasting, a forty percent aqueous glucose solution (5 mL / kg of body weight) was intraperitoneally administered to each mouse (Gao et al., 2007). At 0 and 30 min before and 15, 30, 60, 90 and 120 min after the administration, glucose contents in the blood were measured.

2.5 Determination of the Insulin and C-peptide Levels in Blood

The insulin and C-peptide plasma levels were determined using Mouse Insulin and C-peptide ELISA KITs (U-type) (Shibayagi, Gunma, Japan). Each determination was carried out according to the manufacturer's instructions. Biotin-conjugated anti-insulin antibody (45 μL) was added to each well in an antibody-coated 96-well plate. To the well, 5 μL of the sample or standard solution was added and reacted for 2 hrs. Then 50 μL of peroxidase-conjugated avidin solution was added and reacted for 30 min. Chromogenic substrate solution (50 μL) was added and reacted for 30 min. The reaction was stopped and the absorbance at 450 nm (sub-wave length, 620 nm) was recorded.

2.6 Microscopic Studies of Pancreatic Tissues in the Mice Treated with Alloxan

Pancreatic tissues were isolated, fixed in Bouin's fluid and embedded in paraffin. Serial sections (6 μm) were cut from each paraffin-embedded tissue block, and several sections were stained with hematoxylin-eosin and mouse anti-insulin antibody (Santa Cruz Biotechnology) using the Vectastain Elite ABC Rabbit IgG Kit for visualization by light microscopy. The islets and other cells were recorded with a FX380 CCD Camera and a microscope (Olympus, Tokyo, Japan).

2.7 Statistical Analysis

Student's t-test was used to evaluate the statistical significance of difference. The difference was considered significant when $p<0.05$.

3. Results

3.1 Catalase Activity in Mouse Blood

The catalase activity of acatalasemic mice in blood at 25 °C was 0.15 ± 0.07 μmol/s/g of Hb, and that of normal mice was 6.77± 0.62μmol/s/g of Hb. ($p<0.001$)

3.2 Body Weights Maintained on A. Awamori, the Fermented Burdock and the Control Diets

The average mouse body weights were indicated in **Figure 1**. There is no difference among body weights by feeding control diet, *A. awamori* diet and the fermented burdock diet.

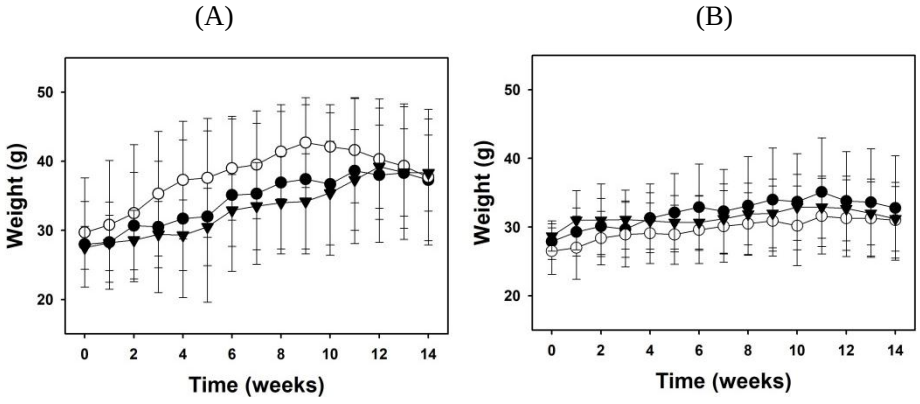

Figure 1. Body weight changes of normal and acatalasemic mice by feeding control, *A. awamori* and the fermented burdock diets

(A) Normal mice, (B) acatalasemic mice. Open circle (○) indicated mice fed control diet, closed circle (●): mice fed *A. awamori* diet, triangle (▼): mice fed fermented burdock diet.

3.3 Incidence of Hyperglycemia Maintained on Each Diet after Alloxan Administration

After feeding each diet for 14 weeks, alloxan was administrated. Incidence of hyperglycemia was examined after 5 days from alloxan administration (Table 2). It indicated that the incidence of acatalasemic mice fed *A. awamori* diet is low as the mice fed fermented burdock diet.

Table 2. Incidence of hyperglycemia after alloxan administration

Mice	Alloxan (mg/kg)	Incidence of hyperglycemia %		
		Control diet (n)	*A. awamori* diet (n)	Fermented burdock diet (n)
Normal	200	0 (4)	0 (4)	0 (5)
Acatalasemia	200	75 (4)	20 (5)	25 (4)

"n" in parentheses indicates number of mice. Hyperglycemia is ＞121 mg/dl of blood.

3.4 Effect of the A. awamori Diet on GTT

GTTs of normal (A) and acatalasemic (B) mice treated with alloxan were indicated in **Figure 2**. GTT of normal mice treated with alloxan suggested that there is essentially no difference among them. However, in the case of acatalasemic mice, blood glucose in mice fed the control diet at 15, 30, 60, 90 and 120 min from glucose administration was higher than mice fed *A. awamori* and the fermented burdock diets. Blood glucose level of acatalasemic mice fed *A. awamori* diet or the fermented burdock diet after 120 min returned to the level before alloxan administration.

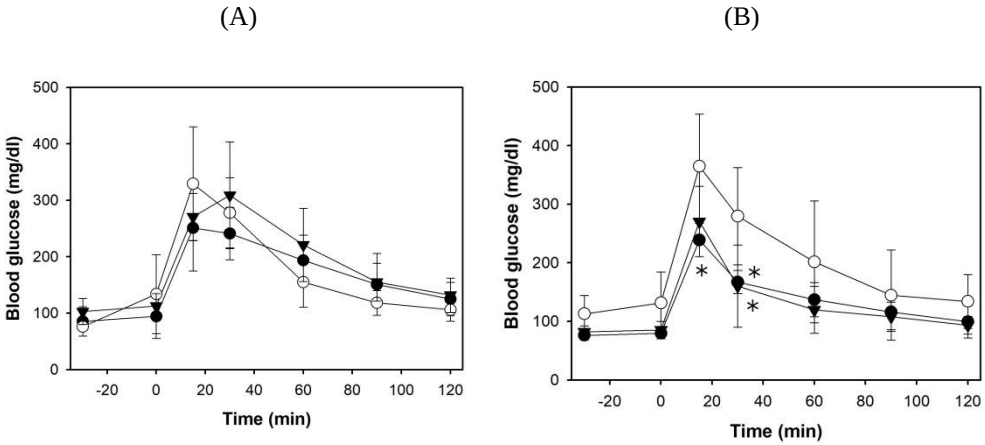

Figure 2. Glucose tolerance tests of alloxan administrated mice

(A): Normal mice, (B): acatalasemic mice. Glucose was loaded at 0 min. Open circle (○) indicated mice fed control diet, closed circle (●): mice fed *A. awamori* diet, triangle (▼): mice fed the fermented burdock diet. * indicated P<0.05, compared to the control diet.

3.5 Insulin and C-peptide Concentrations in Acatalasemic Blood

After alloxan administration, insulin concentrations in acatalasemic mouse blood decreased (data not indicated). Concentration of C-peptide from acatalasemic mice fed the control diet significantly decreased from 740 ± 101 pg/mL to 572 ± 47 pg/mL (P=0.014) but the concentration from mice fed *A. awamori* diet and the fermented burdock diet did not (from 645 ± 95 to 645 ± 65 pg/mL and from 651 ± 61 to 631 ± 52 pg/mL, respectively).

3.6 Microscopic Examination of Pancreatic Tissues in Mice Treated with Alloxan

In the case of acatalasemic mice, insulin-positive cell numbers isolated from the mice were decreased by alloxan administration and were hardly affected by diets (data not indicated). By alloxan administration, cell size from the mice fed the control diet was decreased to 80 ± 10 %, (p=0.055) compared to the size at PBS administration, but the sizes isolated from the mice fed *A. awamori* diet and the fermented burdock diet were not (97 ± 7 and 101 ± 10 %).

4. Discussion

Alloxan induces oxidative stress and causes hyperglycemia. When mice fed *A. awamori*-fermented burdock diet, incidence of hyperglycemia in normal and acatalasemic mice was lower than that in mice fed the control diet after alloxan administration (Doi et al., 2013; Takemoto et al., 2014), and the raw burdock diet does not indicate

anti-hyperglycemia activity (Doi et al., 2015). However, the fermentation of burdock root with *A. awamori* induced increase of DPPH scavenging activity and polyphenol contents, and the increased antioxidant activity may ameliorate alloxan-induced hyperglycemia. A 0.05% *A. awamori* diet was prepared and the feeding was examined to confirm the anti-hyperglycemia activity of *A. awamori*. Feeding of the *A. awamori* diet suppressed the incidence of hyperglycemia and improved the GTT, C-peptide and insulin-positive cell size compared to feeding of the control diet. The result indicated that hyperglycemia induced by alloxan administration was ameliorated by feeding of only *A. awamori*. Several researchers reported that dietary intake of *A. awamori* improved an intestinal environment and induced increase of α-tocopherol (vitamin E) level in their blood and muscles to affect the lipid and protein metabolism (El-Deep et al., 2014; Saleh et al., 2013, 2014). As the intake of vitamin E improved mouse hyperglycemia caused by alloxan and vitamin E improved insulin release from pancreas damaged by alloxan (Kamimura et al., 2013; Takemoto, Doi, & Masuoka, 2016), we deduced that *A. awamori* is an effective probiotic and increases vitamin E to ameliorate hyperglycemia induced by oxidative stress. Therefore, *A. awamori* may be likely to be applicable as a supplement if untoward side effects are not observed.

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