Scientific Research

Effect of *Aspergillus awamori*-Fermented Burdock Root on Mouse Diabetes Induced by Alloxan—Prevention of Cell Apoptosis

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Abstract

Root of burdock contains high amounts of dietary fibers and polyphenols. To improve the functional properties, the root was fermented with *Aspergillus awamori.* Effect of the fermented burdock on alloxan-induced mouse diabetes was examined. A diet containing the 5% fermented burdock powers was prepared to examine effect of the burdock diet on alloxan-induced mouse diabetes. Mice fed the burdock diet and the control diet for 14 weeks. Then, alloxan (200 mg/kg of body weight) was administrated to each mouse. After 5 days from the administration, blood glucose assay and glucose tolerance test were carried out. Incidence of hyperglycemia decreased and the glucose metabolism was improved when mice fed the burdock diet. Insulin, C-peptide, biomarkers of oxidative stress in plasma and apoptosis in pancreas were examined and compared to those obtained from mice fed the control diet. It is deduced that alloxan-induced diabetes is caused to lower insulin concentration. The fermented-burdock diet improves the diabetes and prevents apoptosis in the pancreas.

Keywords

Fermented Burdock, Antioxidant Souce, Alloxan, Diabetes, Apoptosis

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1. Introduction

Catalase (EC1.11.1.6) has a predominant role in removing high concentration of hydrogen peroxide. The catalase deficient animal is called as acatalasemia and is sensitive to the oxidative stress [1]. Alloxan is a diabetogenic drag, and the treatment of animals caused a specific necrosis of pancreatic islets to result in an animal model of insulin dependent diabetes mellitus [2]. We indicated that a smaller amount of alloxan administration to the acatalasemic mouse developed diabetes than that to normal ones [3], and then deduced that acatalasemic animals promoted diabetes faster and more frequently than normal ones [4]. The root of burdock, *Artium lappa*, is taken as a vegetable in Japan and contains high amounts of dietary fibers as well as polyphenols such as chlorogenic and caffeic acids [5] [6]. However, there are few preparations to take considerable amounts of the roots as foods. To improve the functional properties, the roots were fermented with *Aspergillus awamori*, and the fermented burdock was prepared [7]. When rats fed the fermented burdock, their intestinal environments were improved and the obesity was suppressed. As it was known that the injury caused by oxidative stress was ameliorated by the intake of antioxidant [8] [9], we examined effect of fermented burdock as a source of antioxidants on alloxan-induced mouse diabetes.

2. Materials and Methods

2.1. Materials

Male mice of the C3H/AnL CS^{a}CS^a (normal) and C3H/AnL CS^{b}CS^b (acatalasemia) strains established by Feinstein, Braun, & Howard [10] were maintained on a laboratory diet (CE-2 diet, Clea Japan, Tokyo) and water ad libitum until the start of the experiments. Fermented burdock powders were prepared from burdock roots by Ahjikan Co. Ltd (Hiroshima, Japan) and Yaegaki Bio-Industry Inc. (Himeji, Japan) [7]. A fermented burdock diet was consisted of 5% of the burdock powders and 95% of AIN-93M formula diet (w/w) [11], and the control diet was AIN-93M. Pellets of these diets (1.3 cm) were prepared and stored at −20°C until use.

2.2. Animal Experiments

Acatalasemic mice and normal mice (15 weeks old) were divided into two groups, respectively. One group was maintained on the burdock diet and another group the control diet for 14 weeks. Then, alloxan (200 mg/kg of body weight), as an oxidative stress, was intraperitoneally administrated to each mouse [8]. Mice in each group were maintained on the same diet for one more week. After five days from alloxan administration, mice were fasted, and a glucose tolerance test (GTT) was carried out. After seven days, blood was collected from each mouse heart, and heparin was used as the anticoagulant. Oxidative stress markers, as well as the insulin and C-peptide levels in plasma, were examined. Pancreas was also isolated, and the sections were prepared for microscopic studies.

2.3. Determination of Glucose in Blood

Mice were fasted. The 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 [8] [12]. At 0 and 30 min before and 15, 30, 60, 90 and 120 min after the administration, the glucose contents in the blood were measured.

2.5. Measurement of the Oxidative Stress Markers

The measurement of 8-oxo-2'-deoxyguanosine (8-OHdG) in plasma was carried out using an ELISA KIT (JaICA, Shizuoka, Japan). Sample or standard solution was added to each well in a 96-well plate coated with 8-OHdG, and a monoclonal antibody for 8-OHdG was added. The mixture was reacted for 12 hrs. Then, an en-

zyme-conjugated antibody was added and reacted for 1 hr. The reagent solution for the color reaction was added and reacted for 15 min. The absorbance at 450 nm was recorded. Lipid peroxidation in plasma was determined using a Bioxytech LPO-586 KIT (OXIS Health Products Inc, CA, USA). Malondialdehyde and 4-hydroxyalkenals as products of lipid peroxidation were reacted with N-methyl-2-phenylindole at 45°C. The absorbance at 586 nm was recorded. Values of lipid peroxide in blood were calculated as malondialdehyde.

2.6. 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.

2.7. Microscopic Studies of Pancreatic Tissues in the Acatalasemic 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). For apoptosis analysis, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was carried out using the In Situ Cell Death Detection Kit (Roche Diagnostics Japan) [13]. The apoptosis incidence was calculated from the TUNEL-positive cells per the total cells in the Langerhans islets.

2.8. 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

Catalase activity in the mouse erythrocytes was calculated as the difference between the hydrogen peroxide removal rate by hemolysate and the rate (0.73 μmol/s/g of hemoglobin) by hemoglobin [3] [14]. The catalase activity in blood at 25°C was 0.15 ± 0.07 μmol/s/g of Hb in the acatalasemic mice and 6.77 ± 0.62 in the normal mice.

3.2. Biomarkers of Oxidative Stress in Plasma before and after Alloxan Treatment
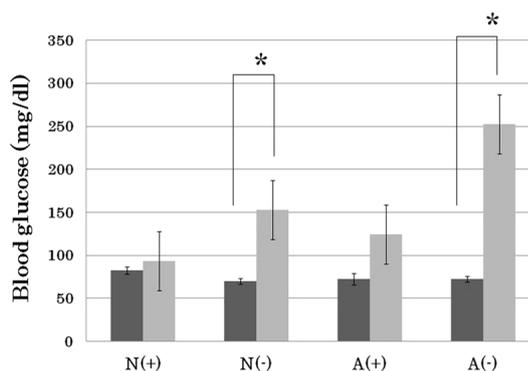
After alloxan administration, 8-OHdG in the mouse plasma increased. The increase in blood was suppressed by feeding the burdock diet but the difference is not significant (data not indicated). On the other hand, increase of lipid peroxide marker in blood after alloxan administration was significantly suppressed by feeding the burdock diet (**Figure 1**).

3.3. Blood Glucose in the Mice Maintained on the Burdock and the Control Diets before and after Alloxan Administration

Before alloxan administration, the average fasting blood glucose in the normal and acatalasemic mice fed a burdock or control diet was scarcely different. After the administration, the average fasting blood glucose of acatalasemic mice was higher than that in normal mice. Furthermore, the average fasting blood glucose of mice fed the control diet was significantly higher than that of mice fed the burdock diet (**Figure 2**).
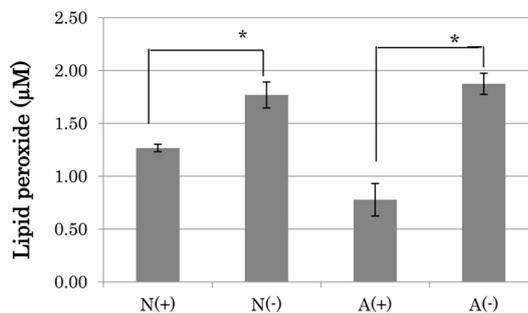
Figure 1. Lipid peroxide in blood after alloxan administration. Mice fed fermented burdock diet (+) or the control diet (−) for 14 weeks. Then, alloxan was administrated. After a week, lipid peroxide in blood was measured. N, Normal mice; A, acatalasemic mice. Vertical lines indicate SE. * indicates p < 0.05.

Figure 2. Blood glucose before and after alloxan administration. Mice fed fermented burdock diet (+) or the control diet (−) for 14 weeks. Then, alloxan was administrated. Black columns, means of blood glucose before alloxan administration; gray columns; means of blood glucose 5 days after alloxan administration. N, Normal mice; A, acatalasemic mice. Vertical lines indicate SE. * indicates p < 0.05.

It was also indicated the incidence of hyperglycemia in both mice fed the burdock diet was lower than that fed the control diet (**Table 1**).

3.4. Effect of the Burdock or the Control Diet on GTT

After alloxan treatment, GTT in the mice was examined. The difference in the blood glucose of the treated acatalasemic mice fed the burdock and control diet was indicated in **Figure 3**. The blood glucose in the mice fed the control diet after 60, 90 and 120 min from glucose administration was higher than mice fed the fermented burdock diet. Blood glucose in the acatalasemic mice fed the control diet after 120 min was higher than that before glucose administration.

3.5. Insulin and C-Peptide Concentrations and the Index of Insulin Resistance

The C-peptide level in the normal mouse blood (data not shown) was significantly higher than that in the acatalasemic blood, and the concentration was hardly affected by each diet. The insulin concentrations in fasting blood after alloxan administration were shown in **Figure 4**. The insulin concentration of mice fed the control diet was lower than the mice fed the burdock diet, and, in them, the concentration of acatalasemic mice fed the control diet was only significantly low. The index of insulin resistance was calculated according to [15] [16].

Table 1. Incidence of hyperglycemia after alloxan administration.

Diet	Incidence of hyperglycemia (%) (*n*)	
	Normal mice	Acatalasemic mice
5% Fermented-burdock	7 (14)	43 (7)
Control	25 (16)	75 (8)

n in parentheses indicates number of mice.
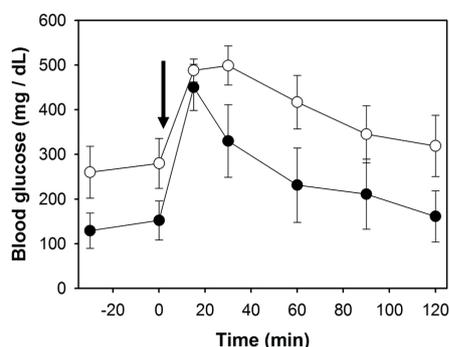
Figure 3. Glucose tolerance test of acatalacemic mice after alloxan administration. Acatalacemic mice fed fermented burdock diet or the control diet for 14 weeks. Then, alloxan was administrated. Glucose tolerance test was carried out after 5 days. Closed circles, blood glucose of mice fed fermented burdock diet; open circles, blood glucose fed the control diet. Arrow indicates glucose loading point. Vertical lines indicate SE.
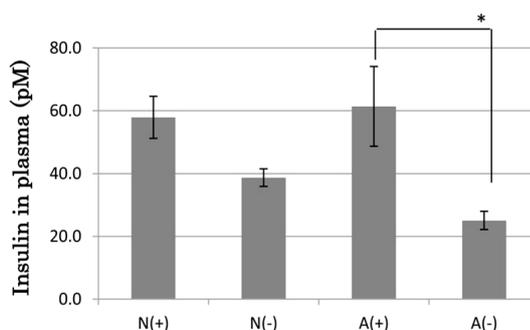
Figure 4. Insulin in blood after alloxan administration. Mice fed fermented burdock diet (+) or the control diet (−) for 14 weeks. Then, alloxan was administrated. Columns indicate means of insulin in plasma after a week. N, Normal mice; A, acatalasemic mice. Vertical lines indicate SE. * indicates $p < 0.05$.

The indexes were almost constant and not affected by the diet, catalase activity and alloxan administration (data not shown).

### 3.6. Microscopic Examination of Pancreatic Tissues in the Acatalasemic Mice Treated with Alloxan

Pancreatic tissues after alloxan administration were subjected to immunohistochemical staining (**Figure 5**). The

numbers of β-cells stained by insulin antibody in the islets of Langerhans were calculated. There was scarcely difference of the numbers in normal mice between the fed the burdock diet and the control one but significant difference in acatalasemic mice (data not shown).

Incidence of apoptosis was calculated using TUNEL-positive cells per the total cells in the islets of Langerhans (**Table 2**). The incidence in acatalasemic mice fed the burdock diet before and after alloxan administration was constant and almost a same level with normal ones, but in acatalasemic mice fed the control diet after alloxan administration was significantly high compared to that fed the burdock diet.

4. Discussion

Alloxan administration to mice caused to increase both oxidative stress markers in blood, and by feeding the fermented burdock diet, lipid peroxide in blood was significantly lowered than that taking the control diet. Incidence of hyperglycemia in normal and acatalasemic mice fed the burdock diet was lower than that in mice fed the control diet. GTT of the acatalasemic mouse group fed the burdock diet was improved compared to that fed the control diet. By alloxan administration, insulin concentration in blood was decreased, and insulin resistance indexes indicated that the burdock diet did not induce insulin resistance. Insulin contents in blood of acatalasemic mice fed the burdock diet were higher than those of the mice fed the control one. These results indicate that hyperglycemia and low insulin concentration induced by alloxan administration are ameliorated by feeding the fermented burdock diet. This study also indicated that acatalasemic mice fed the burdock diet did not induce TUNEL-positive cells in the Langerhans islets after alloxan treatment but acatalasemic mice fed the control diet induced them (**Table 2**). As oxidative stress induced by alloxan caused DNA breaks and activation of poly (ADP-ribose) synthetase to result deterioration of insulin synthesis in pancreas [17], it might be that the DNA breaks caused apoptosis of β-cells in acatalasemic mouse pancreas. Finally, we deduced that feeding the fermented

(a)   (b)
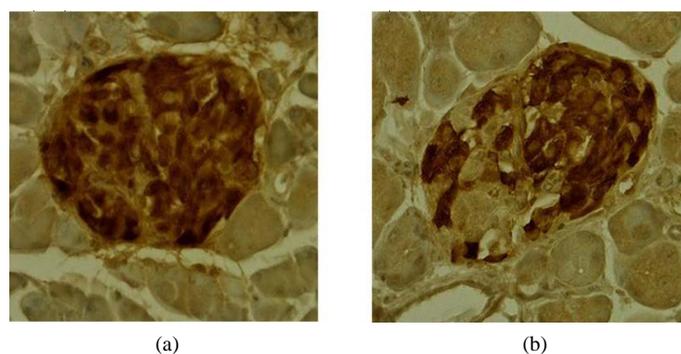
Figure 5. Mouse pancreas stained with insulin-antibody after alloxan administration. Acatalasemic mice fed fermented burdock diet or the control diet for 14 weeks. Then, alloxan was administrated. After a week, mouse pancreas was isolated and stained with insulin-antibody. (a) From mice fed fermented burdock diet; (b) from mice fed the control diet.

Table 2. Incidence of apoptosis in the pancreatic tissues after alloxan administration.

Mice	Diet	Apoptosis (%)
Normal	Control	1.80 ± 0.52
Normal	5% Fermented burdock	1.98 ± 0.31
Acatalasemic	Control	$5.50 \pm 1.23^{*}$
Acatalasemic	5% Fermented burdock	1.22 ± 0.45

Values are expressed as mean $\pm$ SE. *indicates $p < 0.05$ compared to mice fed the 5% fermented burdock diet.

burdock diet prevented cell apoptosis in pancreas of acatalasemic mice and improved the low insulin in blood and hyperglycemia as telmisartan did [4]. However, as the preventing mechanism of diabetes by the fermented burdock diet is still poorly understood, further study is currently underway.

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