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Antioxidant effect of garlic and aged black garlic in animal model of type 2 diabetes mellitus*

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Abstract

Hyperglycemia in the diabetic state increases oxidative stress and antioxidant therapy can be strongly correlated with decreased risks for diabetic complications. The purpose of this study is to determine antioxidant effect of garlic and aged black garlic in animal model of type 2 diabetes. The antioxidant activity of garlic and aged black garlic was measured as the activity in scavenging free radicals by the trolox equivalent antioxidant capacity (TEAC) assay. Three week-old db/db mice were fed AIN-93G diet or diet containing 5% freeze-dried garlic or aged black garlic for 7 weeks after 1 week of adaptation. Hepatic levels of lipid peroxides and activities of antioxidant enzymes were measured. TEAC values of garlic and aged black garlic were 13.3 ± 0.5 and 59.2 ± 0.8 μmol/g wet weight, respectively. Consumption of aged black garlic significantly decreased hepatic thiobarbituric acid reactive substances (TBARS) level compared with the garlic group which showed lower TBARS level than control group ($p<0.05$). Activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) of garlic and aged black garlic group were significantly elevated compared to the control group. Catalase (CAT) activity of aged black garlic group was increased compared with the control group. These results show that aged black garlic exerts stronger antioxidant activity than garlic *in vitro* and *in vivo*, suggesting garlic and aged black garlic, to a greater extent, could be useful in preventing diabetic complications.

Key Words: Aged black garlic, antioxidant, db/db mice, diabetes mellitus, garlic

Introduction

Type 2 diabetes mellitus is characterized by hyperglycemia, resulted from defects in insulin secretion, insulin action, or both. Uncontrolled diabetes leads to diabetic complications, including cardiovascular disease, nephropathy, neuropathy, and retinopathy (Centers for Disease Control and Prevention, 1999). Among them, cardiovascular disease is a major complication and the major cause of mortality in patients with type 2 diabetes. Thus prevention and treatment of diabetic complications remain as critical problems in the management of diabetes mellitus.

It is well known that tight control of hyperglycemia and dyslipidemia is associated with the reduced risk for complications in diabetic patients (American Diabetes Association, 1999; DCCT Research Group, 1993; UKPDS Group, 1998). In addition, there is accumulating evidence that antioxidants may be useful in the prevention of diabetic complications (Ceriello, 2006; Lean *et al.*, 1999; Sinclair *et al*., 1992). Hyperglycemia in the diabetic state generates more reactive oxygen species (ROS) and free radicals (Maritim *et al.*, 2003) and the resulting oxidative stress plays a key role in the pathogenesis and progression of diabetes and diabetic complications (Kaneto *et al*., 2005). It has been reported that both antioxidant nutrients and antioxidant phytochemicals can give an advantage in alleviating diabetes and diabetic complications (Lean *et al.*, 1999, Sinclair *et al*., 1992).

Several studies have reported that garlic (*Allium sativum* L.) could have hypoglycemic (Al-Qattan *et al*., 2008; Eidi *et al*., 2006; Seo *et al*., 2009) and antioxidant effects (Banerjee *et al*., 2003). Consumption of 80% ethanol extract of garlic decreased serum glucose (Eidi *et al*., 2006) and injection of garlic extract attenuated hypoglycemia and structural nephropathy progression in streptozotocin (STZ)-induced diabetic rats (Al-Qattan *et al*., 2008). Consumption of diet containing 5% garlic powder significantly decreased serum glucose and total cholesterol in db/db mice, an animal model of type 2 diabetes (Seo *et al*., 2009). Garlic extract showed 1,1-diphenyl-2-picrylhydrazyl (DPPH)

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radical scavenging activity (Querioz *et al*., 2009) and superoxide dismutase (SOD) activity *in vitro* (Jang *et al*., 2008). Aqueous extract of garlic (500 mg/kg/d IP) increased total serum antioxidant levels in STZ-treated diabetic rats (Drobiova *et al*., 2009).

Antioxidant activity of garlic could be affected by processing (Querioz *et al*., 2009). Aged black garlic recently available on the market in Korea is one of garlic products expected to have strong antioxidant capacity. It is produced by ageing whole garlic at high temperature (70℃) and high humidity (90% RH) (Jang *et al*., 2008; Kang *et al*., 2008). During ageing process, unstable compounds of fresh garlic including alliin are converted into stable compounds including s-allyl cysteine (SAC), the water-soluble compound with potent antioxidant effect (Corzo-Martinez *et al*., 2007; Imai *et al*., 1994). It was reported that aged black garlic showed stronger antioxidant activity *in vitro* than garlic (Jang *et al*., 2008). In the previous study, we reported that consumption of diet containing 5% aged black garlic improved insulin resistance, decreased serum total cholesterol and triglyceride, and increased HDL-cholesterol levels in db/db mice (Seo *et al*., 2009). Therefore, aged black garlic could be more useful than garlic in prevention of diabetic complications. However, the antioxidant effect of garlic and aged black garlic was not compared in animal model of diabetes. Therefore, the purpose of the present study was to investigate the antioxidant effect of garlic and aged black garlic in animal model of type 2 diabetes mellitus to explore their possible use as anti-diabetic agents.

Materials and methods

Preparation of samples

Garlic was obtained from a local market in Namhae-gun and aged black garlic from Injoy Natural Co. (Namhae-gun, Korea). Garlic and aged black garlic were peeled off, mixed with ten volumes of water, and blended. The samples were extracted with water for 1 h at 80℃ and then centrifuged at 14,000 ×g for 15 minutes. The solvent was removed by rotary evaporation at 40℃. The extracts were lyophilized to be used for the trolox equivalent antioxidant capacity (TEAC) assay. Peeled garlic and aged black garlic were freeze-dried and powdered to be used for preparation of animal diets.

TEAC assay

TEAC assay was carried out according to the methods of Re *et al*. (1999). The 7 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution was mixed with 2.45 mM potassium persulfate for 16 h overnight. The solution was diluted with 5 mM phosphate buffered saline (PBS, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. After an aliquot of each sample (10 μL) was mixed with 990 μL ABTS solution at 30℃ for 6 minutes, absorbance at 734 nm was measured. The absorbance of the sample was compared with that of the calibrated Trolox standard. Antioxidant activity was expressed as TEAC (μmol Trolox equivalents/g wet weight). All determinations were carried out in triplicate. All the reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and experimental design

We used the same experimental animals and diets as described in our previous paper (Seo *et al*., 2009). All of the animal experiments were approved by the Animal Resource Center at Inje University, Korea. In brief, three-week-old male db/db (+/+) C57BL/KsL mice (the Korea Research Institute of Bioscience and Biotechnology, Ochang, Korea, n=21) were randomly divided into three groups. Control group was offered a AIN-93G diet, whereas garlic or aged black garlic group were offered the diet containing 5% freeze-dried garlic or aged black garlic *ad libitum* for 7 weeks after one week of adaptation period, respectively. The contents of protein, fat, and dietary fiber of the three diets were the same, respectively (Table 1). The mice were kept under standard lighting conditions (0600-1800 h light and 1800-0600 h dark) at controlled temperature (23-27℃) and humidity (50-60%).

Biochemical analysis

After 7-week experimental period, the mice were sacrificed by heart puncture after an overnight fast. Liver tissues were excised, rinsed, and frozen at -70℃ for further analysis. Antioxidant

Table 1. Composition of experimental diets (%)

Ingredient	Group		
	Control	Garlic	Aged black garlic
Corn starch[1)]	39.75	39.30	38.88
Casein[2)]	20.00	18.99	19.15
Dextrinized cornstarch[3)]	13.20	13.20	13.20
Sucrose[4)]	10.00	6.84	6.96
Alphacel[5)]	5.00	4.64	4.79
Mineral mixture[6)]	3.50	3.50	3.50
Vitamin mixture[7)]	1.00	1.00	1.00
L-Cystine[2)]	0.30	0.30	0.30
Choline bitratrate[5)]	0.25	0.25	0.25
Tert-Butyl hydroquinone[8)]	0.0014	0.0014	0.0014
Soybean oil[4)]	7.00	6.99	6.98
Garlic[9)]	-	5.00	-
Aged black garlic[9)]	-	-	5.00

[1)] Daesang Co., Korea
[2)] ICN Pharmaceuticals Inc., U.S.A.
[3)] Dyets, Inc., USA
[4)] Cheiljedang Co., Korea
[5)] Sigma Co., U.S.A.
[6)] AIN-93G Mineral mixture, ICN Pharmaceuticals, U.S.A.
[7)] AIN-93G Vitamin mixture, ICN Pharmaceuticals, U.S.A.
[8)] Fluka Co., USA.
[9)] Freeze-dried

effect of garlic and aged black garlic was determined by measuring lipid peroxides and activities of antioxidant enzymes in the liver. Lipid peroxides were assessed as thiobarbituric acid reactive substances (TBARS) according to the method developed by Ohkawa *et al.* (1979). Briefly, after 0.1 g liver tissue was mixed with 5 times volume of 10 mM sodium phosphate buffer (pH 7.4), the mixture was homogenized. The homogenate (0.5 mL) was added to 1 mL of solution composed of 15% TCA, 0.4% thiobarbituric acid (TBA), and 2.5% HCl and boiled at 100℃ for 45 minutes. After cooling, the reaction mixture was centrifuged at 3,000 rpm for 10 minutes. The absorbance of the supernatant was measured at 534nm. The protein content was measured by Bradford method (Bradford, 1976) with bovine serum albumin as the standard. The level of lipid peroxides was expressed as nmol malondialdehyde (MDA)/mg protein.

To measure activities of SOD, catalase (CAT) and glutathione peroxidase (GSH-Px), antioxidant enzyme source of liver tissue was prepared. In brief, 1 g of liver tissue was mixed with 10 times volume of 50 mM phosphate buffer (pH 7.4) and homogenized using a glass teflon homogenizer. After the mixture was centrifuged at 3,000 rpm at 4℃ for 10 minutes, the supernatant was used for measurement of CAT and GSH-Px activities. The supernatant was further centrifuged at 13,000 rpm for 20 minutes and the remaining supernatant was used for measurement of SOD activity. SOD activity was assayed according to the method developed by Marklund and Marklund (1974). One unit of SOD was defined as the amount of enzyme that reduces the rate of autoxidation of pyrogallol by 50%. The protein content was measured by Bradford method. CAT activity was measured by Abei method using H_2O_2 as a substrate (Abei, 1974). The amount of the enzyme required to remove 1 μM substrate per minute was defined as one unit activity. GSH-Px was measured by spectrophotometric method developed by Paglia and Valentine (1967). One unit of GSH-Px activity was defined as the amount of the enzyme that converted 1 μM NADPH, substrate to $NADP^+$ per minute. All the assays were carried out in triplicate using a spectrophotometer (Hitachi U-2000, Hitachi Ltd., Tokyo, Japan).

Statistical analysis

All values were expressed as mean ± standard deviation (SD). All statistical analyses were performed using SAS (version 8.02). Statistical differences among the experimental groups were assessed by one-way ANOVA. Tukey's test was used as a follow-up test and significance was defined at $p<0.05$.

Results

Antioxidant activity in vitro

The antioxidant activity of garlic and aged black garlic was measured as the activity in scavenging free radicals by TEAC assay. TEAC values of garlic and aged black garlic were 13.3 ± 0.5 and 59.2 ± 0.8 μmol/g wet weight, respectively (Table 2).

Table 2. TEAC values of garlic and aged black garlic (μmol/g wet weight)

Garlic	Aged black garlic
13.3 ± 0.5	59.2 ± 0.8

[1] Values are mean ± SD of triplicates.
[2] TEAC, trolox equivalent antioxidant capacity

Table 3. Body weight, food intake, and feed efficiency ratio of the animals

	Group		
	Control	Garlic	Aged black garlic
Initial body weight (g)	20.8 ± 1.5[ns]	21.1 ± 1.2	20.7 ± 1.7
Final body weight (g)	41.8 ± 1.8[ns]	42.9 ± 1.9	41.2 ± 2.5
Food intake (g/d)	4.3 ± 0.4[ns]	4.1 ± 0.3	4.2 ± 0.6
Feed efficiency ratio (%)*	10.2 ± 2.2[ns]	10.9 ± 1.2	10.1 ± 2.7

Values represent mean ± SD (n=7 per group).
* Feed efficiency ratio (%) = (body weight gain(g/d)/food intake(g/d)) × 100
NS is not significant.

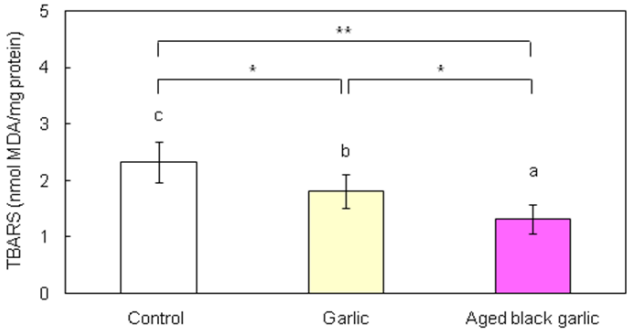

Fig. 1. Effect of garlic and aged black garlic on the levels hepatic TBARS of db/db mice. Values are mean ± SD (n=7 per group). Each bar with different letters is significantly different (*p<0.05, **p<0.01).

Effect on lipid peroxides and antioxidant enzyme activity

Body weight and food intake of the mice were shown in Table 3. Chronic consumption of garlic and aged black garlic at the level of 5% of the diet did not significantly influence body weight, food intake, and feed efficiency ratio in db/db mice.

The effect of garlic and aged black garlic on hepatic levels of lipid peroxides was shown in Fig. 1. Consumption of garlic significantly decreased hepatic TBARS level compared with the control group (1.81 ± 0.30 vs. 2.33 ± 0.36 nmol MDA/mg protein, $p<0.05$). Hepatic TBARS level of aged black garlic group (1.32 ± 0.26 nmol MDA/mg protein) was significantly lower than those of the control ($p<0.01$) and garlic group ($p<0.05$). Fig. 2 showed the effect of garlic and aged black garlic on activities of antioxidant enzymes in the liver. SOD activities of garlic (52.1 ± 5.9 U/mg protein, $p<0.05$) and aged black garlic group (59.9 ± 7.9 U/mg protein, $p<0.01$) were significantly increased compared with the control group (41.1 ± 7.2 U/mg protein). CAT activity of aged

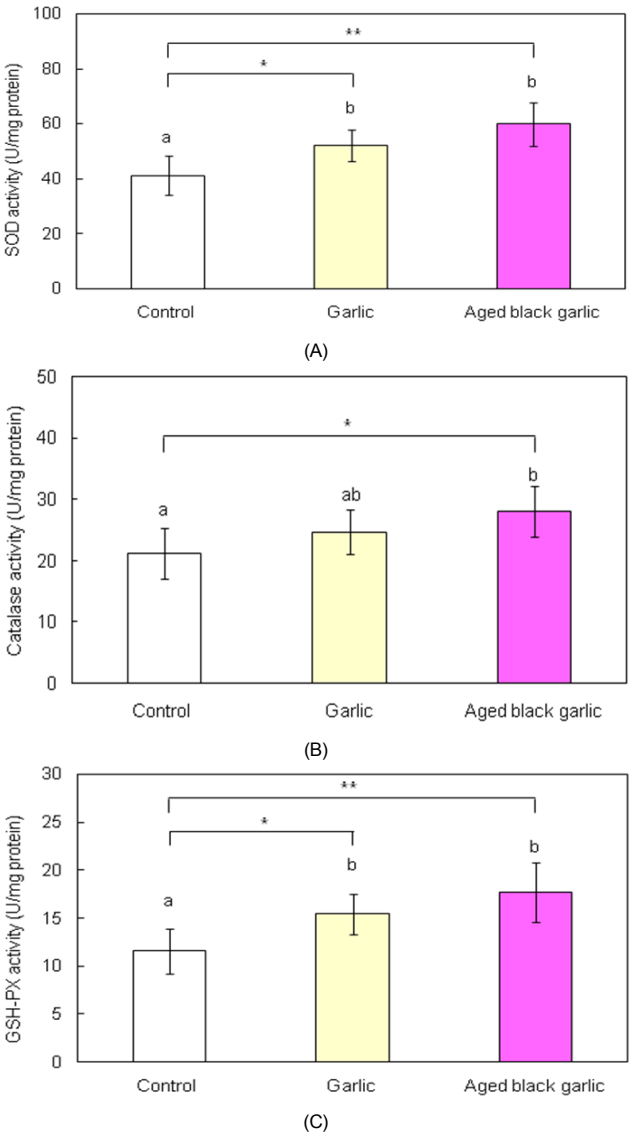

Fig. 2. Effect of garlic and aged black garlic on the hepatic activities (A) SOD, (B) catalase, and (C) GSH-Px of db/db mice. Values are mean ± SD (n=7 per group). Each bar with different letters is significantly different (*p<0.05, **p<0.01).

black garlic group (28.1 ± 4.1 U/mg protein) was elevated compared with the control group (21.2 ± 4.2 U/mg protein, $p<0.05$). CAT activity of garlic group (24.7 ± 3.7 U/mg protein) was not significantly different from the control and aged black garlic group. Consumption of garlic (15.4 ± 2.2 U/mg protein, $p<0.05$) and aged black garlic (17.7 ± 3.1 U/mg protein, $p<0.01$) significantly increased GSH-Px activity compared with the control group (11.5 ± 2.4 U/mg protein).

Discussion

Diabetes mellitus is a severe health problem and the prevalence of diabetes keeps increasing markedly due to an aging population, increased urbanization and more sedentary lifestyles (King *et al.*, 1998). Since perfect cure for diabetes is yet to be found and most of antidiabetic medications could have side effects (Cheng & Josse, 2004), many studies have been conducted to identify natural substances that show potent hypoglycemic activity with fewer side effects (Fujita *et al*., 2001; Park *et al.*, 2006; Youn *et al*., 2004). Garlic and garlic products could be one of the candidates for antidiabetic agents via antioxidant effects (Querioz *et al*., 2009).

We measured free radical scavenging activity of garlic and aged black garlic *in vitro*. TEAC value of aged black garlic was 4.5 times higher than that of garlic, demonstrating that ageing of whole garlic can enhance antioxidant activity. This result is in agreement with the previous reports which demonstrated that aged black garlic showed 3 to 9-fold stronger SOD activities *in vitro* at the concentration of 20-100 mg/mL (Jang *et al*., 2008). The primary sulfur-containing compound of intact garlic bulb is ɣ-glutamyl cysteine which can be hydrolyzed and oxidized to form alliin (Amagase *et al*., 2001). Alliin is converted to odoriferous thiosulfinate allicin by alliinase after processing such as crushing, cutting, chewing or dehydration. During extraction with water or ageing, ɣ-glutamyl cysteine is converted to SAC, a safe compound which contributes heavily to the health benefits of garlic (Amagase, 2006; Amagase *et al*., 2001). It was reported that the total polyphenol content of aged black garlic was increased (10.00 mg/g), although the content of polyphenol compounds of garlic was not high (3.67 mg/g, Jang *et al*., 2008). Therefore, increase in SAC and polyphenol compounds during ageing could be responsible for stronger antioxidant activity of aged black garlic than that of garlic.

We also determined antioxidant effect of garlic and aged black garlic in db/db mice, an animal model of type 2 diabetes that shows insulin resistance, hyperglycemia, and obesity. In the previous study, consumption of lyophilized garlic at 5% level of the diet for 7 weeks significantly increased insulin levels by 12.1% and decreased serum glucose by 8.7% in db/db mice, whereas consumption of aged black garlic significantly decreased homeostasis model assessment for insulin resistance (HOMA-IR) by 11.0% and tended to decrease serum glucose levels (Seo *et al*., 2009).

As shown in Fig. 1, consumption of garlic significantly decreased hepatic lipid peroxides compared with the control group, suggesting that garlic also showed antioxidant effect *in vivo*. This finding was in agreement with the results of previous studies which reported that injection of aqueous garlic extract (500 mg/kg) increased total serum antioxidant levels (Drobiova *et al*., 2009) and incubation of methanol garlic extract (500 mg/kg) decreased hepatic TBARS levels in STZ-treated diabetic rats (Kanth *et al*., 2008). In addition, we found that aged black garlic was more effective in reduction of hepatic TBARS levels than garlic.

Prolonged hyperglycemia of diabetes induces overproduction of ROS and free radicals, which can in turn trigger process of

diabetic complications (Maritim, 2003; Rahimi *et al.,* 2005). Seo *et al.* (2008) reported that TBARS levels in erythrocytes and liver of db/db mice were higher than those of lean heterozygote non-diabetic db/+ mice, demonstrating that hyperglycemia increases oxidative stress. To protect molecules from ROS and free radicals, cells have developed antioxidant defense system including SOD, CAT, and GSH-Px. SOD converts superoxide anions into H_2O_2, which is then further degraded into H_2O by CAT or GSSG by GSH-Px (Haron, 1991). Thus it could be essential to enhance CAT and GSH-Px activities in addition to SOD activity to remove ROS.

As shown in Fig. 2, activities of SOD and GSH-Px of garlic ($p<0.05$) and aged black garlic group ($p<0.01$) were significantly elevated compared with the control group and consumption of aged black garlic significantly increased CAT activity ($p<0.05$) compared with the control group. CAT activity of garlic was not significantly different from those of the control and black aged garlic group. Thus, aged black garlic could be more effective in removing superoxide anions. Jung *et al.* (2006) reported that caffeic acid increased antioxidant enzyme activities in both erythrocytes and liver of db/db mice and decreased hydrogen peroxide and TBARS levels. Garlic and aged lack garlic, to a greater extent, could decrease hydrogen peroxides and contribute to protection against diabetic complications.

It was demonstrated that SAC (Corzo-Martinez *et al*., 2007; Imai *et al*., 1994) and polyphenol compounds exert strong antioxidant effect (Kang *et al.,* 1996). Shin and Ihm (2008) reported that oral administration of SAC (100 mg/rat) decreased TBARS levels and increased GSH levels and mRNA expression of SOD, CAT, and GSH-Px in STZ-treated diabetic rats. It was reported that commercially available aged garlic extract prepared from ageing of garlic at room temperature for 20 months contained approximately 1,000 μg/g SAC, while raw garlic contained 20 μg/g (Ahmad *et al*., 2007). In this study, whole garlic was aged for 3 weeks after heating at 55℃ for 60 min, at 70℃ for 60 min, and then 85℃ for 24 h to produce aged black garlic. Therefore, the concentration of SAC of aged black garlic could be less than that of aged garlic extract. Further study to quantify SAC of aged products of garlic with different ageing condition could be necessary to determine effective dose of SAC and aged black garlic to show beneficial antioxidant effect *in vivo*.

In conclusion, aged black garlic exerts stronger antioxidant effect than garlic *in vitro* and in db/db mice, suggesting that garlic and aged black garlic, to a greater extent, could be useful in preventing diabetic complications.

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