

Antioxidant Effects of the Mixture of Mulberry Leaves and Silkworm Powder on the Plasma and Liver in Streptozotocin-Induced Diabetic Rats

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Abstract

This study was carried out to examine the antioxidant effects of a mixture of mulberry leaves and silkworm powder in plasma and liver of streptozotocin-induced diabetic rats. Sprague-Dawley male rats weighing 100 ± 10 g were used and their diets were supplemented with 0.4% (4 g/kg) of the mixtures. Experimental groups were diabetic rats without supplements (DM group) or with a combination of the supplements: 100% mulberry leaves (M group), 25% silkworm powder mixed with mulberry leaves (25SM group), 50% silkworm powder mixed with mulberry leaves (50SM group), 75% silkworm powder mixed with mulberry leaves (75SM group) or 100% silkworm powder (100S group). The rats were fed experimental diets and water *ad libitum*. All animals were injected with streptozotocin at the 3rd week for inducing diabetes and were sacrificed on 9th day thereafter. Hepatic xanthine oxidase (XOD) activity significantly decreased in the mixture supplemented groups compared to the DM group. Hepatic superoxide dismutase (SOD) activity was not significantly different among any of the experimental groups, but glutathione peroxidase (GSH-px) activity increased in the mixture supplemented groups compared to the DM group. In particular, it was the highest in the 50SM group. The hepatic TBARS values were lower in all the mixture supplemented groups than in the DM group, and it was as lowest when ratio of mulberry leaves to silkworm powder was highest. Hepatic lipofuscin contents were similar with the TBARS value. In conclusion, the mixtures containing silkworm powder reduced oxidative damage by strengthening the antioxidative system and suppressing oxidative stress in the STZ-induced diabetic rat. The 1:1 blend of silkworm powder and mulberry leaves was the most effective combination for antioxidant activity.

Key words: mulberry leaves, silkworm, antioxidant, streptozotocin-induced diabetic rat

INTRODUCTION

Over the last two decade, the prevalence of diabetes rapidly increased and the number of patients is predicted to total 250 million in the world by 2020 (1). Many complications such as cardiovascular diseases, hyperlipidemia, hypertension and arteriosclerosis accompany hyperglycemia (2). It was well-known that cardiovascular disease can result from increased oxidative stress and lipid peroxidation in plasma and tissues (3). Therefore, control of oxidative stress through antiglycemia is needed for the prevention of diabetes and its complications. Recently, many investigators have been developing anti-diabetic material from natural sources such as green tea, mulberry leaves and others ingredients (4-6). Mulberry leaves have been used to feed silkworms (*Bombyx mori*). Studies on the use of mulberry leaves as food additive and functional ingredient increased steadily after it was registered as a plant without virulence in the Korean Food Code in 1998 (5,6). Mulberry leaves have been

reported to have anticancer, antihypertension, anti-oxidative and antihyperlipidemia functions. Mulberry leaves possess various physiological activities because there numerous bioactive components including 25 kinds of amino acids and functional flavonoids such as rutin, quercetin, isoquercetin and kaempferol-3-O- β -D-glucopyranoside (7-9). Among them, rutin has been known to be one of the major capillary strengthening substances and γ -aminobutyric acid (GABA) is a anti-hypertensive material which is 10 times the concentration in mulberry leaves compared to green tea leaves (10-12). Moreover, the mulberry leaf decreases blood cholesterol and triglyceride and increases the levels of HDL-cholesterol. It has a strong antioxidant effect because it contains many phytosterol like β -sitosterol, campesterol, β -sitosterol glycoside, β -ecdysone and inosterone (13-15). Also, attention has been drawn to the presence of deoxyojirimycin (DNJ) in the silkworm, which is known to be a blood glucose lowering substance and a powerful competitive α -glucosidase inhibitor (15). The mulberry leaf

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has various functional properties including the ability to decrease triglycerides and cholesterol and antioxidative action because functional ingredients such as GABA, flavonoids, and phytosterols are present in vast quantities and silkworm powder is very effective at lowering blood glucose levels because of the DNJ content. Therefore, mulberry and silkworm might have a synergic effect if we mix the two at a suitable ratio. We examined the effect of mulberry leaf and silkworm on hyperglycemia in a previous study (16) in order to develop a product which is an antioxidant with hypoglycemic effects by mixing silkworm powder and mulberry leaves.

Consequently, this study was carried out to examine the antioxidant effect of pills produced with mulberry leaves and silkworm powder on the plasma and hepatic tissue of diabetes rats.

MATERIALS AND METHODS

Preparation of plant materials

The mulberry leaves used in this experiment were 'YK-209 mulberry leaves'. This was the most suitable kind to develop for functional foods. The leaves were harvested in Young-Cheon, Korea in May, 2002 and vacuum dried at 60°C after washing. Silkworm powder aged five years and three days was freeze-dried and pulverized. The silkworm powder was cultivated in the fields of Young-Cheon Silkworm Culture Agricultural Cooperative Association.

Production of the mixture

Pills were produced using the method as previously described (16). Five different pills with different amounts of mulberry leaves and silkworm powder, according to the mixing portion of the silkworm powder and mulberry were named; 100% mulberry leaves (M pill), 25% silkworm powder mixed with mulberry leaves (25SM pill), 50% silkworm powder mixed with mulberry leaves (50SM pill), 75% silkworm powder mixed with mulberry leaves (75SM pill) and 100% silkworm powder (100S pill). The moisture contents were 5 to 8% after being dried 4 times with heat at 60°C. Next, the blends were placed in a bottle and sealed.

Experimental animals and diets

Male Sprague-Dawley rats weighing 100 ± 10 g were purchased from Bio Genomics (Seoul, Korea). The rats were acclimated for one week, and then randomly assigned to experimental groups, a diabetic group or the mixture supplemented groups. The mixture supplemented groups were classified as 100% mulberry leaves (M group), 25% silkworm powder mixed with mulberry leaves (25SM group), 50% silkworm powder mixed with

mulberry leaves (50SM group), 70% silkworm powder mixed with mulberry leaves (75SM group), and 100% silkworm powder (100S group). Compositions of diets in experimental groups were shown in Table 1. The diet was supplemented with 0.4% (4 g/kg) of the mixtures. Experimental diets and water were available *ad libitum*. All animals were injected with streptozotocin at the 3rd week for inducing diabetes and were sacrificed on the 9th day thereafter.

Experimental diabetes

Diabetes was induced by an intravenous injection of STZ (50 mg/kg body weight) in a citrate buffer (pH 4.3) via the tail vein. Rats with a blood glucose concentration of 16.7 mmol/L after 9 days were used for the experiment.

Pretreatment of enzyme specimen

After sacrificing the animals under a mild ether anesthetic, their livers were excised, washed with 0.9% NaCl, quick-frozen with liquid nitrogen, and freeze-stored in a -80°C environment. Liver tissue was homogenized by using a potter-elvejhem homogenizer and a solution of 0.25 M sucrose/0.5 mM ethylene diamine tetraacetic acid (EDTA)/5 mM N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES). It was pretreated according to the method developed by Rhee et al. (17).

Measurement of xanthine oxidase (XOD) activity

The hepatic XOD activity was determined according to the method of Stripe and Della Corte (18).

Measurement of antioxidant enzyme activity

The hepatic SOD activity was measured in compliance with the methods of Marklund and Marklund (19). Briefly, SOD was detected on the basis of its ability to inhibit superoxide-mediated reduction. One unit was defined as the amount of enzymes that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as units/mg of protein. The hepatic GSH-px activity was measured in compliance with the methods of Lawrence and Burk (20). GSH-px was measured in a mixture that contained one mmol/L of H₂O₂, and absorbance was measured at 340 nm for one minute. A molar extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to determine the activity. The activity was expressed as nmol of NADPH/mg of protein/minute.

Determination of lipid peroxide in liver tissue

The hepatic lipid peroxide concentration levels were measured according to method of Satoh (21), which measures malondialdehyde produced by a reaction of thiobarbituric acid with lipid peroxides.

Measurement of lipofuscin content

The hepatic lipofuscin content was measured in com-

Table 1. Compositions of diets in experimental groups (g/kg diet)

Ingredients	Groups ¹⁾					
	DM	M	25SM	50SM	75SM	100S
Corn starch ²⁾	698	694	694	694	694	694
Casein ³⁾	150	150	150	150	150	150
DL-methionine ⁴⁾	2	2	2	2	2	2
Corn oil ⁵⁾	50	50	50	50	50	50
Salt mix ⁶⁾	40	40	40	40	40	40
Vitamin mix ⁷⁾	10	10	10	10	10	10
Cellulose ⁸⁾	50	50	50	50	50	50
Pills ⁹⁾						
Mulberry leaves	-	4	3	2	1	-
Silkworm powder	-	-	1	2	3	4
Total	1,000	1,000	1,000	1,000	1,000	1,000

¹⁾DM: Injection of streptozotocin + without any supplement of mulberry leaves or silkworm powder. M: Injection of streptozotocin + 4 g (100%) YK-209 mulberry leaf powder/kg diet, 25SM: Injection of streptozotocin + 3 g (75%) YK-209 mulberry leaf powder + 1 g (25%) silkworm powder/kg diet, 50SM: Injection of streptozotocin + 2 g (50%) YK-209 mulberry leaf powder + 2 g (50%) silkworm powder/kg diet, 75SM: Injection of streptozotocin + 1 g (25%) YK-209 mulberry leaf powder + 3 g (75%) silkworm powder/kg diet, 100S: Injection of streptozotocin + 4 g (100%) silkworm powder/kg diet.

²⁾Pung jin Chem. Co., Seoul, Korea.

³⁾Lactic Casein, 30 mesh, New Zealand Dairy Board, Wellington, N.Z.

⁴⁾Sigma Chem. Co., St. Louis, Missouri, U.S.A.

⁵⁾Dong Bang Oil Co., Seoul, Korea.

⁶⁾AIN-76 likeness (g/kg mixture): Calcium phosphate, dibasic (CaHPO₄ · 2H₂O) 500, Sodium chloride (NaCl) 74, Potassium citrateminohydrate (K₃C₆H₅O₇ · H₂O) 220, Potassium sulfate (K₂SO₄) 52, Magnesium oxide (MgO) 24, Manganous carbonate (45~48% Mn) 3.5, Ferric citrate (16~17% Fe) 6, Zinc carbonate (70% ZnO) 1.6, Cupric carbonate (53~55% Cu) 0.3, Potassium iodate (KIO₃) 0.01, Sodium selenite (Na₂SeO₃ · 5H₂O) 0.01, Chromium potassium sulfate [CrK (SO₄)₂ · 12H₂O] 0.55, filled up to 1,000 with sucrose.

⁷⁾ALN-76 likeness (mg/kg mixture): Thiamin-HCl 600, Riboflavin 600, Pyridoxine · HCl 700, Nicotinic acid (nicotinamide in equivalent) 3,000, D-calcium pantothenate 1,600, Folic acid 200, D-biotin 20, Cyanocobalamin (vitamin B₁₂) 1, Retinyl palmitate or acetate (vitamin A) as stabilized powder to provide 400,000 IU vitamin A activity or 120,000 retinol equivalent, DL- α -tocopheryl acetate 5,000 IU, Cholecalciferol (100,000 IU, may be in powder form) 2.5, Menaquinone (vitamin K, Menadione) 5, filled up to 1,000 with sucrose.

⁸⁾Sigma Chem. Co. CMC (Sodium carboxyl methyl cellulose, non-nutritive fiber), St. Louis, Missouri, U.S.A.

⁹⁾Mulberry leaves: YK-209 mulberry leaf powder. Silkworm powder: Silkworm fed YK-209 mulberry leaves.

pliance with the methods of Fletcher et al. (22).

Protein determination

Protein concentration was measured by the method of Lowry et al. (23) with bovine serum albumin as the standard.

Statistical analysis

Results were analyzed by ANOVA and Tukey's honestly significant difference test (24), if statistical significance was determined by ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS

Measurement of xanthine oxidase (XOD) activity

The result of hepatic XOD activity, which is one of the radical formation systems in the body, are shown in Fig. 1. The activity significantly decreased in the M group, 25SM group, 50SM group, 75SM group and 100S group where the mixture supplemented groups showed 35%, 35%, 34%, 28% and 24%, respectively, compared

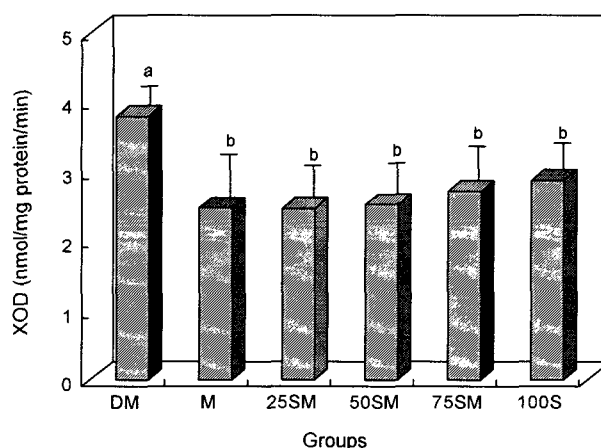


Fig. 1. Effects of the mixtures with mulberry leaves and silkworm powder on hepatic xanthine oxidase (XOD) activity in streptozotocin-induced diabetic rats.

All values are mean \pm SE (n=10). Values with different superscript letters (a,b) are significantly different at $p < 0.05$ by Tukey's test. The Experimental conditions were the same as those described in Table 1.

to the DM group. There were no significant differences

among the mixture supplemented groups.

Superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) activity

The result of hepatic SOD and GSH-px activities are shown in Table 2. SOD activity increased in the mixture supplemented groups compared to the DM group, however the differences were not significant.

GSH-px activity increased significantly in the M group, 25SM group, 50SM group, 75SM group and 100S group where the mixture supplemented groups each showed 21%, 24%, 28%, 24% and 21%, respectively, as compared to the DM group. In particular, there was a high tendency of increased activity in the 50SM group.

Thiobarbituric acid reactive substances (TBARS) and lipofuscin content

The results of the hepatic TBARS values are shown in Fig. 2 (A). The hepatic TBARS values significantly decreased in the M group, 25SM group, 50SM group, 75SM group and 100S group, with the mixture supplemented groups each with 25%, 30%, 30%, 21% and 22%, respectively compared to that of the DM group, but there were no significant differences between the supplemented groups. It was significantly decreased in the 25SM group and 50SM group, with the mixed silkworm powder comprising 25% and 50% of the mixture, and in the M group with 100% mulberry leaves.

The result of the hepatic lipofuscin content is shown in Fig. 2 (B). The hepatic lipofuscin content significantly decreased in the M group, 25SM group, 50SM group, 75SM group and 100S group by 16%, 17%, 18%, 13% and 10%, respectively compared to that of the DM group.

DISCUSSION

This study was carried out to obtain basic data for

Table 2. Effects of the mixtures of mulberry leaves and silkworm powder on hepatic superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) activities in streptozotocin-induced diabetic rats

Groups	SOD (unit/mg protein/min)	GSH-px (nmol NADPH/min/mg protein)
DM	3.76 ± 0.38 ^{1)NS2)}	114.88 ± 09.42 ^{c3)}
M	4.02 ± 0.44	146.26 ± 16.43 ^{ab}
25SM	4.05 ± 0.11	150.83 ± 10.15 ^{ab}
50SM	4.10 ± 0.69	158.93 ± 18.63 ^a
75SM	3.92 ± 0.17	150.23 ± 11.73 ^{ab}
100S	3.89 ± 0.40	145.03 ± 12.42 ^b

¹⁾All values are mean ± SE (n=10).

²⁾Values with different superscript letters (a~c) in the same column are significantly different at p < 0.05 by Tukey's test. The Experimental conditions were the same as those described in Table 1.

³⁾Not significant.

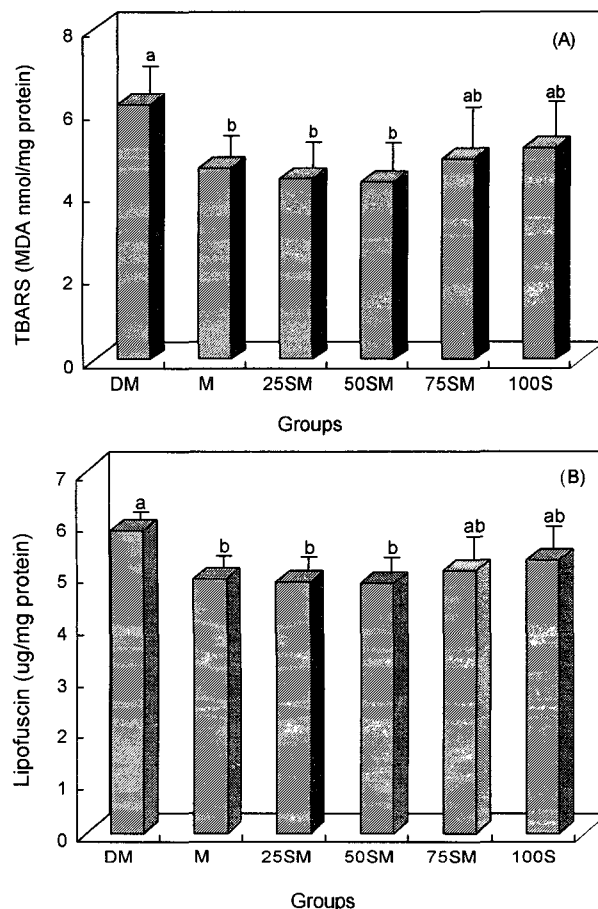


Fig. 2. Effects of the mixtures of mulberry leaves and silkworm powder on hepatic thiobarbituric acid reactive substances (TBARS) value (A) and lipofuscin content (B) in streptozotocin-induced diabetic rats.

All values are mean ± SE (n=10). Values with different superscript letters (a,b,ab) are significantly different at p < 0.05 by Tukey's test. The Experimental conditions were the same as those described in Table 1.

facilitating health food development for diabetic patients by examining the antioxidant effect of the mixture of mulberry and silkworm ingredients. This was done by changing the amount of silkworm powder mixed with mulberry leaves.

Xanthine oxidase (XOD), which mainly exists in the protoplasm, is one of the radical formation systems. The hepatic XOD activity significantly decreased in all the mixture supplemented groups compared to that of the DM group. The functions of mulberry leaf flavonoids as used in this study has been reviewed (12).

Lipid peroxides are constantly produced through normal metabolism, resulting in oxidative stress. There are defense systems in the body, such as SOD or GSH-px, which operate to prevent an accumulation of lipid peroxides (25). The antioxidant enzyme SOD reduces superoxide radicals to H₂O₂, which in turn is excreted as H₂O due to the activity of the GSH-px and catalase,

thereby by protecting the body from oxygen toxicity (26). Changes in enzyme activity occur when oxidative stress increases during a diabetic state (27). Hepatic superoxide dismutase (SOD) activity was not significantly different among any of the experimental groups, but glutathione peroxidase (GSH-px) activity increased in all the mixture supplemented groups compared to the DM group. In particular it was the highest in the 50SM group.

The reason for the low activity in SOD and GSH-px is the sensitivity of the oxidative stress under diabetic state. Enzyme activity fell from accelerated oxidative damage of the cell in a small organs by the promotion of lipid peroxidation in the cell membranes, which has a high content of PUFA. The supplements, however, were supposed to increase enzyme activity by facilitating antioxidative activity, which restrains the superoxide anions, because of the flavonoids in mulberry leaves (28).

The hepatic TBARS values, which are an oxidative damage index, decreased in all the mixture supplemented groups. Among them, the 25SM group and 50SM groups has the lowest value. The oxidative damage was reduced the most when the mix ratio of mulberry leaves was greatest.

Lipofuscin is an index is an age related accumulation of color pigments that is promoted by the generation of free radicals. Hepatic lipofuscin formation was decreased in the M group, 25SM group and 50SM group, which was similar to the TBARS values.

In the same way, the low content of TBARS and lipofuscin in the M group, 25SM group and 50SM group, due to the GSH-px activity which is a free radical clearance system, was increased by the flavonoids in mulberry leaves and by free radical generation, while lipid peroxidation and lipofuscin formation were restrained (29,30).

When the silkworm powder is mixed with the mulberry leaves in the correct way, we could see the effect of antioxidative defense activities that is a free radical clearance system and an induction of defensive power from the oxidative stress increased. The generation of lipid peroxidation or lipofuscin was efficiently restrained in the STZ-induced diabetic rat.

From the result of this study, we could observe an antioxidant effect and an anti-aging effect of the mixtures of silkworm powder with mulberry leaves in STZ-induced diabetic rats. In particular, the 50SM pill, that was mixed with equal amounts of mulberry leaves and silkworm powder (each 50%), was not only effective at lowering blood glucose levels, as in previous research (16), but also had the most comprehensive affect on the antioxidative defence system in this study. Consequently,

supplements prepared with equal amounts of mulberry leaves and silkworm powder may have efficiency for protecting against diabetic induced oxidative damage.

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