

## Antioxidative and Hypolipidemic Effects of Diosgenin, a Steroidal Saponin of Yam (*Dioscorea spp.*), on High-Cholesterol Fed Rats

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**Diosgenin (a steroidal saponin of yam) has long been used as a raw material for the industrial production of steroid drugs, and reported to have a hypocholesterolemic effect by suppressing cholesterol absorption and increasing cholesterol secretion. Oxidative stress has been suggested as a main risk factor in the development of atherosclerosis. The aim of this study is to investigate the possible hypolipidemic and antioxidative effect of diosgenin on rats fed with a high-cholesterol diet supplemented with either 0.1% or 0.5% diosgenin for 6 weeks. We measured the lipid profile in the plasma and liver, lipid peroxidation and antioxidative enzyme activities in the plasma, erythrocyte and gene expression of antioxidative enzymes in the liver, and the oxidative DNA damage in lymphocytes. Diosgenin showed a decrease in the plasma and hepatic total cholesterol levels, but increased the plasma high-density lipoprotein (HDL) cholesterol level. Erythrocyte TBARS and lymphocyte DNA damage measured by the comet assay were decreased in the diosgenin supplemented group. Furthermore, diosgenin feeding enhanced the resistance to lymphocyte DNA damage caused by an oxidant challenge with H<sub>2</sub>O<sub>2</sub>. The antioxidative enzyme activities were also affected by diosgenin supplementation. Total superoxide dismutase (SOD) in the plasma and liver, glutathione peroxidase (GSH-Px) in erythrocytes, and catalase (CAT) in erythrocytes and liver were significantly increased in the 0.5% diosgenin group. The expression of antioxidative enzymes was up-regulated by diosgenin, the expression of GSH-Px being the highest in the 0.5% diosgenin group. These results suggest that diosgenin could be a very useful compound to control hypercholesterolemia by both improving the lipid profile and modulating oxidative stress.**

**Key words:** diosgenin; comet assay; antioxidative enzyme

Mortality from cardiovascular disease is the second leading cause of death in Korea<sup>1</sup> and worldwide.<sup>2</sup> Cardiovascular disease patients have manifested a significant increase in lipid peroxidation and oxidative DNA damage which is correlated to the severity of the hypercholesterolemia.<sup>3,4</sup> Therefore, in respect of hypercholesterolemia, recent interest has been focused on strategies that enhance the removal of reactive oxygen species (ROS), either by using antioxidants or drugs that enhance endogenous antioxidative systems.<sup>5</sup>

Diosgenin is an aglycone of the steroidal saponin, dioscin, in yam (*Dioscorea spp.*), and is a principal raw material for the industrial production of steroid drugs which is obtained after hydrolysis of the yam saponins. Such saponins as dioscin and gracillin, and the prosapogenins of dioscin have been identified from yam. It has been shown that the content of dioscin was about 2.7% (w/w)<sup>6</sup> and of diosgenin about 0.004% in cultivated yam, and 0.12–0.48% in wild yams.<sup>7</sup> It has been reported that diosgenin suppressed cholesterol absorption, increased cholesterol secretion through biliary excretion,<sup>8–11</sup> induced differentiation of the human erythroleukemia cell line by changing lipoxigenase activities,<sup>12</sup> and also induced apoptosis and cell cycle arrest in a human osteosarcoma cell line.<sup>13</sup> In our previous paper, we have reported that *Dioscorea nipponica* Makino, a wild yam, had anti-obesity and hypocholesterolemic effects with lipase inhibitory activity in high-cholesterol and high-fat fed rats.<sup>6</sup> In this present study, we investigated the effect of orally administered diosgenin on the antioxidative systems in high-cholesterol and high-fat fed rats. We measured the lymphocyte DNA damage by the comet assay, lipid peroxidation, and the activities and gene expression levels of such antioxidative enzymes as glutathione peroxidase (GSH-Px), superoxide dismutase (Cu/Zn SOD) and catalase (CAT).

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**Abbreviations:** CAT, catalase; GSH-Px, glutathione peroxidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ROS, reactive oxygen species; SOD, superoxide dismutase; TC, total cholesterol; TEM, tail extent moment; TG, triglyceride; TL, tail length

**Table 1.** Composition of the Experimental Diets

Ingredient	(%)		
	Control	0.1% Diosgenin	0.5% Diosgenin
Casein	20.0	20.0	20.0
Sucrose	15.0	15.0	15.0
Corn starch	39.0	38.9	38.5
Cellulose	5.0	5.0	5.0
Beef tallow	10.0	10.0	10.0
Corn oil	5.0	5.0	5.0
Mineral mixture <sup>1)</sup>	3.5	3.5	3.5
Vitamin mixture <sup>2)</sup>	1.0	1.0	1.0
Choline	0.2	0.2	0.2
Methionine	0.3	0.3	0.3
Cholesterol	1.0	1.0	1.0
Diosgenin (95%)	0	0.1	0.5
Total	100.0	100.0	100.0

<sup>1)</sup>Vitamin mixture (mg/kg): vitamin D<sub>3</sub>, 58.2;  $\alpha$ -tocopherol-acetate, 1200.0; retinol-acetate, 93.2; vitamin K<sub>3</sub>, 6.0; thiamin-HCL, 59.0; vitamin B<sub>12</sub>, 0.2; vitamin C, 588.0; pyridoxine-HCL, 29.0; D-biotin, 1.0; folic acid, 2.0; inositol, 1176.0; Ca-pantothenate, 235.0; riboflavin, 59.0; nicotinic acid, 294.0; sucrose, 96257.017

<sup>2)</sup>Mineral mixture (g/kg): CaCO<sub>3</sub>, 292.9; CaHPO<sub>4</sub>·2H<sub>2</sub>O, 4.3; KH<sub>2</sub>PO<sub>4</sub>, 43.1; NaCl, 250.6; MgSO<sub>4</sub>·7H<sub>2</sub>O, 99.8; Fe(C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>)·6H<sub>2</sub>O, 6.23; CuSO<sub>4</sub>·5H<sub>2</sub>O, 1.56; MnSO<sub>4</sub>·H<sub>2</sub>O, 1.21; (NH<sub>2</sub>)<sub>6</sub>MO<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.025; Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 0.015; ZnCl<sub>2</sub>, 0.005

## Materials and Methods

**Materials.** Diosgenin was purchased from Sigma (St. Louis, MO, USA). The assay kits for triglyceride, and total- and HDL-cholesterol were obtained from Asan Pharmacy Co. (Seoul, Korea). Vitamin and mineral mixtures were from Teklad (Madison, WI, USA). All other chemicals were of reagent grade.

**Animals and diet composition.** Male Sprague Dawley (SD) rats (6-week-old) were obtained from Korea Experimental Animal Center (Umsung, Korea), acclimatized for 1 week on standard feed and then fed on an experimental diet (Table 1). The animals were divided into three groups: control, and 0.1% and 0.5% diosgenin. Each group had 6 animals and was matched for its body weight. Each diet contained 1% cholesterol and 30% of total calories from fat. The animals were housed under a 12-hr light and dark cycle in a temperature and humidity controlled room (22 ± 1 °C), and given free access to the diet and water. The body weight was measured once a week. The animals were killed after 6 weeks of experimental diet feeding. The animal study was approved by the university committee for the appropriate animal research.

**Sample preparations.** Blood samples were collected from the abdominal aorta after sacrifice by CO<sub>2</sub> inhalation and centrifuged at 3,000 rpm for 20 min to separate to plasma and blood cells. To investigate DNA damage by the comet assay, lymphocytes were separated from heparinized whole blood by using Histopaque 1077. The separated lymphocytes were washed and resuspended in PBS, and immediately used for the

comet assay. Liver cytosol and microsome fractions were prepared by centrifugation of the liver homogenate.<sup>14)</sup> Protein concentration was measured by the Bradford method, using bovine serum albumin (BSA) as the standard.<sup>15)</sup>

**Blood and liver lipids.** The plasma triglyceride (TG), total cholesterol (TC), and HDL-cholesterol (HDL) levels were measured by assay kits (Asan Pharm. Co., Korea). The atherogenic index (AI) was calculated as AI = (total cholesterol-HDL cholesterol)/HDL cholesterol. LDL-cholesterol (LDL) was calculated by Friedewald's formula.<sup>16)</sup> Liver total lipids were extracted by the Folch method and quantified. The liver TG and TC levels were measured by assay kits (Asan Pharm. Co., Korea).

**Blood transaminase activity.** The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the plasma were measured by assay kits (Asan Pharm. Co., Korea).

**Lipid peroxidation.** The end product of polyunsaturated fatty acid peroxidation, thiobarbituric acid-reacting substances (TBARS), was determined by the method of Yagi.<sup>17)</sup> In brief, 2.0 ml of a reagent (26 mM thiobarbituric acid and 0.92 M trichloroacetic acid in 0.25 M HCl) and the sample (plasma or homogenized liver) were added to each tube and heated in a boiling-water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at 1000 × g for 10 min. The absorbance of the sample was determined at 535 nm, the breakdown product of 1,1,3,3-tetraethoxypropane being used as a standard.

**Lymphocyte DNA damage by the comet assay.** The comet assay was performed by the procedure of Singh.<sup>18)</sup> Fully frosted slides were covered with 0.65% of normal agarose as the first layer, a mixture of a cell suspension (around 20,000 cells) and 0.65% of low-melting-point agarose (LMA) as the second layer, and finally with 0.65% of LMA (without cells) as the third layer. The slides were allowed to solidify at 4 °C in the dark for 5 min, and then incubated either in a fresh phosphate buffer (unstressed cells) or in a phosphate buffer containing 50 μM freshly prepared H<sub>2</sub>O<sub>2</sub> for 5 min (stressed cells). The slides were then placed in a cold lysis buffer and kept at 4 °C for 1 hr to remove the cellular proteins. After this lysis, the slides were placed in a horizontal electrophoresis tank, and left in the electrophoresis solution for 20 min to allow DNA unwinding and the expression of alkaline labile damage before electrophoresis. Electrophoresis was conducted at 4 °C for 20 min (25 V and 300 mA), and then the slides were immersed in a neutralization buffer, stained with ethidium bromide, and examined under a fluorescence microscope (200×, Leica). Images of 50 randomly selected cells from each slide and two slides per each

**Table 2.** Nucleotide Sequences of PCR Primers Used for Semiquantitative RT-PCR of the Antioxidative Enzymes

Target Name	Primer sequence (5' → 3')	mRNA (bp)
$\beta$ -Actin	Forward: CTA TGA GCT GCC TGA CCG TC Reverse: AGT TTC ATG GAT GCC ACA GG	115
Zn/Cu-SOD	Forward: GTC GTC TCC TTG CTT TTT GC Reverse: TCT GCT CGA AGT GAA TGA CG	131
Mn-SOD	Forward: GAC ATT GTG CCT CTG GGT TT Reverse: GCC CTG CAT ACT TTG TCC AT	114
Catalase	Forward: AAG CTG GTT AAT GCG AAT GG Reverse: CAA GTT TTT GAT GCC CTG GT	75
GSH-Px	Forward: GTC CAC CGT GTA TGC CTT CT Reverse: ATT CTC GAT GAG CAG CAC CT	94

**Table 3.** Body Weight Change, Food Intake and Food Efficiency Ratio of Rats Fed for 6 Weeks on the Experimental Diet Supplemented with Diosgenin

	Control	0.1% Diosgenin	0.5% Diosgenin
Food intake (g/day/rat)	17.8 ± 0.4 <sup>1)ns2)</sup>	18.1 ± 1.0	17.7 ± 0.3
Body weight gain (g/rat)	200.2 ± 12.8 <sup>ns</sup>	207.9 ± 21.2	190.4 ± 21.6
FER (%) <sup>3)</sup>	28.0 ± 1.9 <sup>ns</sup>	28.7 ± 2.1	26.8 ± 2.5

<sup>1)</sup>Each value is the mean ± SD (n = 6).

<sup>2)</sup>ns, not significant

<sup>3)</sup>FER, food efficiency ratio, body weight gain (g)/food intake (g) × 100

treatment were analyzed with a computerized image analysis system (Komet 3.0, Kinetic Imaging, Liverpool, UK). Comets were analyzed by measuring the tail DNA content (%), tail length (TL;  $\mu$ m) and tail extent moment (TEM = tail length × % tail DNA).

**Antioxidative enzyme activities.** The glutathione peroxidase (GSH-Px) activities in the plasma, erythrocytes, and liver were measured spectrophotometrically (Hitachi U-3010, Japan) at 37 °C and 340 nm according to the method of Paglia.<sup>19)</sup> The superoxide dismutase (SOD) activities in the plasma, erythrocytes, and liver were assayed by the method described by Flohe.<sup>20)</sup> Catalase (CAT) activities in erythrocytes and liver were measured spectrophotometrically by the method of Aebi.<sup>21)</sup>

**Gene expression of hepatic antioxidative enzymes by semi-quantitative RT-PCR.** Total RNA was extracted from rat liver with an RNA preparation kit (Quiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2  $\mu$ g of RNA by using SuperScript II RT (Invitrogen Life Tech., Paisley, UK). mRNA levels were analyzed with an Exicycler™ quantitative thermal block (Bioneer Co., Daejeon, Korea), using 10 ng of cDNA as described by the Exicycler™ program. Primers were designed by using Primer3 software from a website (<http://frodo.wi.mit.edu>), and purchased from Bioneer Co. (Daejeon, Korea). The primers used for quantification of the antioxidative enzyme gene expression are shown in Table 2. A quantitative analysis of gene expression was conducted by using the comparative  $C_T$  ( $2^{-\Delta\Delta C_T}$ )

method<sup>22)</sup> with  $\beta$ -actin as an internal control. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

**Statistical analyses.** Data are expressed as the mean ± SD. Multiple comparisons were analyzed by one-way ANOVA and subsequent Duncan's multiple range test at  $\alpha = 0.05$ .

## Results and Discussion

### Food intake, body weight gain and organ weights

As shown in Table 3, diosgenin at the 0.1% and 0.5% levels did not affect the food intake and body weight gain of the animals. However, in our previous study, the animals fed with *Dioscorea nipponica* (DN) powder at the 5% level demonstrated a suppressed body weight gain.<sup>6)</sup> It seems that the administration of DN powder to an animal was more effective in suppressing the weight gain than diosgenin itself. DN powder contains various steroidal saponins and sapogenins (including diosgenin),<sup>6)</sup> and they might have synergistically inhibited the pancreatic lipase activity. The relative organ weight normalized by the body weight is shown in Table 4. The relative liver weight of the rats fed with the 0.5% diosgenin-supplemented diet was about 24% lower than that of the control group. This suggests that the animals fed on the 0.5% diosgenin-containing diet had less fat accumulation in the liver due to the pancreatic lipase inhibitory activity of diosgenin.<sup>6)</sup>

### Plasma AST and ALT activities

The measured plasma AST and ALT activities in the

**Table 4.** The Relative Organ Weights<sup>1)</sup> of the Rats Fed with Diosgenin in the High-Cholesterol Diet for 6 Weeks

Organ	Control	0.1% Diosgenin	0.5% Diosgenin
Heart	0.35 ± 0.03 <sup>2)ns3)</sup>	0.35 ± 0.03	0.35 ± 0.04
Lungs	0.42 ± 0.08 <sup>ns</sup>	0.40 ± 0.04	0.37 ± 0.04
Liver	4.71 ± 0.18 <sup>a4)</sup>	4.26 ± 0.63 <sup>a</sup>	3.59 ± 0.22 <sup>b</sup>
Kidneys	0.71 ± 0.02 <sup>ns</sup>	0.66 ± 0.04	0.68 ± 0.04
Pancreas	0.28 ± 0.08 <sup>ns</sup>	0.26 ± 0.06	0.29 ± 0.09

<sup>1)</sup>Relative organ weight (%) = organ weight (g)/body weight (g) × 100

<sup>2)</sup>Each value is the mean ± SD (n = 6).

<sup>3)</sup>ns, not significant

<sup>4)</sup>Values with different superscripts within a row are significantly different at  $\alpha = 0.05$  by Duncan's multiple-range test.

**Table 5.** AST and ALT Activities of Rats Fed on Diosgenin in the High-Cholesterol Diet for 6 Weeks

	Control	0.1% Diosgenin	0.5% Diosgenin
		Karmen U	
AST	186.0 ± 68.3 <sup>1)a2)</sup>	70.7 ± 43.5 <sup>b</sup>	47.3 ± 8.6 <sup>b</sup>
ALT	106.0 ± 57.5 <sup>a</sup>	38.7 ± 21.7 <sup>b</sup>	24.3 ± 6.0 <sup>b</sup>

<sup>1)</sup>Each value is the mean ± SD (n = 6).

<sup>2)</sup>Values with different superscripts within a row are significantly different at  $\alpha = 0.05$  by Duncan's multiple-range test.

rats that consumed the diosgenin-supplemented high-cholesterol diet are shown in Table 5. A diet high in cholesterol causes an increase in oxidative stress in the liver and results in increases in AST and ALT levels.<sup>23)</sup> Rats fed with the diosgenin-supplemented diet at the level of 0.1% and 0.5% respectively reduced plasma AST by about 62% and 75%, and plasma ALT by 64% and 78% compared to the animals fed on the high-cholesterol control diet. This suggests that the addition of diosgenin at the level of 0.1% or 0.5% may have a liver protective effect under high-cholesterol diet conditions.

#### Plasma and hepatic lipid profiles

The changes of lipid profile in the plasma and liver during the experimental period are presented in Table 6. Feeding rats with the high-cholesterol diet supplemented

with diosgenin for 6 weeks resulted in hypercholesterolemia, as was evident from the total cholesterol level in the plasma. Supplementation of the high-cholesterol diet with 0.1% or 0.5% diosgenin respectively reduced plasma TC by about 26% (88.3 mg/dl) and 32% (81.2 mg/dl) compared to the rats fed on the high-cholesterol diet alone (119.7 mg/dl). Furthermore, the rats that consumed the 0.5% diosgenin-supplemented diet showed an HDL-cholesterol level increased by 1.5 fold (44.1 mg/dl) compared with the control group (28.6 mg/dl), leading to an improvement in the atherogenic index from 3.4 of the control group to 0.9 of the 0.5% diosgenin group. Likewise, HDL/TC was significantly increased by almost twice in the rats fed on the 0.5% diosgenin-supplemented diet compared to the control group. However, diosgenin had no effect on the plasma triglyceride level.

The levels of hepatic total lipids and triglyceride of the 0.5% diosgenin group (56.4 mg/g and 15.4 mg/g) were 45% and 26% less than those of the control group (102.1 mg/g and 20.8 mg/g), respectively. Diosgenin resulted in substantial decrease in the liver cholesterol level of the rats fed on the high-cholesterol diet. The liver cholesterol level was 11.2 mg/g in the rats fed on the 0.5% diosgenin-supplemented diet, which was 39% of that in the rats of the control group (28.7 mg/g). As little as 0.1% diosgenin supplementation sufficed to affect a statistically significant decrease (22.2 mg/g) in the hepatic cholesterol level.

Several studies have reported that dietary diosgenin increased biliary cholesterol secretion several fold in rats without altering the biliary output of bile salts and phospholipids. The alteration of biliary cholesterol secretion in diosgenin-treated rats was related to the induced expression of hepatic *abcg5* and *abcg8* which are very important genes involved in cholesterol transport from hepatocytes to bile.<sup>24–27)</sup> In human hypercholesterolemia, therapy with cholestyramine, an anion-exchange resin that sequesters bile salts in the gut, produces a fall in LDL cholesterol but no increase in HDL cholesterol.<sup>28)</sup> Diosgenin decreased the plasma

**Table 6.** Blood and Liver Lipid Profiles of Rats Fed with Diosgenin in the High-Cholesterol Diet for 6 Weeks

Lipids	Control	0.1% Diosgenin	0.5% Diosgenin
<u>Plasma</u>			
TC (mg/dl)	119.7 ± 15.9 <sup>1)a3)</sup>	88.3 ± 9.8 <sup>b</sup>	81.2 ± 7.0 <sup>b</sup>
HDL-C (mg/dl)	28.6 ± 6.2 <sup>a</sup>	25.2 ± 6.5 <sup>a</sup>	44.1 ± 9.1 <sup>b</sup>
HDL/TC	0.25 ± 0.07 <sup>a</sup>	0.29 ± 0.10 <sup>a</sup>	0.54 ± 0.09 <sup>b</sup>
TG (mg/dl)	50.7 ± 8.9 <sup>ns2)</sup>	44.2 ± 11.9	42.6 ± 10.9
AI <sup>4)</sup>	3.4 ± 1.5 <sup>a</sup>	2.7 ± 0.9 <sup>a</sup>	0.9 ± 0.3 <sup>b</sup>
<u>Liver</u> (mg/g wet wt.)			
Total lipid	102.1 ± 10.0 <sup>a</sup>	95.7 ± 8.9 <sup>a</sup>	56.4 ± 5.2 <sup>b</sup>
TG	20.8 ± 3.0 <sup>a</sup>	21.1 ± 1.2 <sup>a</sup>	15.4 ± 2.3 <sup>b</sup>
TC	28.7 ± 1.7 <sup>a</sup>	22.2 ± 2.7 <sup>b</sup>	11.2 ± 1.2 <sup>c</sup>

<sup>1)</sup>Each value is the mean ± SD (n = 6).

<sup>2)</sup>ns, not significant

<sup>3)</sup>Values with different superscripts within a row are significantly different at  $\alpha = 0.05$  by Duncan's multiple-range test.

<sup>4)</sup>Atherogenic index = (total cholesterol-HDL cholesterol)/HDL cholesterol

**Table 7.** TBARS Levels in Blood and Liver of Rats Fed with Diosgenin in the High-Cholesterol Diet for 6 Weeks

	Control	0.1% Diosgenin	0.5% Diosgenin
Plasma (nmole/ml)	2.55 ± 0.78 <sup>1)ns2)</sup>	2.20 ± 0.38	2.03 ± 0.47
RBC (nmole/mg of Hb)	1.61 ± 0.30 <sup>a3)</sup>	1.36 ± 0.15 <sup>b</sup>	1.35 ± 0.12 <sup>b</sup>
Liver (nmole/g of tissue)	95.4 ± 17.5 <sup>ns</sup>	104.2 ± 7.3	111.1 ± 10.4

<sup>1)</sup>Each value is the mean ± SD (n = 6).

<sup>2)</sup>ns, not significant

<sup>3)</sup>Values with different superscripts within a row are significantly different at  $\alpha = 0.05$  by Duncan's multiple-range test.

total cholesterol level by the same mechanism as that of cholestyramine, but increased the plasma HDL cholesterol level which is quite different from cholestyramine. This marked increase of HDL in the diosgenin-supplemented animals might be associated with a decrease in the cholesteryl ester transfer protein activity.<sup>29)</sup> However, the mechanism for the HDL-raising effect of diosgenin remains to be elucidated. The capability of diosgenin to reduce TC and elevate HDL suggests its potential usefulness as an agent for treating human hypercholesterolemia.

#### *Plasma, erythrocyte and liver lipid peroxidation*

Table 7 shows the level of lipid peroxidation measured by the TBARS concentration in the plasma, erythrocytes and liver. The TBARS concentration in the plasma and liver tended to decrease with diosgenin supplementation, but did not reach statistical significance. However, the erythrocyte TBARS concentration in the diosgenin-supplemented groups was significantly lower than that of the control group. Lipid peroxidation serves as a marker of cellular oxidative stress and has long been recognized as a major causative factor of oxidative damage in such chronic diseases as atherosclerosis and cancer. Some researchers have found that coronary artery disease (CAD) patients showed significant alteration in erythrocyte membranes and antioxidative status. Therefore, in CAD patients, the erythrocyte lipid peroxidation was significantly higher, whereas the erythrocyte SOD and GSH-Px activities and serum vitamin C level were lower than those of healthy subjects.<sup>30)</sup>

The fact that the animals supplemented with diosgenin showed a lower TBARS level in erythrocytes than in the control animals suggests that diosgenin played a role like that of a lipid peroxidation chain-breaking antioxidant in cell membranes and might provide protection against the oxidative damaging effects of polyunsaturated fatty acids.

#### *Lymphocyte DNA damage*

DNA damage is one of the more reliable markers to detect oxidative stress.<sup>31,32)</sup> We used the comet assay to measure the level of DNA strand breaks in freshly isolated blood mononuclear cell fractions from rats fed on the cholesterol diet supplemented with diosgenin. Isolated cells from each group were divided into two and incubated with either a phosphate buffer (unstressed) or

50  $\mu$ M H<sub>2</sub>O<sub>2</sub> (stressed) for 5 min. As shown in Table 8, diosgenin did not induce any damage per se, and seemed to protect DNA against oxidative damage in the lymphocytes stressed with H<sub>2</sub>O<sub>2</sub>. The control rats showed greater lymphocyte DNA damage than the diosgenin-supplemented rats in both the unstressed and stressed cells. The protective effect of diosgenin against DNA damage was observed to be much stronger in the cells stressed with H<sub>2</sub>O<sub>2</sub> than in the unstressed cells. Thus, diosgenin appears to have exerted a greater antioxidative effect in the oxidant-challenged state.

It is known that DNA damage contributed significantly to the development and the progression of atherosclerosis<sup>33)</sup> and that there is a relationship between the level of DNA damage and the severity of coronary artery disease.<sup>34)</sup> Excessive oxidative stress and/or inadequate defense against antioxidants are implicated in the pathogenesis of cardiovascular disease.<sup>35,36)</sup> It has been shown that increased DNA damage in hypercholesterolemia was caused by the increased production of reactive oxidative species and decreased antioxidative capacity.<sup>37)</sup> In this study, the control animals fed on the high-cholesterol diet without diosgenin supplementation showed increased lymphocyte DNA damage associated with hypercholesterolemia. However, this lymphocyte DNA damage was less in the diosgenin-supplemented animals fed on the high cholesterol diet.

An increase of oxidative DNA damage has recently been found in human atherosclerotic plaque.<sup>38)</sup> It seems likely that the reactive oxygen species generated by many routes could cause DNA damage in atherosclerosis,<sup>39)</sup> so that the accumulation of somatic mutations may be an important risk factor for the initiation and progression of atherosclerotic disease.<sup>40)</sup> The inhibition of oxidative DNA damage is an interesting objective point that should be evaluated for testing the efficacy of new therapies for preventing the development and progression of atherosclerosis.

There are various intracellular antioxidative mechanisms for DNA protection. These include scavenging damaging ROS, enzymatic inactivation of ROS, and binding iron. Activation and altered transcription of antioxidative enzymes are also important to maintain a low level of DNA damage under the normal intracellular conditions of continuous oxidative challenge of varying intensity.<sup>41)</sup>

Our results from the comet assay indicate that lymphocyte DNA was more resistant to oxidative

**Table 8.** Lymphocyte DNA Damage Measured by the Comet Assay in Rats Fed with Diosgenin in the High-Cholesterol Diet for 6 weeks

Parameter	Control	0.1% Diosgenin	0.5% Diosgenin
<u>Unstressed lymphocytes</u>			
Tail DNA (%)	7.98 ± 3.72 <sup>1)a2)</sup>	7.68 ± 3.72 <sup>a</sup>	6.94 ± 3.70 <sup>b</sup>
Tail length (um)	9.71 ± 5.71 <sup>a</sup>	8.53 ± 4.81 <sup>b</sup>	8.71 ± 5.30 <sup>b</sup>
Tail extent moment	0.75 ± 0.54 <sup>a</sup>	0.62 ± 0.40 <sup>b</sup>	0.57 ± 0.42 <sup>b</sup>
<u>Lymphocytes stressed with H<sub>2</sub>O<sub>2</sub></u>			
Tail DNA (%)	9.86 ± 5.53 <sup>a</sup>	8.49 ± 3.76 <sup>b</sup>	8.50 ± 4.53 <sup>b</sup>
Tail length (um)	15.3 ± 8.69 <sup>a</sup>	12.5 ± 6.65 <sup>b</sup>	12.6 ± 7.56 <sup>b</sup>
Tail extent moment	1.66 ± 1.81 <sup>a</sup>	1.04 ± 0.71 <sup>b</sup>	1.10 ± 0.99 <sup>b</sup>

<sup>1)</sup>Each value is the mean ± SD (n = 6).

<sup>2)</sup>Values with different superscripts within a row are significantly different at  $\alpha = 0.05$  by Duncan's multiple-range test.

**Table 9.** Blood and Liver Antioxidative Enzyme Activities of Rats Fed with Diosgenin in the High-Cholesterol Diet for 6 Weeks

Antioxidative enzyme	Control	0.1% Diosgenin	0.5% Diosgenin
<u>Plasma</u>			
T-SOD (unit/ml)	5.82 ± 3.62 <sup>1)a3)</sup>	7.29 ± 3.09 <sup>a</sup>	12.5 ± .304 <sup>b</sup>
GSH-Px (μmole/min/ml)	1.48 ± 0.29 <sup>ns2)</sup>	1.54 ± 0.24	1.65 ± 0.18
<u>Erythrocyte</u>			
T-SOD (unit/mg of Hb)	21.6 ± 7.26 <sup>ns</sup>	21.4 ± 6.70	21.3 ± 4.54
GSH-Px (μmole/min/mg of Hb)	1.72 ± 0.34 <sup>ab</sup>	1.94 ± 4.14 <sup>bc</sup>	2.18 ± 3.49 <sup>c</sup>
CAT (k/mg of Hb)	25.0 ± 1.41 <sup>a</sup>	26.1 ± 3.42 <sup>ab</sup>	28.3 ± 2.29 <sup>bc</sup>
<u>Liver</u>			
T-SOD (unit/mg of prot.)	50.9 ± 6.70 <sup>a</sup>	64.9 ± 11.7 <sup>b</sup>	69.3 ± 6.65 <sup>b</sup>
GSH-Px (μmole/min/mg of prot.)	11.3 ± 1.44 <sup>ns</sup>	11.5 ± 2.78	13.2 ± 1.15
CAT (k/mg of prot.)	19.8 ± 3.87 <sup>ab</sup>	18.4 ± 1.76 <sup>a</sup>	24.2 ± 0.52 <sup>b</sup>

<sup>1)</sup>Each value is the mean ± SD (n = 6).

<sup>2)</sup>ns, not significant

<sup>3)</sup>Values with different superscripts within a row are significantly different at  $\alpha = 0.05$  by Duncan's multiple-range test.

challenge by H<sub>2</sub>O<sub>2</sub> with the dietary supplementation by diosgenin to hypercholesterolemic rats. Diosgenin supplementation at the 0.1% or 0.5% level conferred significant protection against lymphocyte DNA damage in both unstressed and stressed cells. The role of dietary diosgenin in DNA protection and the mechanism for this protection are not yet clear.

#### *Plasma, erythrocyte and liver antioxidative enzyme activities*

The plasma, erythrocyte and liver antioxidative enzyme activities are shown in Table 9. In respect of the plasma, the T-SOD activity was significantly higher in the 0.5% diosgenin group (12.5 U/ml) than control group (5.82 U/ml). However, the GSH-Px activity was no different among the groups. Diosgenin supplementation (0.5%) caused a 27% and 13% increase in GSH-Px and CAT in erythrocytes, respectively. In the hepatic tissues, T-SOD was increased by 28% and 36% in the 0.1% and 0.5% diosgenin groups, respectively, and CAT was increased by 22% in the 0.5% diosgenin group. The GSH-Px activity in liver was not significantly affected.

The biological effects of free radicals are controlled *in vivo* by a wide range of antioxidants such as vitamins E and C, carotenoids, glutathione and antioxidative enzymes. Among these enzymes, SOD catalyzes dismutation of the superoxide anion into hydrogen peroxide,

while GSH-Px both detoxifies hydrogen peroxides and converts lipid hydroperoxides to non-toxic alcohols.<sup>42)</sup> A reduced antioxidative defense status in the plasma and erythrocytes might result in increased peroxidation of cell membrane lipids and hence an increased plasma concentration of lipid peroxides. This, in turn, might play an important role in atherogenesis. The activities of plasma SOD and erythrocyte GSH-Px and CAT in the 0.5% diosgenin group were higher than those in the control group. Erythrocytes normally contain enough scavengers such as CAT and GSH-Px to protect against free radical injury. The decreased activity of the antioxidative system in the control animals might be responsible for the increased peroxidation of membrane lipids. It has been reported that the increased peroxidation of membrane lipids caused a reduction in the activity of antioxidative enzymes.<sup>43)</sup> It might be assumed that the increased activity of antioxidative enzymes in the diosgenin group played an important role in restricting the production of the active oxygen species induced by hypercholesterolemia.

#### *Expression of hepatic antioxidative enzymes*

In response to oxidative stress, an organism strives to modulate the gene expression of several antioxidative enzymes. In the present research, we aimed to evaluate changes in the mRNA expression of the antioxidative

**Table 10.** Mean  $\Delta C_T$  Value and Relative mRNA Expression ( $2^{-\Delta\Delta C_T}$ ) of the Hepatic Antioxidative Enzymes in Rats Fed with Diosgenin in the High-Cholesterol Diet for 6 Weeks

Antioxidant enzymes	Control	0.1% Diosgenin	0.5% Diosgenin
GSH-Px			
$\Delta C_T$	5.52 ± 1.06 <sup>1)a3)</sup>	3.94 ± 0.96 <sup>ab</sup>	1.82 ± 0.54 <sup>c</sup>
$2^{-\Delta\Delta C_T}$	1	2.99	13.0
CAT			
$\Delta C_T$	1.49 ± 0.71 <sup>a</sup>	1.12 ± 0.33 <sup>a</sup>	-0.10 ± 0.04 <sup>b</sup>
$2^{-\Delta\Delta C_T}$	1	1.29	3.03
Cu/Zn SOD			
$\Delta C_T$	-6.55 ± 1.98 <sup>a</sup>	-7.80 ± 2.01 <sup>ab</sup>	-8.47 ± 2.66 <sup>b</sup>
$2^{-\Delta\Delta C_T}$	1	2.38	3.78
Mn SOD			
$\Delta C_T$	0.45 ± 0.12 <sup>ns2)</sup>	0.19 ± 0.05	-0.50 ± 0.17
$2^{-\Delta\Delta C_T}$	1	1.19	1.93

$C_T$  = threshold cycle for PCR at which the amplified product was first detected;  $\Delta C_T$  = difference of the  $C_T$  values derived from the target gene being assayed and  $\beta$ -actin considered as the reference gene;  $2^{-\Delta\Delta C_T}$  = relative gene expression.

<sup>1)</sup>Each value is the mean ± SD (n = 6).

<sup>2)</sup>ns, not significant

<sup>3)</sup>Values with different superscripts within a row are significantly different at  $\alpha = 0.05$  by Duncan's multiple-range test.

enzymes, GSH-Px, CAT, Cu/Zn SOD and Mn SOD, in hepatic tissues, and to determine whether the diosgenin-supplemented diet could modulate their expression. The semi-quantitative RT-PCR data are shown as  $\Delta C_T$  and the relative gene expression in Table 10.

The relative mRNA expression of the antioxidative enzymes in the diosgenin-supplemented groups was increased. GSH-Px expression was increased by 2.99-fold in the 0.1% and by 13.0-fold in the 0.5% diosgenin-supplemented groups in comparison to the control group. CAT expression was increased by 1.29-fold and 3.03-fold, and Cu/Zn SOD increased by 2.38-fold and 3.78-fold in the 0.1% and 0.5% diosgenin-supplemented groups in comparison to the control group. Mn SOD was increased 1.93-fold in the 0.5% diosgenin group. The effect of diosgenin supplementation on oxidative stress was also evident for GSH-Px, with an increase of 13-fold compared to the control group. The results obtained in this experiment suggest that dietary supplementation with diosgenin might control oxidative stress by modulating GSH-Px expression.

Mn SOD is known as a mitochondrial enzyme, and its mRNA expression is easily induced by exposure to superoxide and hydroxyl radicals, while Cu/Zn SOD is not easily altered by a variety of stimuli.<sup>44)</sup> The level and balance of antioxidative enzymes modulates the susceptibility of a cell to oxidative injury. The enhanced SOD activity without concomitant increases in the catalase and GSH-Px activities may result in an excessive accumulation of hydrogen peroxide which in turn, reacts with metals to produce hydroxyl radical through the Fenton reaction.<sup>45)</sup> Our results for the gene expressions of antioxidative enzymes by diosgenin administration showed that Cu/Zn SOD and catalase were slightly induced, while GSH-Px was markedly induced. This modified balance between the antioxidative enzymes might be able to remove superoxides more efficiently. Although there are several reports showing

that diosgenin suppressed the activation and gene expression of NF $\kappa$ B, which has been identified in the regulatory regions of all of the antioxidative enzyme genes, we do not clearly understand which cellular mechanisms may account for these changes to the antioxidative system.<sup>46-48)</sup> Further investigations on the mechanism for the altered antioxidative enzyme gene expression by diosgenin treatment would therefore be necessary.

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