Suppression of Lipid-Hydroperoxide and DNA-Adduct Formation by Isoflavone-Containing Soy Hypocotyl Tea in Rats

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Abstract

Objective: Phytoestrogen isoflavones (IFs) are considered to suppress estrogen-related cancers through their antiestrogenic activity. The antioxidant effect of IFs, however, has not been confirmed in an *in vivo* system, so suppression of hydroperoxide formation and resultant DNA adduct formation were studied.

Methods: The antioxidant effects of the soya-hypocotyl tea (SHT), which contained daidzein (14+/-1.5 mg/l) and genistein (3+/-0.5 mg/l), were examined in Wistar rats fed the AIN-76 control diet or iron deficient diet (FeD) for 4 weeks. The intake amount of the diet and IFs were measured daily. Urinary excretion of IFs was measured for 3 days before sacrifice. In addition to the serum lipid analyses, phosphatidylcholine hydroperoxide (PCOOH), and phosphatidylethanolamine hydroperoxide (PEOOH) production in red blood cells and the liver were measured as a biomarker of oxidants. Production of DNA adducts by oxidative stress was measured by the amount of 8-hydroxy-2'-deoxyguanosine (oh⁸dG) in the liver and kidney, and urine. Histological changes were checked by H&E staining and immunohistochemistry for oh⁸dG.

Results: FeD rats showed anemia, growth retardation, hyperlipidemia. IFs only lowered the triacylglycerol level and did not change the cholesterol level. Rats fed the normal diet did not show suppression of PCOOH and PEOOH production in either red blood cells or the liver, while groups administered SHT showed suppressed production of PCOOH and PEOOH in the liver. The cumulative intake of daidzein, genistein and the total amount of IFs showed significant inverse associations with urinary excretion of oh⁸dG. oh⁸dG in the kidney showed an inverse association with the amount of oh⁸dG in the urine. Enzyme-histochemically, a strong localization of oh⁸dG was found in the epithelial cells of the bile canaliculi and proximal tubules of the kidney.

Conclusion: IFs and SHT showed antioxidant effects at physiological concentrations in an *in vivo* system. The antioxidant effects of IFs decreased oxidation stress to the nuclear DNA, which was shown by the decreased oh⁸dG production. It is suggested that to prevent various cancers, in addition to the known antiestrogenic, antityrosin kinase, and other effects. IFs appeared to promote excretion of oh⁸dG.

Key words: daidzein, genistein, antioxidant, DNA adduct, rat experiment

Introduction

There have been many epidemiological studies describing the human health effects of soy rich food on the prevention of estrogen-related cancers and cardiovascular diseases, the decrease of climacteric symptoms, and the prevention of osteoporosis (1–3). Japanese people consume a lot of soybean products containing phytoestrogens, such as daidzein and genistein, which may lead to the low incidence of hormone-related cancers (4–6). Murlies et al. (7) found that urinary daidzein excretion was significantly lower

in breast cancer patients (0 nmol/24 hr vs 1,558 nmol/24 hr in controls). The most plausible mechanism was suggested to be the antiestrogenic effects of isoflavones in soybeans. Various parts of soybeans contain different components of IFs, and the hypocotyls are characterized by its high amount of daidzein (8). IFs also have various pharmacological activities, such as inhibitory activities on tyrosine kinase, aromatase, and endothelial proliferation (1, 3). The hypocotyl, which is the germ part of soybeans, contains five-fold more ratio of isoflavones (IFs) than a soybean as a whole (8). Ordinary soy protein contains more genistein than daidzein, but in hypocotyls, the daidzein to genistein ratio was about 5:1. IFs have various pharmacological activities, such as inhibitory activities on tyrosine kinase, aromatase, and endothelial proliferation, in addition to the antiestrogenic activity. Their polyphenol groups suggest their antioxidative activity, but it has not been confirmed by an *in vivo* system.

It was reported that an iron deficiency yielded lipid hydroper-

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oxides in rats (9), so the antioxidant effects of isoflavonoids appeared to be more easily confirmed by this system. The purpose of this study was to find proof of the suppression of hydroperoxide production by soya-hypocotyl tea (SHT) drinking and its effect on DNA adduct formation using a biomarker of 8-hydroxy-2'-deoxyguanosine (oh⁸dG) in the liver, kidney and urine. The safety or toxicity of SHT was also checked histologically and immunohistochemically.

Materials and Methods

1. Animals and soya-hypocotyl tea (SHT)

Thirty-six male Wistar-strain rats, 3 weeks of age, were purchased from Clea Japan Inc, Tokyo, and housed individually in screen-bottom stainless steel cages in a room with controlled temperature (22±1°C) and lighting 12 h light/dark cycle. Distilled water and a basal semi-purified diet were provided *ad libitum* during a 5-day acclimation period. Half of the rats (C group) were fed the control diet (AIN-76), and the other half (FeD group) were fed the iron deficient diet. The composition of the control diet used in this study was as follows: casein milk, 20.0%; corn starch, 15.0%; sucrose, 50.0%; cellulose, 5.0%; corn oil, 5.0%; vitamin mixture (AIN-76), 1.0%; mineral mixture (AIN-76), 3.5%; DL-methionine, 0.3%; chorine bitartrate, 0.2%. The iron deficient diet used mineral mixture (AIN-76) that excluded Fe(C₆H₅O₇)·5H₂O (Table 1).

The control and FeD groups were each further divided into three groups: the first group (C0 and D0, respectively) drank water, the second group (C10 and D10, respectively) drank ordinary extracted soya hypocotyl tea (IF10) and the third group (C50 and D50, respectively) drank concentrated tea (IF50). These were prepared by Fuji Oil Co., Ltd, Osaka. The ordinary extracted 1 l of IF10 contained daidzin 11.3±1.1 mg, genistin 2.6±0.5 mg, daidzein 2.7±0.4 mg, and genistein 0.4±0.0 mg, total 17.0±1.8 mg and concentrated IF50 contained daidzin 51.7±2.6 mg, genistin 12.0±1.2 mg, daidzein 11.2±0.1 mg, and genistein 1.3±0.0 mg, total 76.2±3.4 mg. Water and SHT were given to the rats *ad libitum*, and the consumption was recorded for 4 weeks. Body weight and diet intake were recorded daily, in addition to the observation of general condition.

Three days before sacrifice, each rat was delivered to the metabolic cage, and urine and feces were collected. The rats were killed after Nembutal[®] anesthesia. All experiments reported herein were approved by the TUA Animal Care and Use Committee in our university.

Table 1 Composition of diets (%)

Contents	Control	FeD ²⁾
Casein milk	20.0	20.0
Corn starch	15.0	15.0
Sucrose	50.0	50.0
Cellulose	5.0	5.0
Corn oil	5.0	5.0
Vitamin mixture ¹⁾	1.0	1.0
Mineral mixture ¹⁾	3.5	3.5
{Fe(C ₆ H ₅ O ₇)·5H ₂ O}	0.6	0.0}
DL-Methionine	0.3	0.3
Chorine bitartrate	0.2	0.2
Total	100.0	100.0

¹⁾ This mixture was identical to AIN-76.

²⁾ Iron deficient diet. Fe(C₆H₅O₇)·5H₂O in mineral mixture was excluded.

2. Biochemistry

Hemoglobin was measured by the cyan-methohemoglobin method (hemoglobin-test kit, Wako Pure Chemical Industries, Osaka, Japan). Total cholesterol was measured by the cholesterol oxidase-phenol method (Cholesterol E-test kit, Wako Pure Chemical Industries, Osaka, Japan), free cholesterol by the cholesterol oxidase phenol method (Free cholesterol E-test kit, Wako Pure Chemical Industries, Osaka, Japan). HDL-cholesterols was measured by the precipitation method (HDL-Cholesterol E-test kit, Wako Pure Chemical Industries, Osaka, Japan). Ester cholesterol was calculated by extraction of free cholesterol from total cholesterol. Triacylglycerol (TG), phospholipid and free fatty acids were measured by the enzymatic method (TG E-test kit, phospholipid E-test kit, free fatty acid E-test kit, Wako Pure Chemical Industries, Osaka, Japan). Beta-lipoprotein was also measured by the precipitation method (Detaminer BLP kit Kyowa Medex Co. Ltd, Tokyo, Japan). Beta-lipoprotein was considered to be sum of VLDL and LDL.

3. Analysis of urinary isoflavones

The urinary daidzein, genistein and equol were analyzed according to the method of Adlercreutz et al. combined with the modified HPLC method described by Gamache et al. (10). For the recovery calculation 20 µl of ³H-estradiol glucuronide was added to the tubes containing 1 ml of urine. The recovery rate was calculated from the rate of the count before and after extraction using a liquid scintillation counter. After mixing and equilibrating for 30 min at room temperature, 0.5 ml of enzyme solution [0.5 ml Helix pomatia juice (Type HP-2S, Sigma, St. Louis, Missouri, USA) in 10 ml 0.2 M acetate buffer (pH 4.0) containing 0.15 g ascorbic acid] was added to the tubes. After mixing, the sample was hydrolyzed overnight at 37°C. From the hydrolyzed sample IFs were extracted twice with 5 ml diethyl ether, and the ether fraction was evaporated to dryness under a nitrogen gas flow. The residue was dissolved in 0.2 ml methanol, and 20 µl sample was injected into the HPLC with a diode-array UV detector, scanning from 250 to 400 nm (Beckman Coulter K.K., Tokyo, Japan). Peaks were detected at 254 nm for daidzein and genistein and at 280 nm for equol and O-DMA. The HPLC column was ODS-80Ts-Qa (150×4.6 mm I.D. 5 µm particle size) (Tosoh, Tokyo, Japan) with a guard column (TSK guard-gel ODS-80Ts, 1.5×3.2 mm I.D. 5 µm particle size) (Tosoh, Tokyo, Japan), and was maintained temperature at 25°C using a column oven. HPLC analysis was carried out by linear gradient, from 1.5/0.5/8.0 [methanol, acetonitrile, 0.2 M acetate buffer (pH 4.0), v/v/v] to 6.0/3.0/1.0 for 45 min, and returned to the initial condition for 5 min. The flow rate was 1.0 ml/min. Quantification was performed by measuring the peak areas based on calibrations (from 4 to 160 µmol/l) and corrected for losses during hydrolysis and extraction based on the recovery data. All solvent and chemicals were of HPLC grade or analytical grade (10).

4. Whole antioxidant activity of plasma

The auto-oxidation inhibition of linoleic acid was measured by the modified Miller's method (11, 12). Mixture of 150 µl beta-carotene solution (1.0 mg/ml chloroform), 100 µl linoleic acid solution (0.1 g/ml chloroform) and 300 µl Tween 40 solution (0.2 g/ml chloroform) were moved to the Erlenmeyer flask. The chloroform in the flask was completely evaporated with nitrogen gas, and the 30 ml distilled water was poured to the flask to

prepare the linoleic acid-beta-carotene solution (LC solution). The 5 µl plasma was added to each well in a 96-well microplate, and then 200 µl LC solution was added. The plate was moved to the 50°C incubator immediately, and the rate of fading color of beta-carotene during 5 and 60 min was calculated from the absorption rate at OD 490 nm (12).

5. Analysis of PCOOH and PEOOH

The amounts of hydroperoxides, PCOOH and PEOOH, in the red blood cells and the liver were measured by the fluorescent chemiluminescence HPLC method (13, 14). Briefly, total lipids were extracted by the mixture of EDTA, 2-propanol and chloroform from a 1 ml red blood cell pack. Evaporated extract was resolved in chloroform:methanol (2:1) with 0.002% butylated hydroxytoluene (BHT), and 20 µl was injected into the HPLC column. The chemiluminescence detection high performance liquid chromatography (CL-HPLC) method incorporated cytochrome c and luminol as a post-column hydroperoxide-specific luminescent reagent. An elute from a n-propylamine-bound silica column with hexan-2-propanol-methanol-water, 5:7:2:1 (v/v/v/v) was monitored at 240 nm UV detector (Shodex UV4, Showa, Denko, Co., Ltd., Japan), and then mixed with a chemiluminescence's reagent at a flow rate of 1 ml/min. Generated chemoluminescence was detected using a chemoluminescence detector (Shodex CL2, Showa, Denko, Co., Ltd., Japan), which determined both PCOOH and PEOOH on a Sekonic ss-250s multiple recorder. Sensitivity was 10 pmol hydroperoxide-O₂ with a mixture of 10 µg/ml cytochrome c and 2 µg/ml luminol in 50 mM borate buffer (pH 10.0) (15).

6. Determination of 8-hydroxy-2'-deoxyguanosine (oh⁸dG) in DNA

DNA was extracted from the liver and kidney homogenates with the DNA Extractor WB kit (Wako, Japan) according to the method of Nakae et al. (16). The extracted DNA (about 100 µg) was dissolved in 100 µl of water and denatured by heating at 100°C for 3 min. To the solution of denatured DNA, 2 µl of 1 M sodium acetate (pH 4.8) and 8 µg of nuclease P1 (5 mg/ml, Yamasa Co. Choshi, Chiba) were added, and then the mixture was digested into nucleotides at 37°C for 1 h. To each sample, 16 µl of 1 M Tris-HCl (pH 7.2) was added and the mixture was treated with 1.3 units of alkaline phosphatase (type III from sigma P-4252) at 37°C for 2 h. The oh⁸dG in the digested DNA was assayed by HPLC with an electrochemical detector (ECD). A column (Puresil C18 5 µ 120 A column; 7 µm, 4.6 mm×25 cm, Waters) was connected to an HPLC apparatus (CCPM; Tosoh Co., Tokyo) coupled with an ECD (model 5100A; ESA, Inc., Bedford, MA) equipped with an analytical cell (model 5010; detector 1: 0.10 V, Detector 2: 0.45 V) and a guard cell (model 5020; 0.50 V). The column was eluted with 0.2 M NaH₂PO₄ containing 8% methanol at a flow rate of 1.0 ml/min. The amount of deoxyguanosine (dG) was calculated from the absorbance at 290 nm measured with a UV monitor (model Waters 486; Waters Co.), while the amount of oh⁸dG was simultaneously assayed from the ratio of the peak height of oh⁸dG to the peak height ×10⁵ of dG (17, 18).

7. Analysis of urinary oh⁸dG

Urinary oh⁸dG was measured using an ELISA kit (Nikken Food Co. Ltd, Fukuroi, Shizuoka) (19). Briefly, 96-well microtiter

plates were filled with 100 µl of 1 mg/ml oh⁸dG and incubated at 4°C overnight. After three washes with PBS containing 1% BSA and 0.5% Tween 20, each well was filled with 200 µl urine for 1 h at room temperature. After three washes with PBS, 50 µl of monoclonal antibody, N45.1, was added to each well and incubated for 1 h at room temperature. After washing, 100 µl of 0.05 M citrate buffer (pH 5.0) containing 0.4 mg/ml of o-phenylenediamine and 0.015% H₂O₂ was added and incubated for several minutes and the reaction was terminated by the addition of 2 M sulfuric acid. The oh⁸dG was quantified by absorbance at 490 nm with a microtiter plate reader.

8. Histological and immunohistochemical examination

The liver, heart, kidney, intestines, adrenals, femoral bone, testis and epididymis were fixed in 4.5% buffered paraformaldehyde, and the liver, prostatic gland, seminal vesicles and adrenals were fixed in cooled methanol for immunohistochemistry. The tissue sections were routinely stained for hematoxylin-eosin by the ordinary method.

Immunohistochemistry of oh⁸dG: Paraffin embedded cut sections were deparaffinized in xylene and alcohol. Pretreatment with 2N-HCl for 30 min, neutralization with 0.1 M sodium borate, and additional pretreatment with 1% H₂O₂ in methanol were carried out to increase stainability and to decrease the endogenous peroxidase reaction. After these pretreatments on the tissue sections, oh⁸dG was stained by the ABC method. The primary antibody was monoclonal anti-oh⁸dG mouse IgG, the secondary antibody was biotin conjugated anti-mouse Ig, and the third reaction was horseradish peroxidase labeled avidin. The sections allowed to color by DAB solution mixed with 0.03% H₂O₂ after 30 min reaction at room temperature. The antibody dilution used to detect the antigen was 1: 200. Anti-oh⁸dG antibody kit was obtained from Nikken Food Co. Ltd., Fukuroi, Shizuoka (19).

9. Statistical analysis

All statistical analyses were performed by the program package, SPSS ver 10 (SPSS, Tokyo). Comparisons among groups was performed by ANOVA, and correlation analysis was done by calculating Pearson's correlation coefficient. A two sided p-value<0.05 was considered significant.

Results

1. Body weight, amount of feeding, and SHT on nutritional effects

The cumulative diet intake diet and SHT for 4 weeks, and the calculated cumulative intake of IFs by each subgroup are shown in Table 2 in addition to the body weight and hemoglobin concentration at sacrifice. The hemoglobin level of the FeD group dropped to 6.5+/-2.0 g/dl compared with 14.5+/-1.4 g/dl in the control group. The ferritin level was also low in the FeD group. The FeD group showed both lower total diet intake and amount drunk than those of the control group. The FeD group showed anemic eye color, growth retardation, and the final body weight was about 50 g lower than that of the control group (Fig. 1). The SHT group did not show any morbid condition throughout the experiment. Neither diarrhea nor constipation was observed. There was no significant difference in the body weight among the subgroups treated by IF10 and IF50 in each group.

Multiple regression analysis by variables, such as total intake of diets and drinks, isoflavone intake and ferritin level at sacrifice,

Table 2 Cumulative amount of dietary intake, drink, IFs for 4 weeks, and body weight, hemoglobin and ferritin at sacrifice

	Group											
	C0		C10		C50		D0		D10		D50	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
Total diet intake (g)	386	8	382	24	376	14	321	29	316	25	318	31
Total drink intake (mg/day)	494	30	470	28	475	49	393	60	406	30	417	22
cumulative daidzin intake (mg)	0	0	5,305	319	24,575	2,561	0	0	4,586	344	21,574	1,151
cumulative genistin intake (mg)	0	0	1,268	76	5,324	555	0	0	1,096	82	4,674	249
cumulative daidzein intake (mg)	0	0	1,221	73	5,704	594	0	0	1,055	79	5,007	267
cumulative genistein intake (mg)	0	0	188	11	618	64	0	0	162	12	542	29
cumulative IFs intake (mg)	0	0	7,982	480	36,220	3,774	0	0	6,900	518	31,797	1,696
Body Weight at 4 th week	222 ^{a,b,c}	10	222 ^{d,e,f}	18	218 ^{g,h,i}	15	181 ^{a,d,g}	34	179 ^{b,e,h}	29	182 ^{c,f,i}	21
Hemoglobin (g/dl)	15.3 ^{a,b,c}	1.4	14.1 ^{d,e,f}	1.0	15.3 ^{g,h,i}	1.5	6.7 ^{a,d,g}	2.1	6.5 ^{b,e,h}	3.0	5.6 ^{c,f,i}	0.6
Ferritin (ng/ml)	127.8	156.8	111.8	92.2	117.1	191.2	61.7	57.4	114.8	185.6	33.9	10.7

C0; control group, C10; soya hypocotyl tea (SHT) low dose, C50; SHT high dose, D0; iron deficient group (ID), D10; ID+SHT low dose, D50; ID+SHT high dose.

Iron deficient diet groups (D0, D10, D50) showed significant low hemoglobin and body weight compared with the control diet groups (C0, C10, C50).

Ferritin also showed a low value in the D0 and D50 group, but there was no significant difference.

Subscripts in the columns show significant difference ($p < 0.05$) by ANOVA between the subscript letters.

Fig. 1 Growth curve of the control and FeD groups. Soya-hypocotyl tea administration did not show growth inhibition in either group.**Table 3** Urinary excretion of IFs and intake of daidzein and genistein on the corresponding 3 days

group	Intake from SHT (nmole/day)				Urinary excretion of IFs (nmole/day)						Recovery ratio					
	Genistein		Daidzein		Genistein		Equol		Daidzein		Genistein		Daidzein		Daidzein+Equol	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
C0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ND		ND		ND	
C10	157.6	16.8	900.6	95.9	106.8	20.8	529.5	312.6	339.9	113.7	0.68	0.10	0.37	0.11	0.98	0.33
C50	599.0	61.1	3,414.6	348.1	319.3	51.6	2,538.8	619.0	1,758.4	261.8	0.54	0.09	0.52	0.09	1.26	0.19
D0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ND		ND		ND	
D10	123.9	12.9	707.9	73.7	104.6	25.1	510.2	288.2	388.5	80.1	0.86	0.23	0.56	0.13	1.28	0.43
D50	505.6	74.0	2,882.2	421.7	265.3	115.6	1,882.4	875.8	1,505.4	681.8	0.52	0.22	0.52	0.24	1.18	0.43

C0; control group, C10; soya hypocotyl tea (SHT) low dose, C50; SHT high dose, D0; iron deficient group (ID), D10; ID+SHT low dose.

D50; ID+SHT high dose.

ND; not done.

showed that the only significant contribution to the body weight was the total diet intake ($\text{Beta}=0.847$, $p=0.000$), while beta of ferritin was -0.104 ($p=0.133$), IF intake was 0.029 ($p=0.664$), and the total amount of drink was 0.127 ($p=0.169$).

2. Intake and excretion of isoflavones in the urine

The cumulative amount of IF intake was calculated from the consumption and content of IFs in SHT (Table 1). The C10 group consumed 8,000 mg IFs, whereas C50 consumed 36,000 mg IFs,

D10 consumed 6,900 mg IFs and D50 consumed 32,000 mg. The excreted amounts of IFs in the urine are shown in Table 3. The recovery ratio (urinary excretion for 3 days/intake for 3 days) of was higher in genistein than daidzein, but if daidzein metabolite, equol, was added to daidzein, it became 1.17. The daidzein to genistein ratio in the recovery ratio was 0.6 in IF10 and 1.0 in IF50, respectively. In the IF10 group, the daidzein to genistein ratio in the urine was about 3, and in IF50 it was about 5. The equol concentration in the urine was high in both the C and FeD

Table 4 Serum lipids by subgroup

	Triglyceride		Free fatty acid		Total Cholesterol		HDL-cholesterol		Esterified cholesterol		Free cholesterol		Phospholipid		Beta-Lipoprotein	
	(mg/dl)		(μEq/l)		(mg/dl)		(mg/dl)		(mg/dl)		(mg/dl)		(mg/dl)		(mg/dl)	
group	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
C0	107.4 ^a	10.4	1,498.3	149.5	97.3 ^{a,b}	11.3	33.7 ^{a,b,c}	4.5	73.1 ^{a,b,c}	8.2	24.1	3.3	154.1 ^a	13.4	250.6 ^a	53.3
C10	95.3 ^b	13.9	1,352.4	149.3	97.4 ^{c,d}	8.3	30.7 ^{d,e,f}	4.2	74.5 ^{d,e,f}	8.0	22.9 ^a	2.8	150.2 ^b	15.9	240.9 ^b	32.8
C50	105.9 ^c	15.6	1,361.1	128.8	103.6 ^{e,f}	15.1	35.8 ^{g,h,i}	2.4	83.8 ^{g,h,i}	22.5	23.7 ^b	3.4	150.9 ^c	14.5	320.5 ^c	46.4
D0	353.6 ^{a,b,c,d,e}	289.2	1,550.0	327.7	91.7 ^g	32.2	20.4 ^{a,d,g}	6.5	52.9 ^{a,d,g}	9.0	38.7 ^{a,b,c,d}	28.2	207.3 ^{a,b,c,d,e}	92.2	928.5 ^{a,b,c,d}	798.7
D10	166.8 ^d	79.6	1,490.5	398.5	71.1 ^{a,c,e,g}	4.8	17.6 ^{b,e,h}	5.2	49.5 ^{b,e,h}	5.0	21.6 ^c	5.6	150.9 ^d	20.1	514.3 ^d	210.7
D50	151.3 ^e	58.9	1,317.4	137.4	74.1 ^{b,d,f}	4.4	18.4 ^{c,f,i}	5.2	56.4 ^{c,f,i}	9.7	22.0 ^d	3.3	145.2 ^e	5.5	548.4	128.5

C0; control group, C10; soya hypocotyl tea (SHT) low dose, C50; SHT high dose, D0; iron deficient group (ID), D10; ID+SHT low dose, D50; ID+SHT high dose.

Triglyceride ($p=0.011$), free fatty acid ($p=0.481$), total cholesterol ($p=0.04$), HDL-cholesterol ($p=0.000$), esterified cholesterol ($p=0.000$), free cholesterol ($p=0.141$), Phospholipid ($p=0.004$), Beta-Lipoprotein ($p=0.013$) shows significant differences among the groups by ANOVA.

Subscripts in the columns show significant differences ($p<0.05$) by ANOVA between the subscript letters.

groups. The equol:daidzein ratio was about 1.5 in the C group, and 1.3 in FeD group. The ratio of daidzein+equol:genistein was about 8 in the C group and 13 in FeD group. Daidzein to genistein ratio in the drinking amount for the corresponding 3 days was 5.7.

3. Effects of SHT on lipid metabolism

Serum triacylglycerol was significantly higher in the FeD group than in Controls (353 ± 118 vs. 107 ± 4 mg/dl) (Table 4, Fig. 2). Among the FeD group, IF administration showed a decreasing trend in the TG level. The median of D0, D10 and D50 was 200, 140.2 and 128.7 mg/dl, respectively. V-LDL was also high in the FeD group, but IF50 appeared to decrease the serum level. Beta-lipoprotein, which corresponded to the LDL+VLDL, also increased in FeD, but the IF tea appeared to lower the value, although the reverse tendency was found in the control group. The free fatty acid of C0 and D0 was 1.4 and 1.5, respectively, and it appeared to decrease slightly by SHT.

In contrast, the total cholesterol level of the FeD group was lower than that of the control group. There was no significant difference in the free cholesterol levels among the subgroups, but esterified cholesterol was lower in the FeD group. The phospholipid level did not differ significantly among the subgroups.

4. Effects of SHT on hydroperoxide production

PCOOH and PEOOH of RBC did not show a difference among the subgroups. However, PCOOH and PEOOH in the liver were low in the C50 and D50 subgroups (Table 5, Fig. 3).

5. Effect of SHT on oh⁸dG production

oh⁸dG production in the liver was higher in D0 compared with C0, and it became low in D10. There was no effect of SHT in the control group, rather C10 showed higher oh⁸dG production. oh⁸dG in the kidney showed a similarly decreasing trend in both the control and the FeD group, and the decreasing trend was more obvious in the FeD group. oh⁸dG in urine did not show a significant difference in the control group, but an increase was observed in D50 (Fig. 4).

6. Correlation among SHT drinks, hydroperoxides and oh⁸dG

The concentration of PCOOH and PEOOH from RBC (Pearson's $CC=0.85^{**}$) and from the liver (Pearson's $CC=0.475^{**}$) showed significant correlation, respectively. Amount of hydroperoxides from the RBC and the liver, however, did not show a significant correlations (Table 6). oh⁸dG of the kidney correlated with body weight (Pearson's $CC=0.391^*$), total drink (Pearson's

$CC=0.387^*$) and total diet (Pearson's $CC=0.354^*$). It also correlated with total cholesterol, free cholesterol, and phospholipid, (Pearson's $CC=0.354^*$). Urinary oh⁸dG significantly correlated with the cumulative amount of daidzein intake (Pearson's $CC=0.497^{**}$), genistein intake (Pearson's $CC=0.492^{**}$) and total

Fig. 2 Serum triacylglycerol and total cholesterol concentration by subgroup. The FeD group showed increased TG and decreased total cholesterol. Upper margin of the box shows 75th percentile, and the lower margin shows 25th percentile. Bar in the box shows median value. Length of the bar shows 1.5-fold the length of the box, and a star shows a sample greater than 3-fold the length. Box plot figure.

Table 5a Serum anti-auto-oxidation, production of hydroperoxides by subgroup

group	Anti-auto-oxidation			PCOOH			PEOOH			PCOOH			PEOOH		
	(%)			(pmol/ml packed RBC)			(pmol/ml packed RBC)			(pmol/g liver)			(pmol/g liver)		
	mean	sd	median	mean	sd	median	mean	sd	median	mean	sd	median	mean	sd	median
C0	65 ^a	5	62	145	87	118	145	214	49	1,218	470	1,159	118 ^a	80	126
C10	58 ^{b,c}	12	56	355	338	268	197	274	91	1,872 ^{a,b}	842	1,690	296 ^{a,b,c,d}	193	231
C50	64 ^{d,e}	7	66	412	602	157	192	359	56	810 ^a	340	833	82 ^{b,e}	60	57
D0	77 ^{a,b,d}	18	85	171	101	135	98	81	62	2,023 ^c	1,174	1,868	72 ^{c,f}	43	69
D10	70	26	79	167	42	163	42	24	36	1,562 ^d	932	1,316	211 ^{e,f,g}	81	231
D50	81 ^{c,e}	3	82	223	147	157	87	62	60	700 ^{b,c,d}	299	614	90 ^{d,g}	51	99

C0; control group, C10; soya hypocotyl tea (SHT) low dose, C50; SHT high dose, D0; iron deficient group (ID), D10; ID+SHT low dose, D50; ID+SHT high dose.

Anti-auto-oxidation ($p=0.032$), PCOOH in the liver ($p=0.015$) and PEOOH ($p=0.002$) in the liver show significant differences among the groups by ANOVA. Subscripts in the columns show significant differences ($p<0.05$) by ANOVA between the subscript letters.

Table 5b DNA adduct by subgroup

group	liver oh ⁸ dG			kidney oh ⁸ dG			urinary oh ⁸ dG		
	(oh ⁸ dG/10 ⁵ dG)			(oh ⁸ dG/10 ⁵ dG)			(ng/mg creatinine)		
	mean	sd	median	mean	sd	median	mean	sd	median
C0	1.02 ^a	0.18	0.97	2.74 ^a	1.44	2.79	0.83 ^a	0.21	0.79
C10	1.49	0.34	1.48	3.38 ^{b,c}	2.90	2.43	1.04 ^b	0.09	1.06
C50	1.07	0.82	1.08	1.97	0.80	1.78	0.91 ^c	0.12	0.89
D0	1.57 ^b	0.57	1.78	2.79 ^d	1.43	2.34	0.35 ^d	0.12	0.29
D10	0.63 ^{a,b,c}	0.19	0.69	1.17 ^b	0.42	1.17	0.60 ^e	0.21	0.60
D50	1.23 ^c	0.43	1.21	0.94 ^{a,c,d}	0.93	0.68	3.02 ^{a,b,c,d,e}	1.70	2.75

C0; control group, C10; soya hypocotyl tea (SHT) low dose, C50; SHT high dose, D0; iron deficient group (ID), D10; ID+SHT low dose, D50; ID+SHT high dose.

Liver oh⁸dG ($p=0.013$), kidney oh⁸dG ($p=0.037$), urinary oh⁸dG ($p=0.000$) show significant differences among the groups by ANOVA.

Subscripts in the columns show significant differences ($p<0.05$) by ANOVA between the subscript letters.

IF intake (Pearson's $CC=0.498^{**}$). Although the hydroperoxides of the kidney were not measured, liver PCOOH negatively associated with the urinary oh⁸dG (Pearson's $CC=-0.341^{*}$). The ferritin and hemoglobin levels did not show any significant correlation with either the hydroperoxides or oh⁸dG level.

7. Histological and immunohistochemical examination

Histology: Rats fed iron deficient diets showed focal fatty infiltration in the liver, and slightly increased hematopoiesis in the spleen, but lymphoid tissues were generally well preserved. Epithelial cells of the renal tubules showed slightly granular cytoplasm, but hyaline droplets were not present. The adrenals showed normal appearance. The heart muscles were slightly hypertrophic. The pancreas, and other organs looked normal.

oh⁸dG staining: oh⁸dG was better stained in the methanol fixed tissues. In general, nuclei of smooth muscle cells of the artery were well stained. In the liver, the nuclei of the bile ducts were strongly stained (Fig. 5a). Hepatic cell nuclei were weakly stained, but Kupffer cells were not stained. Endothelial cell nuclei of the portal vein were moderately stained, but staining of the nuclei of the central vein was weak. In the kidney, nuclei of epithelial cells of the pelvis were strongly stained. Nuclei of glomerular epithelial cells and of proximal tubular nuclei showed focal staining (Fig. 5b). Nuclei of Henle's loop showed moderate to weak staining. In the FeD group, more diffusely stained nuclei of the tubular epithelial nuclei were found as well as faintly stained Henle's loop. Nuclei of transitional cells of the bladder were positively stained, but more strongly in prostatic cell nuclei. In the testes, nuclei of the basal layer spermatogonia were stained in addition to or similar to nuclei of mesothelial cells. In the heart, nuclei of cardiac muscles were positively

stained, and the cytoplasm showed weak granular staining. In the spleen, all nuclei of spleen cells, such as lymphocytes, myeloid cells, and megakaryocytes, were moderately to weakly stained. In the FeD group, staining of the liver, testes, heart, and spleen were similar to controls, although it was slightly faint. Each SHT subgroup showed similar staining patterns to their corresponding group.

Discussion

The presence of polyphenol groups in IFs suggests their anti-oxidative effects, because antioxidant activity of flavonoids, such as quercetin and kaempferol, are well known. However, antioxidant activity of IFs has not been confirmed in an *in vivo* system. In addition, large amounts of genistein and daidzein intake required checking for unpredictable side effects before human usage as supplements.

Iron deficiency was usually considered to decrease the production of free radicals, because of the lowered Fenton reaction rate. Uehara et al. (9), however, reported that iron deficiency increased lipid hydroperoxide formation in both liver and serum, probably due to the substitution of copper to iron, which compensated the process of the Fenton reaction. In the present study, iron-deficient rats increased their serum antioxidation level as expected. Plasma proteins appeared to stabilize the plasma redox system. FeD showed many alterations in the lipid metabolism; e.g. increased triacylglycerol and lowered cholesterol level. These were influenced by the complicated oxidation-reduction response among these lipid components. Iron-deficient rats showed increased XOD activity without a significant change in SOD, and as a result, increased PCOOH in the serum and the liver (9). SHT

Fig. 3 PCOOH in the red blood cells and liver in each subgroup. Box plot figure.

Fig. 4 oh⁸dG in the kidney and urine by subgroup. Box plot figure.

Table 6 Correlation Coefficient between IFs, hydroperoxides, and oh⁸dG in various tissues

		nmole/d intake		nmole/d urine			pmol/ml packed RBC		pmol/g tissue		oh ⁸ dG/10 ⁵ dG		ng/mg cr
		Genistein	Daidzein	Genistein	Daidzein	Equol	PCOOH	PEOOH	PCOOH	PEOOH	oh ⁸ dG liver	oh ⁸ dG kidney	oh ⁸ dG urine
Genistein (intake)	Pearson's CC	1.000	1.000**	0.921**	0.923**	0.909**	0.183	0.033	-0.453**	-0.210	-0.009	-0.291	0.469**
	p		0.000	0.000	0.000	0.000	0.270	0.843	0.005	0.212	0.957	0.077	0.003
Daidzein (intake)	Pearson's CC	1.000**	1.000	0.921**	0.923**	0.909**	0.183	0.033	-0.453**	-0.210	-0.009	-0.291	0.469**
	p	0.000		0.000	0.000	0.000	0.270	0.842	0.005	0.213	0.957	0.077	0.003
Genistein (urine)	Pearson's CC	0.921**	0.921**	1.000	0.984**	0.845**	0.242	0.017	-0.396*	-0.148	-0.038	-0.271	0.343*
	p	0.000	0.000		0.000	0.000	0.143	0.919	0.015	0.383	0.820	0.100	0.035
Equol (urine)	Pearson's CC	0.909**	0.909**	0.845**	0.842**	1.000	0.085	-0.016	-0.403*	-0.104	-0.066	-0.336	0.332*
	p	0.000	0.000	0.000	0.000		0.610	0.925	0.013	0.539	0.694	0.039	0.042
Daidzein (urine)	Pearson's CC	0.923**	0.923**	0.984**	1.000	0.842**	0.204	0.000	-0.424**	-0.245	-0.039	-0.273	0.395*
	p	0.000	0.000	0.000		0.000	0.218	0.998	0.009	0.144	0.818	0.098	0.014
PCOOH (RBC)	Pearson's CC	0.183	0.183	0.242	0.204	0.085	1.000	0.850**	0.042	0.123	0.187	0.257	-0.008
	p	0.270	0.270	0.143	0.218	0.610		0.000	0.803	0.468	0.260	0.119	0.962
PEOOH (RBC)	Pearson's CC	0.033	0.033	0.017	0.000	-0.016	0.850**	1.000	0.043	0.157	0.218	0.306	0.036
	p	0.843	0.842	0.919	0.998	0.925	0.000		0.801	0.354	0.188	0.062	0.831
PCOOH (liver)	Pearson's CC	-0.453**	-0.453**	-0.396*	-0.424**	-0.403*	0.042	0.043	1.000	0.475**	0.217	0.171	-0.345*
	p	0.005	0.005	0.015	0.009	0.013	0.803	0.801		0.003	0.197	0.312	0.036
PEOOH (liver)	Pearson's CC	-0.210	-0.210	-0.147	-0.245	-0.104	0.123	0.157	0.475**	1.000	0.013	0.132	-0.162
	p	0.212	0.213	0.383	0.144	0.539	0.468	0.354	0.003		0.938	0.438	0.337
oh ⁸ dG (liver)	Pearson's CC	-0.009	-0.009	-0.038	-0.039	-0.066	0.187	0.218	0.217	0.013	1.000	0.265	0.129
	p	0.957	0.957	0.820	0.818	0.694	0.260	0.188	0.197	0.938		0.108	0.442
oh ⁸ dG (kidney)	Pearson's CC	-0.291	-0.291	-0.271	-0.273	-0.336*	0.257	0.306	0.171	0.132	0.265	1.000	-0.173
	p	0.077	0.077	0.100	0.098	0.039	0.119	0.062	0.312	0.438	0.108		0.298
oh ⁸ dG (urine)	Pearson's CC	0.469**	0.469**	0.343*	0.395*	0.332*	-0.008	0.036	-0.354*	-0.162	0.129	-0.173	1.000
	p	0.003	0.003	0.035	0.014	0.042	0.962	0.831	0.036	0.337	0.442	0.298	

** : Pearson's correlation coefficient is significant at the 1% level (two sided)

* : Pearson's correlation coefficient is significant at the 5% level (two sided)

Fig. 5 Immunohistochemical localization of oh⁸dG in the liver and the kidney. (Left) Bile nuclei and cytoplasm of the bile canaliculi in the liver are strongly stained, in addition to the granular staining of the liver cell nuclei. (Right) The nuclei of the distal tubules in the kidney were strongly stained, in addition to the weak stain of the nuclei and cytoplasm of proximal tubules and glomerular mesangial cell nuclei. in the liver, and proximal tubules in the kidney are strongly stained. Lower pictures are control stains of the liver and kidney without anti-oh⁸dG monoclonal antibody.

did not cause significant change in lipid metabolism, except for a positive association with LDL+VLDL and a marginal reverse association with free fatty acids. The dose-dependent inhibitory effect of SHT was not significant in the production of lipid hydroperoxides. Absorption of IFs appeared to be changed in FeD group compared with the control.

The excreted amount did not significantly differ between the C10 and D10 groups, but D50 showed lower excretion than C50. Equol production was high in rats, but appeared to be low in the

FeD group. These values, however, were much higher than that of humans (20). Large volumes of the cecum may increase the absorption of IFs, and intestinal bacterial flora may be affected by the iron deficient condition. Further studies are necessary in this regard.

oh⁸dG was one of the DNA adducts which reflected oxidative DNA damage (21). IFs in SHT appeared to act as scavengers of free radicals with resultant decreased production of oh⁸dG in the tissues. By biochemical analyses, liver was the main target for

measuring oh⁸dG (13). The amount of oh⁸dG in the liver decreased as the intake of SHT increased. We did not measure hydroperoxides in the kidney, so the association between PCOOH and oh⁸dG could not be directly compared. We found characteristic localization of oh⁸dG in the liver and kidney. The epithelial cells in the proximal convoluted tubules were stained deeper than distal tubules in the kidney. This suggested that oh⁸dG could be excreted from these tubules.

A reverse association between PCOOH in the kidney and the amount of oh⁸dG in the urine suggested that the urinary excretion was an important route to discard DNA adducts. Enzyme-histochemical localization of oh⁸dG in the epithelial cells in the proximal convoluted tubules in the kidney coincided well with the above findings. Or, if oh⁸dG filtered at the glomeruli, reabsorption at the proximal tubules might be affected by IFs in the urine. It is interesting that the epithelial cells in the intrahepatic bile duct were also well stained. This suggested that the bile system appeared to be another important pathway to discard damaged

DNA adducts into the feces.

In this experiment, antioxidant activity of SHT was confirmed. DNA adduct, oh⁸dG, in the liver and kidney decreased by the intake of IFs, but the relation was stronger in the kidney. These findings suggest that the hypocotyl supplement could be available for the prevention of cancer and other life-style related diseases (22, 23).

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