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• *RAPID COMMUNICATION* •

Effects of fermented soy milk on the liver lipids under oxidative stress

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Abstract

AIM: To investigate the effects of fermented soy milk powder on the antioxidative status and lipid metabolism in the livers of CCl_4-injected rats.

METHODS: Forty-five healthy male Sprague-Dawley rats were randomly assigned to five groups according to five different diets: control (AIN-76), AIN-76+high-dose fermented soy milk powder, AIN-76+low-dose fermented soy milk powder, AIN-76+high-dose milk yogurt powder and AIN-76+low-dose milk yogurt powder. The experiment lasted for 8 wk. After 4 wk, all the rats received intraperitoneal administration of CCl_4 (0.2 mL/100 g body weight) every week. Total cholesterol (TC), triglyceride (TG), TBARS, ALP, and antioxidative enzymes in the liver were evaluated.

RESULTS: There was also no significant difference in TBARS and antioxidative enzymes in the liver. TC and TG in the groups fed with fermented soy milk powder were generally lower than those fed with casein powder.

CONCLUSION: Consumption of fermented soy milk was positive in lowering total cholesterol and TG accumulation in the liver under CCl_4-induced oxidative stress.

Key words: Soy; Fermented soy milk; Antioxidative; Liver protection

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INTRODUCTION

Soybeans contain valuable nutritional attributes. It has been found that the intake of soy foods is closely related to lowering the occurrences of chronic diseases[1]. There are many functional ingredients contained in soy foods such as soy protein, isoflavones, saponins, phytic acid, phytosterol, and phenolic acid[2-4]. Compared to casein, soy protein showed a greater antioxidative ability in preventing lipid oxidation[5]. Isoflavones have been found to increase the activities of some antioxidative enzymes in the liver[6]. Soy foods in Oriental countries can be divided into two categories: unfermented and fermented products. Fermented soy products have been greatly researched recently because their nutritional attributes may be changed due to the metabolism of microorganisms. Fermented soy milk, unlike fermented milk or yogurt drinks, contains no lactose or cholesterol and may have the health benefits from both soy itself and the fermentation. Cheng *et al*[7] suggested that consumption of fermented soy milk was beneficial to human intestinal health. Our purpose was to investigate the effect of fermented soy milk on liver protection under induced oxidative stress.

MATERIALS AND METHODS

Fermented soy milk

Fermented soy milk was provided by Taiwan Tobacco & Liquor Cooperation (TTL, Taipei, Taiwan, China) Spray drying was applied to produce fermented soy milk powder.

Animals

Forty-five 6-wk-old healthy male Sprague-Dawley (SD) rats (National Laboratory Animal Center, Taiwan, China) were randomly assigned to five groups according to different diets. Guidelines for the ethical care and treatment of animals from the Animal Care Committee at Taipei Medical University were strictly followed. Rats were individually housed and maintained in a temperature-controlled (23±2 ℃) room with a 12-h light/dark cycle. They were fed a chow diet for 1 wk before switching to the experimental diet. Five rats were killed to acquire the baseline biological values of liver. Water and food were available *ad libitum*.

Treatment and sample collection

Fasting blood samples from the tail vein were collected in tubes containing heparin on the last day of wk 0, 2, 4, and 6.

Table 1 Composition of different diets (g/25 g)[1]

	CC[2]	HCS	LCS	HCM	LCM
Corn starch	13.2	4.8	9.0	6.7	9.8
Casein	5.0	4.4	4.7	2.1	4.0
Cellulose	1.6	1.1	1.4	1.1	1.4
Soybean oil	1.5	1.5	1.5	1.5	1.5
Mineral mixture	1.5	0.9	1.2	0.9	1.2
Sucrose	1.5	0.9	1.2	0.9	1.2
Vitamin mixture	0.5	0.3	0.4	0.3	0.4
Methionine	0.1	0.1	0.1	0.1	0.1
Fermented soy milk		10.0	5.0		
Milk yogurt				10.0	5.0

[1]Diets were iso-caloric; [2]CC, control group; HCS, high-dose fermented soy milk powder; LCS, low-dose fermented soy milk powder; HCM, high-dose fermented milk powder; LCM, low-dose fermented milk powder.

The blood was then centrifuged for 15 min at 1 400 r/min to separate the plasma and erythrocytes and stored at -80 ℃. Starting from the 4th wk, rats received an abdominal injection of CCl_4 (0.2 mL/100 g body weight) once a week until the end of the experiment. The body weight of each rat was recorded every 2 d. At the end of wk 8, rats were killed, and liver samples were collected, weighed, and stored at -80 ℃.

Diets

The diets were iso-energetically formulated (Table 1). The control diet was AIN76 (CC). Four experimental diets were prepared by mixing 10 g (HCS), 5 g of fermented soy milk powder (LCS), 10 g (HCM), and 5 g of milk yogurt powder (LCM).

Preparation of liver homogenates and lipid extraction

For preparing liver homogenate, 1.5 g of liver samples was mixed with 2.5 mL of buffer (0.25 mol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl, pH 7.4) and blended at 4 ℃ (avoiding light), followed by centrifugation at 4 500 r/min for 6 min. The isolated liver cells were stored at -80 ℃.

For extracting liver lipid, 1 g of the liver samples was mixed well with 12 mL of chloroform/methanol (2:1) and then filtered. The filtrate was evenly distributed into two test tubes and chloroform/methanol/H_2O (3:48:47) was added to each tube to 10 mL. Then 2 mL of 0.05% $CaCl_2$ was added to each tube, followed by degassing for 15 min and centrifugation at 2 500 r/min and 4 ℃ for 3 min. The supernatant was removed and methanol was added to the sample to 10 mL. The sample in each tube was then mixed together, to which chloroform/methanol (2:1) was added to 25 mL.

Alkaline phosphatase (ALP) determinations

ALP was evaluated using commercial kits (ALP reagent L-type, Wako).

Activities of antioxidant enzymes in the liver

The activities of antioxidative enzymes were determined using commercial kits: superoxide dismutase (SOD, Calbiochem), glutathione peroxidase (GPx, Randox), and glutathione reductase (GR, Randox).

Table 2 Body weights of each group fed with different diets (mean±SD, g)

	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8
	(Before injection of CCl_4)			(After injection of CCl_4)	
CC[1]	223.6±13.61	267.4±16.51	317.0±30.2	371.9±36.5	402.1±25.2
HCS	237.7±11.81	276.5±11.11	338.7±14.11	395.3±17.91	429.0±27.01
LCS	231.4±14.31	272.1±11.01	329.2±9.41	395.0±17.91	435.4±26.71
HCM	231.6±9.71	264.7±9.41	314.3±11.0	367.4±12.82	403.1±10.3
LCM	225.2±10.8	260.6±14.3	309.9±19.9	364.5±17.02	405.3±17.4

[1]CC, HCS, LCS, HCM, LCM, as in Table 1. n = 7-9.

Table 3 Plasma ALP concentration (IU/dL) of each group fed with different diets[1]

	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8
	(Before injection of CCl_4)			(After injection of CCl_4)	
CC	33.8±0.0	51.3±9.5	43.8±14.0	194.4±65.6	303.7±119.9
HCS	50.2±11.1	70.5±8.9	57.8±6.6	472.6±235.1	338.6±130.3
LCS	49.9±21.3	64.2±4.4	47.1±3.6	278.6±205.7	360.7±206.1
HCM	35.1±6.9	56.4±4.9	39.2±6.7	577.8±74.2	548.0±150.8
LCM	37.7±6.8	57.3±8.0	43.0±3.5	255.1±170.3	592.8±115.6

Thiobarbituric acid reactive substances (TBARS)

A blank (0.1 mL of water), a standard (0.1 mL of 4.2 μmol/L malonaldehyde), or 0.1 mL of the sample was mixed well with the reagent (a mixture of 4 mL of 22% sulfuric acid, 0.5 mL of 10% phosphotungstic acid, and 1 mL of 0.67% 2-thiobarbituric acid). Then the mixture was heated in a water bath to 95 ℃ for 1 h. After being cooled with ice water, 2 mL of butanol was added to the sample, followed by centrifugation at 3 000 r/min for 15 min. The optical density of the pinkish upper part was determined using a fluorescence spectrometer with excitation at 515 nm and emission at 555 nm.

Statistical analysis

All the results were presented as mean±SD. Two-way analysis of variance (ANOVA) and the least significant difference test were performed using SAS® 6.13 to analyze the time and diet effects.

RESULTS

Body weights of the animals

During the feeding period, body weights in each group of all the rats increased (Table 2). At the end of the feeding period, the body weights of rats fed with fermented soy milk powder (HCS and LCS) were significantly higher than those in the other groups (P<0.05). None of the rats showed any abnormal condition.

Alkaline phosphatase (ALP) determinations

ALP activity in the plasma was used to evaluate the liver function. Before injection (i.e. before wk 4) of CCl_4, the ALP (Table 3) in any group did not significantly differ. After injecting CCl_4, ALP activities in all the groups increased (P<0.05). HCS and HCM had higher ALP activities than did the other groups. After wk 6, the ALP activities in the HCS and HCM groups had significantly decreased (P<0.05). In wk 8, the ALP activity in the HCS

Table 4 Activities of antioxidative enzymes and concentrations of TBARS, TC, and TG of the liver in CCl_4-treated rats[1] (mean±SD)

	Baseline	CC[1]	HCS	LCS	HCM	LCM
CAT[2]	0.92±0.19	0.25±0.14	0.30±0.123	0.45±0.25	0.47±0.16	0.44±0.27
GPx	703±154	615±166	680±141	857±179	734±94	762±120
GR	613±288	591±160	768±94	906.4±158	772±283	668±125
SOD	0.118±0.035	0.094±0.015	0.082±0.007	0.088±0.026	0.111±0.035	0.084±0.024
TBARS	6.32±0.71	5.53±1.00	6.08±0.77	4.79±0.67	6.24±0.97	5.92±0.97
TC	5.54±0.73	6.69±2.77	5.37±2.71	4.16±1.55	7.07±2.83	7.06±2.85
TG	12.82±0.69	15.86±3.93	18.633±5.62	16.76±0.067	19.60±0.79	20.62±5.21

group had decreased even lower than those in the HCM group ($P<0.05$).

Antioxidative enzymes, TBARS, TC, and TG in the liver (Table 4)

Results showed that at the end of the feeding period, the catalase activity in the liver in all the groups decreased ($P<0.05$), while the activities in CC and HCS rats were significantly lower than those in the other groups ($P<0.05$). The activity of GPx in all the groups did not significantly change through the experiment. The activity of GR in the LCS group significantly increased at the end and was higher than those in the other groups ($P<0.05$), while those in the other groups did not differ from each other and from the baseline. The activities of SOD in all the groups decreased at the end but only those in the HCS and LCS groups were significantly lower than that in the baseline ($P<0.05$).

At the end of feeding period, the TABRS in all the groups did not differ from each other but tended to be lower or significantly lower (CC, LCS, and LCM; $P<0.05$) than that in the baseline. Rats in the LCS and HCS groups had lower TC value than did those in the HCM, LCM, and control groups ($P<0.05$). Rats in the LCS and HCS groups tended to have lower TG than did those in the HCM and LCM groups.

DISCUSSION

The isoflavone contents of HCS and LCS groups were 0.97 and 0.47 mg/d, respectively in our experiment. Converting the amounts for rats to that for a 70-kg adult, values were about 54 and 27 mg of isoflavones daily although the isoflavone contents in the plasma were either trace or undetectable. It has been pointed out that a daily intake of 50 mg of isoflavones is beneficial to health[8]. The reason why the isoflavones were not detected was due to that all rats were fasted for 8 h before their blood samples were collected. It was found that daidzein and genistein in the plasma were undetectable in rats after 8 h of ingestion[9-11]. The time might have been too long for the isoflavones to be detected.

We found that under such doses of CCl_4, all rats in this experiment had similar activities of GPx and GR in the liver no matter if they had consumed fermented soy milk powder or not. This could be due to a relatively high dose of CCl_4. Zavate[12] pointed out that injecting a dose of 0.2 g/100 g BW of CCl_4 in rats resulted in decreases or deactivation in the activities of catalase, SOD, and GPx, and increases in lipid oxidation in the liver. In addition, the CCl_4 injection produced acute damage to the liver. Protection of the liver through food consumption may not be effective enough in a short period.

In the diets of HCS and LCS groups, we found 3-hydroxyanthranilic acid (3-HAA; data not shown). 3-HAA is a by-product of soy fermentation[13]. It has been found as effective as α-tocopherol and was more effective than genistein[14,15] in inhibiting lipid oxidation *in vivo*. The effect of 3-HAA on protecting liver lipids from oxidation needs further research. Other than 3-HAA, many components in soy are antioxidative. Soy isoflavones, soy protein, and saponins all possess antioxidative abilities[16]. Our data did not clearly suggest that consumption of fermented soy powder was effective in reducing TBARS. This could be due to the limitation when applying TBARS to evaluate lipid oxidation in the liver. Massie *et al*[17] pointed out that the storage time of liver samples before measuring TBARS affected the results. In addition, the antioxidants such as BHT and EDTA added to the test tubes during the preparation of the liver homogenate also affected the outcomes[18]. In our experiment, addition of EDTA was included in the procedure. In addition, Morrow *et al*[19] found that under high oxidative stress, the oxidative damages to the liver resulted in an increase in F2-isoprostane (F2-IsoPs) which could not be evaluated with TBARS method.

Our result suggested that consumption of fermented soy milk powder slightly reduced the accumulation of TG in the liver caused by CCl_4. Pencil *et al*[20] indicated that CCl_4 inhibited the secretion of lipoproteins in the liver and altered the metabolism of fatty acids and resulted in fatty livers.

In conclusion, consumption of fermented soy milk was positive in lowering total cholesterol and TG accumulation in the liver under oxidative stress. Fermented soy milk drink is relatively new to the market. The results open an opportunity to further research on the amount and formula of fermented soy milk needed to achieve health benefits.

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