Red Clover (*Trifolium pratense* L.) Sprout Prevents Metabolic Syndrome

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Summary We examined the prevention effect of red clover (*Trifolium pratense* L.) sprout on metabolic syndrome using a high-carbohydrate and high-fat diet (Western diet; WD)-induced male C57BL/6J obese model mouse. Red clover sprout-lyophilized powder (RC) contained 3.5 mg/g dry-weight of formononetin as a major phenolic compound, as analyzed by high performance liquid chromatography. Supplementation of 0.3% (w/w) RC in a WD (WD+RC) showed an anti-obesity effect and ameliorated lipid metabolism in the obese model mice. Additionally, fasting plasma glucose levels were significantly reduced in the WD+RC group. Administration of 0.1 mg/kg formononetin reduced the postprandial blood glucose level, as assessed using the oral maltose tolerance test. However, no significant formononetin intake effect was observed on the plasma insulin level. These results suggest that the formononetin contained in red clover sprout inhibits α -glucosidase and thereby contributes to reducing the postprandial blood glucose response in mice.

Key Words red clover sprout, anti-metabolic syndrome, formononetin, postprandial blood glucose, α -glucosidase inhibitor

Red clover (*Trifolium pratense* L.) is a leguminous plant that is native to Europe and Asia, and it is also a herbal plant (1). Although the market size is small, red clover sprouts are also used as a vegetable, such as in salads. Red clover is known to be rich in isoflavonoids such as formononetin and biochanin A (2, 3), which is considered to be the reason for its usefulness as an herb.

Metabolic syndrome is set as a group of several characteristics caused by hypernutrition, a lack of exercise, and abdominal fat accumulation, and the prevalence is rising throughout the world (4, 5). Recently, in vivo experiments using C57BL/6 mice and 0.1 to 10 mg/kg/d of formononetin added to a high-fat diet for 12 wk resulted in weight gain, visceral fat accumulation, hyperlipidemia, and bone density loss. Formononetin also had a strong effect on preventing obesity and bone loss caused by the high fat diet (6).

In this study, we examined the effect of red clover sprout intake on metabolic syndrome prevention using a high-fat and high-carbohydrate diet (Western diet; WD)-induced obese model mouse. We also found that red clover sprout has blood glucose lowering effect, and that this effect may be due to formononetin in red clover.

MATERIALS AND METHODS

Materials. Red clover seed was purchased from Suba Seeds Co. S.p.A. (Longiano, Italy). Red clover sprouts

were germinated for 5 d, and red clover sprout-lyophilized powder (RC) was prepared as described below. Briefly, after freezing at -60°C , red clover sprout was lyophilized for 16 h using a FDU-1200 freeze dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The dried standard product was crushed using a Vita-Mix Absolute Mill (Osaka Chemical Co., Ltd., Osaka, Japan) at magnitude 6 for 3 min, and this was used as RC. The feed used in this study was CRF-1 (commercial diet), AIN93G, or WD, which was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). AIN93G and WD ingredients are shown in Table 1. Formononetin and biochanin A was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

Isoflavonoid determination. To measure isoflavonoids, 500 μL of 1 N HCl was added to 20 mg of the RC, and the mixture was stirred and then incubated at 98°C for 2 h (7). After neutralization with NaOH, it was lyophilized again. The dried product was suspended in 400 μL of 80% (v/v) methanol solution, stirred at room temperature for 5 min, and centrifuged at 15,000 rpm for 10 min at 10°C . Thereafter, the supernatant was filtered with Dismic 13HP020AN (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and high performance liquid chromatography (HPLC) was performed to quantify formononetin and biochanin A. The obtained polyphenol aglycones were analyzed using the Alliance HPLC system (Nihon Waters K.K., Tokyo, Japan), which was equipped with a Myghtysil RP-18 GPII column (5 μm , $4.6\phi\times 250$ mm, Kanto Chemical). The conditions were as follows: sol-

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vent A, 0.1% acetic acid in water; solvent B, 0.1% acetic acid in acetonitrile; gradient, 0–30 min for 25% B isocratic, 30–40 min for 25–70% B linear (curve number 6 in Waters Empower software), and 40–44 min for 70% B isocratic; flow rate, 1.0 mL/min; column temperature, 40°C; detection, UV at 260 nm; and injection volume, 10 μ L. The isoflavonoids were identified on chromatograms based on their retention time by comparison with the authentic compounds.

Animal experiments. Animal experiments were approved by the Kobe BM Laboratory Animal Welfare Committee in accordance with the “Law on the Protection and Management of Animals,” “The Ordinance on Protection and Management of Animals,” and “Guidelines on How to Dispose of Animals” (Permission nos. 17K010-4 and 17K010-5). Mice used in these experiments were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). The mice were housed

under the following conditions: room temperature, 23°C; and humidity, 55%. The light period was from 8:00–20:00, and the dark period was from 20:00–8:00.

Eight-week-old male C57BL/6J mice were randomly assigned to three groups (control, WD and WD+RD groups; six mice per group), housed individually, and fed ad libitum for 8 wk with AIN-93G (control group), WD (WD group), or 0.3% (w/w) RC in WD (WD+RC group). Mouse body weight and food consumption were monitored. After fasting for 16 h, mice were sacrificed under isoflurane anesthesia, and organ weights (epididymal white adipose tissue and liver) and plasma markers (triglyceride, total cholesterol, very low-density lipoprotein (VLDL) cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and blood glucose) were measured. Blood was collected into Capiject II tubes (with heparin lithium and plasma separating agent; Terumo Co., Ltd., Tokyo, Japan). After 30 min of inversion and mixing, the mixture was centrifuged at 5,000 rpm for 20 min under 4°C to collect plasma. Plasma lipoproteins were analyzed using an on-line dual enzymatic method for simultaneous quantitation of cholesterol and triglycerides by HPLC at Skylight Biotech (Akita, Japan), in accordance with the procedure described by Usui et al. (8). Plasma glucose was measured using a Glucose CII-Test Wako kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Oral sugar tolerance test. In the oral maltose tolerance test (OMTT), 12-wk-old male C57BL/6J mice (eight animals in each group) were acclimated to CRF-1, and after fasting for 16 h, a 15% (w/v) aqueous solution of maltose (control group) or a 0.01 mg/mL solution of formononetin in the maltose aqueous solution (+FMN group) was orally administered at a dose of 10 mL/kg body weight (0.1 mg/kg formononetin). Blood was collected 0 to 120 min after administration, and blood glucose levels were measured using GlutestAce R (Arkray Factory, Inc., Shiga, Japan). Plasma insulin was analyzed by an enzyme-linked immunosorbent assay at

Table 1. Experimental diet ingredients.

Constituent	Control (AIN-93G)	Western diet (WD)
Content (%)		
Corn starch	39.7486	13.5458
Casein	20	20
α -Corn starch	13.2	1.25
Sucrose	10	34
Soybean oil	7	1.15
Lard	0	20
Cellulose powder	5	5
AIN-93G mineral mix	3.5	3.5
AIN-93G vitamin mix	1	1
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
BHT	0.0014	0.0042
Total	100	100
Energy (kcal/g)	4.0	4.7

Fig. 1. High-performance liquid chromatography profile of red clover solution after acid degradation. Chemical structures of compounds corresponding to the peaks are shown.

Fig. 2. Feeding efficiency (A) and body weight gain (B) in mice during the 8-wk experimental period. Each value represents the mean $\pm$ SE ($n=6$). ** $p<0.01$ and * $p<0.05$ vs. control group.

Fig. 3. Liver (A) and epididymal white adipose tissue (EWAT) (B) weight comparison. Each value represents the mean $\pm$ SE ($n=6$). §§ $p<0.01$ vs. control group and ## $p<0.01$ vs. WD group.

Nagahama Lifescience Laboratory (Shiga, Japan). Based on the obtained data, the blood glucose area under the curve from 0 to 120 min (Δ AUC) and the difference between the maximum value and the initial value of blood glucose and plasma insulin concentration ($\Delta C_{\max}$) were calculated.

Statistics. Tukey–Kramer tests were performed using Microsoft Excel, with Statcel4 ver. 4 as an add-in software (The Publisher OMS Ltd., Saitama, Japan). Differences were considered significant at $p<0.05$.

RESULTS

After lyophilizing of 203.8 g of red clover sprout, 10.6 g of RC was obtained, and the moisture content

was calculated as 94.8%. HPLC analysis showed that formononetin is detected as a major peak in RC (Fig. 1), and its content was calculated as 3.5 mg/g dry weight (DW). The content of Biochanin A was 0.45 mg/g DW. Additionally, no other remarkable peaks were observed. These results showed that RC used in this study contained formononetin as a major phenolic compound.

First, we examined the anti-obesity effect of RC supplementation on high-carbohydrate and high-fat diet-induced obesity. Feeding efficiency was $2.7 \pm 0.21\%$ in the control group, and it was significantly higher in the WD groups. There was no difference between the WD group ($8.3 \pm 0.36\%$) and the WD+RC group ($7.0 \pm 0.65\%$) (Fig. 2A). Although there was no signifi-

Table 2. Plasma biochemistry.

Test group	Control	WD	WD+RC
TG	28.4±4.2	42.7±2.7 ^{\$}	28.8±2.9 [#]
Total-C	84.4±1.9	124.3±3.1 ^{\$\$}	100.2±2.6 ^{\$\$,##}
VLDL-C	3.17±0.36	4.15±0.22 ^{\$}	3.41±0.18
LDL-C	8.93±0.73	28.6±1.03 ^{\$\$}	20.3±1.12 ^{\$\$,##}
HDL-C	71.8±1.2	92.7±1.9 ^{\$\$}	73.6±1.9 ^{##}
L/H	0.124±0.01	0.295±0.01 ^{\$\$}	0.279±0.02 ^{\$\$}
FPG	165±10	213±5.4 ^{\$\$}	184±6.9 [#]

WD, Western diet; WD+RC, 0.3% (w/w) red clover containing WD.

^{\$\$} $p < 0.01$ and ^{\$} $p < 0.05$ vs. control; ^{##} $p < 0.01$ and [#] $p < 0.05$ vs. WD.

Each value represents the mean ± SE ($n = 6$).

TG, triglyceride; Total-C, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; L/H, LDL-C/HDL-C ratio; FPG, fasting plasma glucose.

Fig. 4. Fluctuation of the blood glucose level in the oral maltose tolerance test (OMTT). Each value represents the mean ± SE ($n = 8$).

cant change in body weight gain, a decreasing tendency was observed in the WD+RC group compared with the WD group (Fig. 2B). There was no significant difference in liver weight among the three groups (Fig. 3A). However, there was a marked difference in epididymal white adipose tissue (EWAT) in organ weight between WD and WD+RC (Fig. 3B). These results indicate that RC supplementation showed anti-obesity effects in a WD-induced obesity model mice.

Next, we analyzed a plasma marker that was mainly related to lipid metabolism (Table 2). In the WD+RC group, plasma triglyceride, total cholesterol, and LDL cholesterol levels were decreased compared with the WD group. However, there was no change in VLDL cholesterol levels, and no improvement in the low-density lipoprotein cholesterol/high-density lipoprotein cholesterol (L/H) ratio by lowering HDL cholesterol. Thus, RC supplementation ameliorated lipid metabolism effects in

Table 3. Aggregated value of OMTT.

		Control	+FMN
Blood glucose	Δ AUC (min*mg/dL)	8,140±698	6,320±364*
	$\Delta C_{\max}$ (mg/dL)	162±9.91	111±6.36***
Insulin	$\Delta C_{\max}$ (ng/mL)	0.12±0.075	0.356±0.089

*** $p < 0.001$ and * $p < 0.05$ vs. control.

Each value represents the mean ± SE ($n = 8$).

obesity model mice.

Fasting plasma glucose levels were significantly reduced in the WD+RC group compared with the WD group (Table 2). Additionally, the OMTT showed that formononetin suppresses the increase in postprandial blood glucose (Fig. 4). The aggregated data are presented in Table 3. A significant decrease in the blood glucose level was observed in the +FMN group compared to the control group for both Δ AUC and $\Delta C_{\max}$. However, $\Delta C_{\max}$ for the plasma insulin level showed only an increasing tendency ($p = 0.062$) in the +FMN group.

DISCUSSION

RC supplementation was shown to have anti-obesity and dyslipidemia ameliorating effects in WD-induced obesity model mice. Additionally, the main phenolic compound of RC was shown to be formononetin. There are various reports of bean components that are related to anti-obesity and anti-hypolipidemia (9), and it is natural to assign the cause of these RC effects to formononetin. The trends after RC administration seem to be similar, although there are some differences such as no change in VLDL cholesterol and low L/H efficacy compared to formononetin administration alone (6).

In this study, we focused on the plasma glucose lowering effect of RC. Dietary fiber, protein, phytic acid, and glutamine have been reported to be characteristic constituents of legumes that control postprandial blood glucose, and whole bean intake of 196–338 g/d has been proposed to be an effective serving size (10). The average daily food intake in mice (body weight, 30 g) is about 3 g/d. The RC used in this experiment was calculated as about 0.024 g/kg/d (or 1.5 g/d for a weight of 60 kg) based on the human equivalent dose (HED) (11). This is much lower than the previously proposed value, even considering the water content of legume beans (about 10% w/w). These findings suggest that other RC-specific components besides the well-known candidates, such as dietary fiber and protein (10), are involved in the glyce-mic response.

Recently, in vivo experiments using rats showed that intake of genistein (an isoflavonoid) suppresses elevated blood glucose levels via stimulating GLP-1-insulin signal (12). Clinical trials showed that intake of soy beverage stimulates insulin secretion and suppresses the rise in blood glucose levels (13), but the active component

has not been identified. Ma et al. (14) reported that formononetin does not affect blood glucose and insulin levels. Our OMTT results also support this previous result because no significant difference in the plasma insulin level was found between the control and +FMN groups. This suggests that absorbed endogenous formononetin does not lower blood sugar via increasing insulin levels.

Two working groups showed that formononetin is a potent α -glucosidase inhibitor in in vitro experiments (15, 16). The IC_{50} of formononetin was reported as 0.031 mM or 0.027 mM in these two studies, while that of acarbose was 0.382 mM (15). The OMTT showed the glucose absorption inhibitory effect by formononetin that was presented in these authors' reports.

These authors reported that the inhibitory effect on the increase in postprandial blood glucose largely depends upon inhibition of formononetin by α -glucosidase. Biochanin A is also a potent inhibitor ($IC_{50}=0.020$ mM) (15), but its contribution is considered to be minor in the RC because its content is low, at about one-eighth of that of formononetin in RC.

Thus, these results suggest that the ability of formononetin in red clover sprout to inhibit α -glucosidase might contribute to lowering blood glucose and to anti-obesity and ameliorating lipid metabolism. Red clover sprouts are, therefore, expected to be effective in preventing metabolic syndrome.

Formononetin drastically reduced postprandial blood glucose levels in the OMTT, but this reduction is considered to be because of the aglycone shape, which is rare in raw plants. In our experiments, the non-hydrolyzed RC contained formononetin aglycone at a concentration of 0.2 mg/g DW. It has been reported that some polyphenol glycosides become aglycones via digestive enzymes in the oral cavity (17, 18) or lactase phloridrin hydrolase in the small intestine epithelium (19). Some studies reported that formononetin glycosides (such as ononin) are present in red clover (2, 3), but their aglyconization has not been reported in vivo. In this study, we did not investigate the relationship between the aglyconization efficiency and α -glucosidase inhibitory activity of formononetin glycosides, which are derived from red clover, in the small intestine. Further research is required on this issue.

This study showed that 0.3% (w/w) of RC supplementation exhibits anti-obesity, plasma lipid-lowering, and glucose-lowering effects. It corresponds to a formononetin intake of about 1.05 mg/kg/d, and the HED was calculated to be 0.085 mg/kg/d. This supplementation also corresponds to a human (60 kg body weight) consumption of about 30 g of fresh red clover sprouts per day. This means that incorporation of red clover sprout as a food into a usual diet can prevent metabolic syndrome. Further research is expected in the future, including clinical trials.

Disclosure of state of COI

AH is an employee of Saladcosmo Co. Ltd. MN was an employee of Saladcosmo Co. Ltd. and the present affiliation is Yasaidekenkou R&D Co. Ltd. All the other authors

declared no competing interests.

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