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Hypocholesterolemic effect of daily fisetin supplementation in high fat fed Sprague–Dawley rats

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ABSTRACT

We aimed to test whether fisetin could modulate cholesterol homeostasis in rats with diet-induced hypercholesterolemia, and further investigated the underlying mechanisms by which fisetin exerts its cholesterol lowering effect. Blood lipid profile, hepatic cholesterol content, as well as gene expressions in cholesterol metabolism were examined. Elevated levels of total cholesterol and LDL-cholesterol, along with hepatic cholesterol content in a high fat group were found to be significantly reduced by fisetin. The high fat diet significantly decreased hepatic mRNA levels of LDLR, SREBP2, HMGCR and PCSK9 in comparison to the control diet, however, fisetin did not further elicit any changes in mRNA levels of the same genes. The high fat diet dramatically increased the transcript levels of CYP7A1, which was subsequently reversed by the fisetin. In HepG2 cells, fisetin was found to increase the levels of a nuclear form of SREBP2 and LDLR. In conclusion, fisetin supplementation displayed hypocholesterolemic effects by modulating the expression of genes associated with cholesterol and bile acid metabolism.

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1. Introduction

Epidemiological studies have shown that the consumption of diets rich in fruit and vegetables is associated with lower risks of many chronic diseases such as cardiovascular disease (CVD) and cancer, as well as reducing the risk of stroke (O'Byrne et al., 2002; Hertog et al., 1997; Geleijnse et al., 2002; Lee et al., 2011). It is mainly attributed to the antioxidant capacities derived from flavonoid contents in fruits and vegetables (Halliwell, 1994). Strawberries have been reported to have the highest total antioxidant activity among fruits (Wang et al., 1996), which is conferred by the wide variety of flavonoids contained therein (Hannum, 2004). In fact, strawberry consumption either as an individual component (Tsuda et al., 2004), or as whole strawberries (Prior et al., 2008), has been specifically implicated in the effects on risk factors for CVD. For example, several human studies have consistently reported the effects of strawberry consumption on the blood lipid profiles (Zunino et al., 2011; Basu et al., 2009; Jenkins et al., 2008), inflammation (Basu et al., 2010; Edirisinghe et al., 2011) and oxidative stress (Basu et al., 2009; Jenkins et al., 2008). Fisetin (Fig. 1) is one of the most predominant flavonols occurring in strawberries, its content being as high as 160 μg/g (Kimira et al., 1998). Despite the abundance of fisetin in strawberries, there is limited information on the potential metabolic effects of fisetin. A few studies have reported that fisetin could display hypoglycemic activities *in vitro* (Constantin et al., 2010) and in animals (Prasath and Subramanian, 2011a, 2011b). However, there was found to be no previously published data on the effects of fisetin on cholesterol metabolism.

In the present study, we aimed to test whether fisetin could modulate cholesterol homeostasis in rats with diet-induced hypercholesterolemia, and further investigated underlying mechanism by which fisetin exerts cholesterol lowering effect.

2. Materials and methods

2.1. Materials

Fisetin and BCA assay kit were purchased from Sigma–Aldrich (St. Louis, MO, USA). RNA lysis buffer and RNeasy Lipid Tissue Mini Kit for RNA extraction were purchased from Qiagen (Hilden, Germany). To perform the semi-quantitative RT-PCR, oligo-dT and Superscript™ II reverse transcriptase were used (Invitrogen, Carlsbad, CA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 100,000 units/L of penicillin and 100 mg/L of streptomycin were from Gibco-Invitrogen (Grand Island, NY). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for cell viability assay was purchased from Amresco (Solon, OH). For immunoblot analysis, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), enhanced chemiluminescence kit (Young In Frontier,

Abbreviations: CYP7A1, cytochrome P450 family 7 subfamily A polypeptide 1; FXRα, farnesoid X receptor α; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP2, sterol regulatory element binding protein 2.

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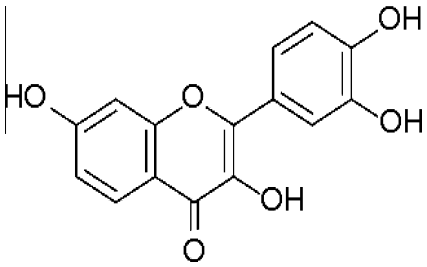

Fig. 1. Chemical structure of fisetin.

Seoul, Korea) and specific antibodies such as LDLR (Cayman, Ann Arbor, MI), SREBP2 (Abcam, Cambridge, MA), HMGCR and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

2.2. Animals and study design

The study was designed to examine the effects of fisetin supplementation on blood lipid profiles in high fat fed rats. Five weeks old Sprague–Dawley (SD) rats were used for an experiment of fisetin supplementation in high fat diets. After a 1 week adaptation period, the animals were randomly assigned to one of the three experimental groups (control: C, $n = 8$, high fat group: HF, $n = 8$, and high fat group with fisetin supplementation: HF + F, $n = 8$). After randomization, 6 week old rats were grown for further 8 weeks. The control diet was based on AIN-76 rodent diet composition (Table 1). Fisetin of the high fat diet was provided at as dose of 10 mg/kg of body weight. Dosage of fisetin was established based on the previous studies (Ragelle et al., 2012; Shia et al., 2009). All experimental animals were purchased from Koatech (Pyungtek, Korea), and were grown in a pathogen-free environment and housed in a temperature (18–24 °C) and humidity (50–60%) controlled room. Animals were fed daily, and *ad libitum* for all three diets. Test diets were stored at 4 °C. Animal body weight was measured every week. All the experimental procedures applied to the animals were approved by the Committee on Animal Experimentation and Ethics of Korea University.

2.3. Animal blood and liver tissue collection and measurements of biochemical parameters

Animals were starved for 12 h prior to sacrifice. Blood samples were collected in EDTA-containing polystyrene via the abdominal inferior vena cava. Plasma was obtained by centrifugation at 3,000 rpm for 30 min at 4 °C and stored at −80 °C, after which analysis for biochemical parameters, including blood lipid profiles, was carried out. The liver was extracted from each sacrificed rat within 1 min after death, and the weight of the liver was recorded. Extracted livers were immediately frozen in liquid nitrogen and stored at −80 °C Plasma cholesterol, LDL cholesterol, and HDL cholesterol were measured with the commercially available Wako test kits (Wako, Osaka, Japan) which use enzymatic methods of determination. Hepatic lipids were extracted using the method developed by Folch et al. (1957), and dried lipid residues were dissolved in 2 mL ethanol. The concentrations of cholesterol in the hepatic-lipid extracts were measured using the same enzymatic test kits used for the plasma analysis.

Table 1
Composition of experimental diets.

Ingredients	Groups[1]		
	CTL ($n = 8$)	HF ($n = 8$)	HF + F ($n = 8$)
Corn starch	15	15	15
Casein	20	20	20
Sucrose	50	34	33.99
Corn oil	5	3	3
Mineral mix[2]	3.5	3.5	3.5
Vitamin mix[3]	1	1	1
Cellulose	5	5	5
DL-methionine	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2
Lard	–	17	17
Cholesterol	–	1	1
BHT[4]	0.001	0.001	0.001
Fisetin	–	–	10 mg/kg BW
Total (g)	100.001	100.001	100.001

[1] CTL: control, $n = 8$; HF: high fat group, $n = 8$; HF + F; high fat group with fisetin supplementation, $n = 8$.
[2] Mineral mix: AIN-76 mineral mix.
[3] Vitamin mix: AIN-76 vitamin mix.
[4] BHT: tert-Butyhydeoquinone.

2.4. RNA extraction from animal liver and semi-quantitative RT-PCR

Liver samples (0.05 g), which were obtained from the animals were homogenized in 0.4 mL of lysis buffer using a Dounce homogenizer. Total RNA from animal liver was extracted from liver tissue using RNeasy Lipid Tissue Mini Kit according to the manufacturer's protocol. The cDNA was synthesized from 1 μg of RNA using oligo-dT and Superscript™ II reverse transcriptase. Rat primer sequences tested were presented in Table 2. The PCR conditions for rat primers were 1 min 30 s at 97 °C, followed by 30 cycles of 97 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. The final extension was done at 72 °C for 15 min. The PCR conditions for human primers were 15 min at 95 °C, followed by 26 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final extension was performed at 72 °C for 10 min. Semi-Quantitative RT-PCR and quantification of gene expression were performed by using an StepOnePlus™ Real-Time PCR System (Applied Biosystems, Grand Island, NY). Values were expressed in arbitrary units. The mRNA levels were determined by relative values to that of an endogenous GAPDH gene, and expressed as fold change over the control.

2.5. Cell culture and MTT assay

Fisetin was purchased from Sigma–Aldrich (St. Louis, MO). Human hepatoma HepG2 cells from American Type Culture Collection (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% of heat inactivated FBS containing 100,000 units/L of penicillin and 100 mg/L of streptomycin Gibco-Invitrogen. The cells were maintained in an incubator with 5% CO_2 at 37 °C. A density of 1×10^6 - cells was seeded in each well within 6-well culture plates. Fisetin stock solutions were prepared in DMSO. The cells were serum-starved overnight prior to the addition of fisetin. Cell viability was determined by measuring the levels of lactate dehydrogenase in the incubation media, using a commercially available kit after 24 h of treatment of MTT according to the manufacturer's instruction.

2.6. Immunoblot analysis

To obtain total cell extracts, the human hepatoma HepG2 cells (1×10^6 cells) were treated with various concentrations of fisetin, harvested, and lysed in a lysis buffer (40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail. After resting on ice for 30 min, the homogenates were centrifuged at 14,000 rpm for 1 h at 4 °C, and then the supernatants were collected. To extract total protein from the rat livers, tissue samples of each were homogenized at 4 °C in a lysis buffer. After resting on ice for 1 h, the tissue homogenates were centrifuged at 14,000 rpm for 30 min at 4 °C three times and then at 14,000 rpm for 1 h at 4 °C. The resulting supernatants (total lysates) were used for immunoblot analysis. Protein concentrations were determined by BCA method. Immunoblot analysis was performed using specific antibodies for LDLR, SREBP2, HMGCR and β-actin. The bands were visualized using enhanced chemiluminescence kit and quantified by densitometry using an Alphaview® software (Alpha Innotech).

Table 2
Primers used for quantitative real-time RT-PCR.

Gene description	Primers	Sequences(5′ → 3′)	Annealing temperature (°C)
LDLR	F	CAGCTCTGTGTGAACCTGGA	58
	R	TTCTTCAGGTTGGGGATCAG	
SREBP2	F	AGACTTGGTCATGGGGACAG	58
	R	GGGGAGACATCAGAAGGACA	
HMGCR	F	TGCTGCTTTGGCTGTATGTC	58
	R	TGAGCGTGAACAAGAACCAG	
CYP7A1	F	CTGCAAGAGAGGGATGAAGG	58
	R	ACAGGAGGGTTGTTGACCAG	
PCSK9	F	CTTGTGTCTAGCCAAAGGTG	58
	R	TGTAGCAAGTGCTCTCAGGT	
FXRα	F	TATGCAGGGAGAAAACTGAA	58
	R	CTG AAACCCTGGAAGTCTTT	
GAPDH	F	TCTGACATGCCGCCTGGAGAA	58
	R	TGGAGGCCATGTAGGCCATGA	

LDLR, low density lipoprotein receptor; SREBP2, sterol regulatory element binding protein 2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; CYP7A1, cytochrome P450 family 7 subfamily A polypeptide 1; PCSK9, proprotein convertase subtilisin/kexin type 9; FXRα, farnesoid X receptor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward primer; R, reverse primer.

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