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Fenugreek Seeds Affect Intestinal Cholesterol Transporters in Caco-2 Cells

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The seeds of fenugreek（*Trigonella foenum-graecum* L.）, which have a long history for use in traditional Chinese medicine, are reported to possess some hypocholesterolemic effect. To clarify the mechanism for cholesterol-lowering action, effects of fenugreek on the function and mRNA expression of intestinal cholesterol transporters, Niemann-Pick C1-Like 1（NPC1L1）and ATP binding cassette（ABC）transporters: ABCA1, ABCG5, and ABCG8, were examined in Caco-2 cells. Fenugreek seed water extract significantly inhibited both the net transport of cholesterol from the apical to basolateral side and the accumulation into the cells. Significant suppression in mRNA expression of the cholesterol transporters NPC1L1, ABCA1, ABCG5, ABCG8 and the transcription factor liver X receptor was observed in the cells treated with fenugreek. These results suggest that fenugreek possibly has inhibitory effects on mRNA expression and transport activity of the active cholesterol transporters, leading to the decreased net absorption of cholesterol from the intestine.

Key words: Fenugreek seeds, Caco-2 cells, cholesterol transporter, intestinal absorption

INTRODUCTION

Fenugreek（*Trigonella foenum-graecum* L.）belonging to Fabaceae family has a long history of medical uses in Middle East, India, and China［1］. Nowadays, it is also widely cultivated in these areas. Its leaf, bean, and germinated seeds can be used as vegetable and to feed cattle. Traditional Chinese medicine has been using fenugreek as a tonic for weakness, as well as for leg edema, colic pain, gastroenteritis, and warm coldness. Some beneficial pharmacological effects of fenugreek have been reported, including antidiabetic［2］, lipid-lowering and antioxidant［3］, antiinflammatory［4］, antimicrobial［5］, and cancer-preventive activities［6］. The hypolipidemic properties of oral fenugreek seed powder, ethanol extract, and of water-soluble extract have been demonstrated by several animal studies using rats, rabbits, and dogs［7-11］. Sowmya and Rajyalakshmi［12］reported that consumption of germinated fenugreek seeds resulted in a significant reduction in total cholesterol in human subjects. A clinical trial by Sharma *et al*.［13］showed significant reduction of total cholesterol and triglycerides by the defatted fenugreek seeds in type I diabetes patients. Recently, Vijayakumar *et al*.［14］suggested that the lipid-lowering effect of thermostable extract of fenugreek seeds is due to inhibition of fat accumulation and upregulation of the low-density lipoprotein receptor. However, very little is so far known about the mechanisms for lipid-lowering and hypocholesterolemic effects of fenugreek seeds.

Intestinal absorption of dietary cholesterol contributes as a main regulator of serum cholesterol homeostasis［15］. Many investigators demonstrated that some active transporters distributed in the gastrointestinal tract; Niemann-Pick C1-Like 1（NPC1L1）, ATP-binding cassette（ABC）transporter A1（ABCA1）, ABCG5, and ABCG8 mediate the intestinal cholesterol absorption［16］. NPC1L1

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is an influx transporter, which transfers intestinal cholesterol from the lumen into the enterocytes[17], whereas ABCG5 and G8 function together as an efflux pump excreting cholesterol out to the lumen [18]. ABCA1 plays a role in intestinal cholesterol absorption on the basolateral membrane of the enterocytes [19]. Additionally, it is well known that these intestinal cholesterol transporters are regulated by transcription factors, such as a nuclear receptor: liver X receptor (LXR)[16].

The purpose of this study is to clarify the mechanism for hypolipidemic effect of fenugreek in the absorption process at the intestine. Focusing on the cholesterol transporters expressing on the enterocytes, we used Caco-2 cell monolayers as an *in-vitro* model of the intestinal membrane [20, 21]. The Caco-2 cells are the human colon carcinoma cell, which is known to exhibit intestinal characteristics and express several carrier-mediated transport systems including NPC1L1 and the ABC transporters [22, 23]. In this study, effects of fenugreek seeds on the membrane transport activity for cholesterol were examined. Furthermore, we determined the mRNA expression of the major cholesterol transporters: NPC1L1, ABCA1, ABCG5, and ABCG8, and also of the nuclear receptors: LXR and farnesoid X receptor (FXR), in Caco-2 cells treated with or without fenugreek.

MATERIALS AND METHODS

Materials

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle medium (DMEM), non-essential amino acids (NEAA), fetal bovine serum (FBS), L-glutamate, trypsin-EDTA, and antibiotic-antimycotic mixture (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL of amphotericin B in 0.85% NaCl) were purchased from Invitrogen Co. Ltd. (Carlsbad, CA, USA). [H^3]-Cholesterol was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Reagents for real-time RT-PCR assay were purchased from Takara Bio Inc. (Otsu, Japan). All other chemicals of the highest purity were purchased from Wako Pure Chemicals Industries, Ltd (Osaka, Japan), Nacalai Tesque, Inc. (Kyoto, Japan) or Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

The culture medium consisted of DMEM containing 10% FBS, 1% NEAA, and 1% L-glutamate, and of 5% antibiotic-antimycotic mixture (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B in 0.85% NaCl). Cells were harvested with 0.25% trypsin-1mM EDTA. Caco-2 cells were grown in 5% CO_2/95% air at 37ºC. The cell suspension (1.5 mL) containing 3.0×10^5 cells/mL was seeded onto a polycarbonate filter (3.0 μm pore, 4.2 cm^2 growth area) set inside a 6-well plate (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan) for a "transport and accumulation" study [24]. For an mRNA expression study, Caco-2 cells were seeded at a density of 2.0×10^5 cells/well in a 6-well multi-plate. The culture medium was replaced with the fresh one on alternate days. "Transport and accumulation" experiments were performed between 16 and 21 days after seeding at the cell passage of 37 to 43, when the monolayers had reached confluence.

To evaluate the integrity of the Caco-2 cell monolayer, the transepithelial electrical resistance (TEER) of the monolayer was measured using EVOM (World Precision Instruments, Sarasota, FL, USA) before the transport and accumulation experiments. The monolayers, which exhibited TEER values over 750 ohms · cm^2, were used in the study.

Preparation of fenugreek seed water extract

Fenugreek seeds were purchased from a local pharmacy in Ningxia, China. The seeds were parched for 5 min until the color became deep, and then left at room temperature to be cooled down. The parched seeds were comminuted into the coarse grain with a mortar and a sieve (12 mesh). The coarse-ground seeds (10 g) were soaked in 200 mL of distilled water for 2 h, boiled for 20 min at 100ºC, and filtered with 4-ply gauze to collect the extraction solution. The above extraction procedure from the coarse-ground seeds was repeated three times, and the extract solution was concentrated to 100 mL to make fenugreek seed water extract (fenugreek extract) with a concentration of 100 mg/mL

as the amount of the coarse-ground seeds.

Cell viability

The viability of Caco-2 cells after exposure to the fenugreek extract was tested by Cell Titer-Blue Cell Viability Assay (Promega Co., Madison, WI, USA) [25]. Caco-2 cells were seeded at a density of 2.0×10^4 cells/well in 96 wells plate and incubated for 24 h at 37°C. The culture medium with or without the fenugreek extract (0.01 to 10 mg/mL) was added to final volume of 100 μl/well and incubated for 48 h at 37°C. After exposure, cells were washed by Hanks' Balanced Salt Solutions (pH 7.4) and cells were incubated with the culture medium containing Cell Titer-Blue reagent at 37°C for 2-3 h, and then, fluorescence was measured at 560/590 nm with fluorescence multi-well plate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Transport and accumulation study of cholesterol

The transport and accumulation experiments were performed in the transport medium as follows: KCl, 5.36 mM; NaCl, 137 mM; $Na_2HPO_4 \cdot 7H_2O$, 0.34 mM; KH_2PO_4, 0.44 mM; $NaHCO_3$, 4.17 mM; $CaCl_2$, 1.26 mM; $MgCl_2 \cdot 6H_2O$, 0.49 mM; $MgSO_4 \cdot 7H_2O$, 0.41 mM; glucose, 19.45 mM; and Hepes (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]) [20].

Initially, the Caco-2 cell monolayer was pre-incubated with the culture medium with or without fenugreek extract (1 mg/mL) in the apical chamber for 24 or 48 h. After removal of the culture medium, the pre-warmed transport medium containing cholesterol (1 μM) with [^{3}H]-cholesterol as a tracer and the cholesterol-free transport medium were applied to the apical and the opposite chambers, respectively. Aliquots of samples (0.1 mL) were collected from the basolateral chamber periodically and replaced with equal volumes of the transport medium. The apical-to-basolateral permeability coefficient (P_{app}) of cholesterol was calculated as its flux rate by the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

where dQ/dt is the permeation rate, A is the surface area of the monolayer, and C_0 is the initial concentration of cholesterol [21]. The cholesterol transport experiment was also performed in the intact Caco-2 cells using the transport medium containing ezetimibe (50 μM, apical chamber only), an inhibitor of NPC1L1.

To evaluate the cholesterol accumulation into cells, the cell monolayer was separated from the filter and was added 1 M NaOH solution (1 mL) to lyse the cells. Radioactivities of [^{3}H]-cholesterol in the collected lysate and the medium were measured by Packard Tri-Carb 1500 liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL, USA). The protein amount of the cell lysate was measured by the Bradford method [26]. Accumulation of cholesterol into cells was expressed as the cell/medium ratio calculated by the following equation:

$$\text{Cell/medium ratio } (\mu L/mg \text{ protein}) = \frac{\text{dpm of } ^3H \text{ in cells / protein amount in cells (mg)}}{\text{dpm of } ^3H \text{ in medium / medium volume } (\mu L)}$$

mRNA expression of transporters and nuclear receptors

The cells were incubated on a 6-well plate in the culture medium with or without 1.0 mg/mL of fenugreek extract for 24 or 48 h. Total RNA was isolated from the incubated cells using a GenElute Mammalian Total RNA Kit (Sigma Chemical Co.) with Amplification Grade DNase I treatment. The concentration of RNA was measured by using a spectrophotometer, NanoDrop (Thermo Fisher Scientific Inc.). RT-PCR was performed using AMV RT-PCR Kit (Takara Bio Inc.). Quantitative real-time PCR assays for the cholesterol transporters and an internal reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were performed using TaqMan Assays-on-Demand, Gene Expression Assay (ABI: Applied Biosystems, Foster City, CA, USA) with an ABI PRISM 7000 Sequence Detection System. Relative quantification values expressed as cycle threshold were averaged and subsequently used to determine the relative expression ratios between control and other groups. To adjust for the variations in starting template, the expression levels of several cholesterol transporter genes in all samples were normalized by GAPDH.

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test or one-way analysis of variance (one-way ANOVA). In all cases, a p value of 0.05 or less was considered statistically significant.

RESULTS

The fenugreek extract did not change the Caco-2 cell vitality within the concentration range of 0.01-1.0 mg/mL after the exposure for 48 h (Fig. 1). At the concentrations of 5.0 and 10 mg/mL, however, the cell viability significantly decreased by addition of the fenugreek extract in the medium. We, therefore, adopted the fenugreek extract concentration of 1.0 mg/mL to evaluate the effect of exposure to fenugreek on cholesterol transporters.

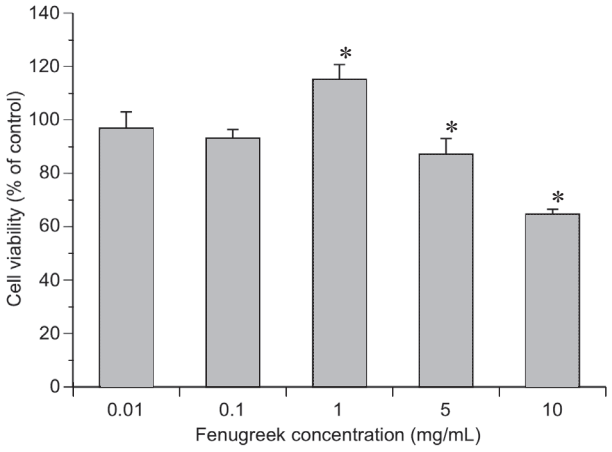

Fig. 1. Effects of exposure to fenugreek extract on Caco-2 cell viability

Each column represents the mean and S.D. of 4 independent experiments.

Significant difference was observed when compared with the control by one-way ANOVA with Dunnett test (* p<0.05).

Fig. 2 shows the time course of the apical-to-basolateral transport of cholesterol in Caco-2 cells with or without ezetimibe. Cholesterol was linearly translocated from the apical to basolateral side across the cell monolayer up to 24 h. When ezetimibe was added in the apical side, the permeated amount of cholesterol was decreased. Significant decrease of about 35% was observed at 24 h.

The effects of fenugreek extract on the cholesterol transport in the apical-to-basolateral direction across the Caco-2 cell monolayers are shown in

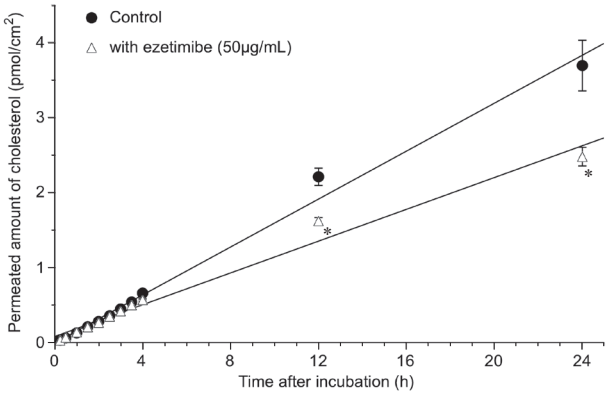

Fig. 2. Effects of ezetimibe (50 μM) on the apical-to-basolateral transport of cholesterol in Caco-2 cells

Ezetimibe was added in the transport medium of the apical side. Each symbol represents the mean ± S.D. of 3 independent determinations. The solid lines are based on the linear least-squares regression analysis. Significant difference was observed when compared with the control by Student's *t*-test (* p<0.05).

Fig. 3A. The exposure of the cells to fenugreek extract (1.0 mg/mL) for 24 and 48 h significantly reduced the relative P_{app} of cholesterol in a time-dependent manner. The cell/medium ratios of cholesterol after incubation with or without fenugreek extract for 24 and 48 h are shown in Fig. 3B. Accumulation of cholesterol in cells was significantly decreased to about 70% of the control by 48 h treatment with fenugreek extract, whereas no change was observed in cells treated for 24 h.

Effects of the fenugreek extract on the mRNA expression levels of cholesterol transporters: NPC1L1, ABCA1, ABCG5, and ABCG8, and of nuclear receptors: LXRα and FXR, in Caco-2 cells were demonstrated in Figs. 4 and 5, respectively. After 24 h exposure to fenugreek extract, mRNA expression levels of these transporter genes were significantly decreased except for the ABCA1 gene. In the cells treated with fenugreek extract for 48 h, more potent suppression was observed in mRNA levels of these cholesterol transporters, although the NPC1L1 mRNA level were not altered obviously. In the nuclear receptors, fenugreek-treated cells showed 2- or 3-fold higher expression levels of FXR. In contrast, LXRα was significantly suppressed to less than 50% of the control by the exposure to fenugreek extract.

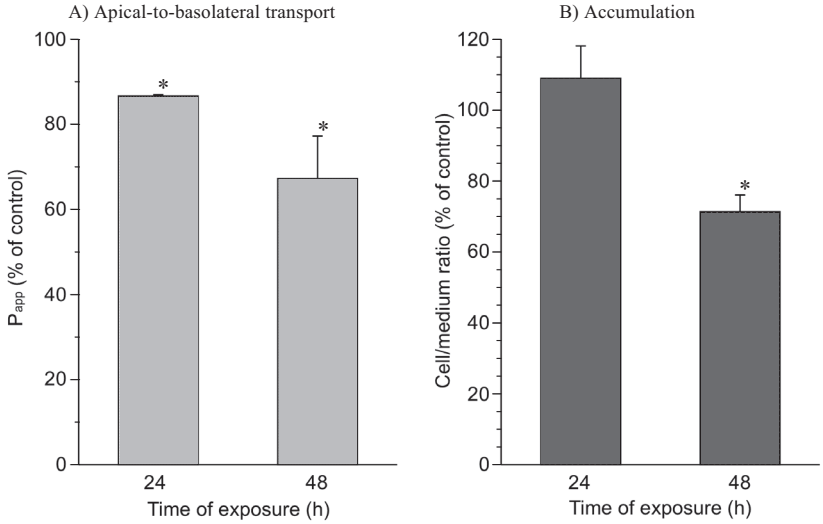

Fig. 3. Effects of fenugreek extract (1 mg/mL) on the apical-to-basolateral transport (panel A) and on the accumulation (panel B) of cholesterol in Caco-2 cells

The P_{app} and cell/medium ratio were measured at 24 h after addition of cholesterol in the apical chamber. Each column represents the mean and S.D. of 3 independent determinations. Significant difference was observed when compared with the control by one-way ANOVA with Dunnett test (* p<0.05).

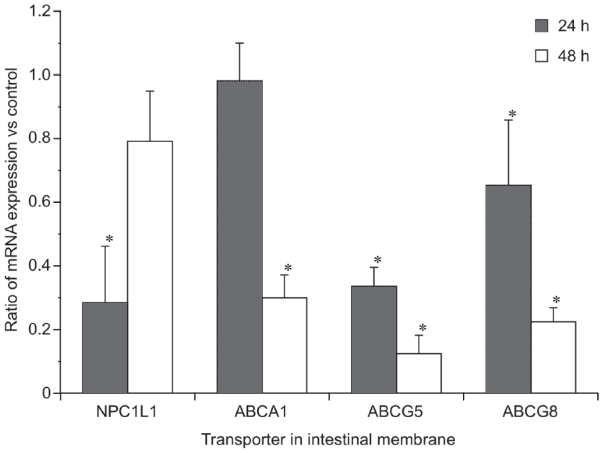

Fig. 4. Effect of fenugreek extract (1 mg/mL) on mRNA expression of cholesterol transporters in Caco-2 cells

NPC1L1: Niemann-Pick C1-Like 1; ABCA1, ABCG5, ABCG8: ATP-binding cassette transporters A1, G5, G8. Each column represents the mean and S.D. of 3 independent determinations. Significant difference was observed when compared with the control by one-way ANOVA with Dunnett test (* p<0.05).

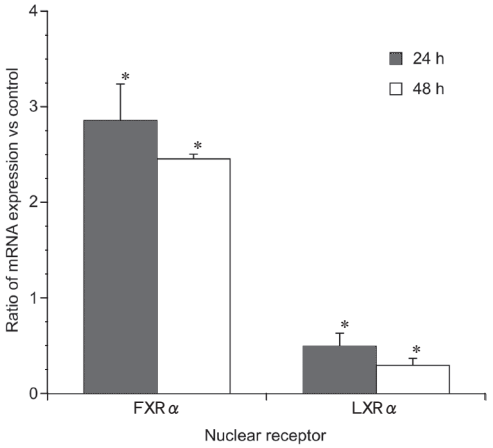

Fig. 5. Effect of fenugreek extract (1 mg/mL) on the mRNA expression of nuclear receptors in Caco-2 cells

FXRα: farnesoid X receptor; LXRα: liver X receptor. Each column represents the mean and S.D. of 3 independent determinations. Significant difference was observed when compared with the control by one-way ANOVA with Dunnett test (* p<0.05).

DISCUSSION

Cholesterol is essential for life and also a risk factor in the development of heart disease. Exogenous cholesterol is known to be taken up from the intestine *via* active transport systems [16, 27]. The known major cholesterol carrier proteins are NPC1L1, ABCA1, ABCG5, and ABCG8. NPC1L1 is a carrier protein localized on the brush border membrane of jejunal enterocytes. Altmann *et al*. [28] reported that NPC1L1-deficient mice showed a significant 70% reduction in cholesterol absorption, so that NPC1L1 is considered to have a critical role for cholesterol transport from the intestinal lumen into the epithelial cells. Also, NPC1L1 is known to be a direct molecular target of ezetimibe, a potent cholesterol and phytosterol uptake inhibitor [29].

Several ABC transporters are also involved in the

cholesterol translocation at the apical and basolateral membranes in enterocytes. ABCA1 is located on the basolateral surface of intestinal cells, which contributes to cholesterol homeostasis by mediating cholesterol efflux across the cell membrane and combining with free plasma apolipoprotein A-I to form nascent high-density lipoprotein. Other ABC transporters, ABCG5 and ABCG8, are predominantly expressed on the mucosal surface of the enterocytes as a heterodimer ABCG5/G8. This heterodimeric transporter can export cholesterol and phytosterols into the bile and intestinal lumen, regulating the absorption of cholesterol and plant sterols and bile secretion [30]. These ABC transporters are regulated by the nuclear receptor, LXR. In the enterocytes, LXR upregulates ABCG5/G8 and ABCA1, leading to increased efflux of cholesterol into the lumen and lymphatics, respectively [27]. Additionally, ABCA1 is regulated by another nuclear receptor FXR as well. The synthesis of ABCA1 would be stimulated by downregulation of FXR [31].

Based on the above aspects about the role of several transporters and nuclear receptors in cholesterol homeostasis, we examined whether fenugreek extract may change the transport activity and mRNA expression of these proteins.

In the apical-to-basolateral transport study (Fig. 2), cholesterol linearly permeated across the Caco-2 cell monolayer along with time up to 24 h. We, therefore, evaluated the cholesterol transport activity for 24 h after addition of cholesterol in the apical side. As shown in Fig. 2, ezetimibe significantly reduced cholesterol translocation from the apical to basolateral side of the cell monolayer. As ezetimibe is a positive control for the NPC1L1-mediated transport, this result suggests that NPC1L1 possibly functions in the Caco-2 cell monolayer used in our study. As shown in Fig. 3A, the relative P_{app} of cholesterol was significantly reduced when the cells were treated with fenugreek extract. This result implicates that the net absorption of cholesterol from the intestine into the body may be inhibited. Net absorption from the apical to basolateral side depends on the balance of the three transporters: NPC1L1, ABCA1, and ABCG5/G8. Reduced net transport of cholesterol suggests that fenugreek extract inhibits the transport activity of the absorption transporters, NPC1L1 and ABCA1, more strongly than inhibits the efflux transporter ABCG5/G8. Alternatively, fenugreek extract may stimulate the efflux transport mediated by ABCG5/G8 from the cell to the apical side. Cholesterol accumulation into the Caco-2 cells was also decreased when the cells were exposed to fenugreek extract for 48 h (Fig. 3B). This change is consistent with the result of decreased net transport of cholesterol.

As mentioned above, the cholesterol transporters are regulated by several nuclear receptors. To explore the possible mechanism how fenugreek extract affects the cholesterol transport in Caco-2 cells, we have performed the mRNA expression study for genes of the cholesterol transporters and nuclear receptors. Fenugreek extract suppressed the expression of NPC1L1, ABCA1, ABCG5, and ABCG8 (Fig. 4). Interestingly, the effects of fenugreek extract on NPC1L1 and ABCA1 mRNA were time-dependent. This may partly explain the time-dependent inhibition of the net transport and accumulation of cholesterol, which is observed in Fig. 3. The mRNA expression levels of the cholesterol transporters in Caco-2 cells were all decreased by fenugreek extract. From these aspects, the decreased net transport of cholesterol may be due to inhibition of NPC1L1 and/or ABCA1 and not to stimulation of the efflux transporter ABCG5/G8. Fenugreek extract probably has a suppressive efficacy on the mRNA expression of the carrier proteins transporting cholesterol from the intestine into the body. Considering that the accumulated amount of cholesterol in the cells was reduced (Fig. 3B), it is likely that fenugreek extract may influence the function of the influx transporter NPC1L1 rather than ABCA1, which moves cholesterol out of the cells. This assumption may be supported by the results that mRNA expression of NPC1L1 was significantly repressed by fenugreek extract as compared with no effect on ABCA1 at the shorter time of exposure, 24 h.

There are many aspects that cholesterol homeostasis is regulated by the nuclear receptors [27]. LXRs of the nuclear receptor superfamily are transcription factors that primarily act as a cholesterol sensor coordinating the expression of genes involving in cholesterol metabolism [32]. It is reported that LXR regulates gene expression of various cho-

lesterol transporters. For example, the NPC1L1 gene expression is downregulated by LXR activation in the enterocytes [33] and LXR stimulates cholesterol excretion mediated by ABCG5/G8 [34]. The expression of the ABCA1 gene is upregulated by LXR/retinoid X receptor activation, resulting in enhanced basolateral efflux of cholesterol from Caco-2 cells [35, 36]. As shown in Fig. 5, fenugreek significantly decreased the LXRα gene expression. Decreased expression of ABCA1, ABCG5, and G8 may be explained by LXRα-mediated downregulation, because the inhibitory effects on these transporters were time-dependent. In contrast, the change observed in NPC1L1 mRNA expression could not be implied by the decreased expression of LXRα gene. Other nuclear receptors such as peroxisome proliferator-activated receptors may be involved in the fenugreek effect on the NPC1L1 gene [37].

Lambert *et al*. [38] revealed that FXR is a negative regulator of dietary cholesterol absorption in the study using FXR-knockout mice. Thus, we examined whether fenugreek could change the expression of FXR in Caco-2 cells. As the results (Fig. 5), upregulation of FXR was observed in the fenugreek-treated cells. It is reported that FXR inhibits the LXRα activity partly *via* small heterodimer partner expression [39], so that decreased mRNA expression of ABCA1 and ABCG5/G8 observed in Fig. 4 may be due to LXRα inhibition caused by FXR activation. Neimark *et al*. [40] demonstrated that FXR mediates a negative feedback regulation of the apical sodium-dependent bile acid transporter in response to bile acid in enterocytes. This aspect suggests that fenugreek may inhibit bile acid reabsorption through FXR upregulation, leading to a hypocholesterolemic effect *in vivo*.

The previous chemical analysis indicates that fenugreek seeds abundantly contain proteins, carbohydrates as well as saponins [8]. Ribes *et al*. [41] showed that the saponin-rich fraction of fenugreek had a hypolipidemic effect, while no effect on high cholesterol levels was observed in the protein fraction. Additionally, Petit *et al*. [42] demonstrated that steroid saponins extracted from fenugreek seeds decreased total plasma cholesterol in rats. These findings suggest that saponins likely contribute to the hypolipidemic activity of fenugreek seeds. Further studies are required to elucidate the underlying mechanism for up- and downregulation of the genes of cholesterol transporters and the regulating nuclear receptors.

In conclusion, the present study represents that fenugreek seed water extract possibly contains some constituent(s) which has inhibitory effects on mRNA expression and transport activity of the active transporters: NPC1L1, ABCA1, and ABCG5/G8, leading to decreased net transport of cholesterol from the apical to basolateral side in Caco-2 cells. This may be one of the mechanisms for fenugreek's lipid-lowering effects that have been reported in the basic and clinical studies.

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