Coleus forskohlii Extract Attenuated the Beneficial Effect of Diet-Treatment on NASH in Mouse Model

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Summary Obesity is one of the main causes of non-alcoholic steatohepatitis (NASH), which is associated with impaired liver functions including drug metabolism. Coleus forskohlii extract (CFE) is a popular ingredient of weight loss dietary supplements in Japan. In this study, we examined the effect of CFE on the treatment of NASH. C57BL/6 mice (male, 10-wk-old) were fed a NASH diet (high-fat, low-methionine, and choline-deficient diet) for 12 wk to establish NASH. Then, we examined the effect of 0.5% (w/w) CFE in diet during diet-treatment (change to control diet) and/or treadmill-exercise (45 min at 20 m/min, 5 d/wk) to improve NASH for 3 wk. After experimental period, lipids profiles and liver functional markers in the blood, and hepatic lipid content and major CYP subtype mRNA expression and activity in liver were measured. Diet-treatment, but not exercise decreased liver weight and hepatic lipid contents in NASH induced mice. CFE attenuated the effects of diet-treatment which reduced liver weight, even though body weight and adipose tissue weight were reduced. Further, CFE significantly increased liver microsomal CYP1A1, CYP1A2, CYP2C, and CYP3A activities in each condition, and CYP inductions were greater in diet-treatment group compared to those in exercise group. These results suggest that taking CFE should be avoided during diet-treatment of NASH, especially in patients under medication.

Key Words body weight, Coleus forskohlii extract (CFE), cytochrome P450 (CYP), dietary supplement, exercise, forskolin, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH)

Non-alcoholic fatty liver disease (NAFLD) and the more progressive non-alcoholic steatohepatitis (NASH), which shows similar characteristics observed in alcoholic liver disease, have recently been recognized as liver diseases in developed countries (1, 2). NASH may further progress from steatosis and fibrosis to cirrhosis and hepatocellular carcinoma (3, 4). Prevention and treatment of NASH are therefore important to prevent progression to severe clinical conditions. Currently, effective medication does not exist for NASH, and diet-treatment and exercise are recommended. However, NASH is associated with obesity and metabolic syndrome, such as dyslipidemia, insulin resistance, and diabetes mellitus (5, 6), therefore medication for these diseases combined with diet-treatment and exercise are prescribed to NASH patients (7, 8).

The use of dietary supplements including herbal products has increased not only in Japan (9–12), but also worldwide (13–15). Coleus forskohlii grows in warm subtropical temperate areas such as India, Nepal, Sri Lanka, and Thailand. C. forskohlii root extract (CFE) contains forskolin, which plays a major role in mediating the pharmacological action of C. forskohlii. Forskolin activates adenylate cyclase and induces cAMP synthesis (16, 17). It has been used as an Ayurvedic remedy to treat various diseases, such as cardiovascular disease, abdominal colic, respiratory disorders, painful micturition, insomnia, and convulsions (18). In Japan, C. forskohlii is the most popular herbal ingredient for commercial weight-loss supplements (19). Increased cAMP synthesis by CFE may be associated with enhancement of CAMP-dependent lipolysis in the adipose tissue. It has been reported that forskolin induces CAMP accumulation and lipolysis in fat cells isolated from rat adipose tissue (20, 21). In addition, CFE suppressed body weight gain and lipid accumulation by ovariectomy in rats (22). In humans, forskolin slightly decreased the body fat mass in overweight/obese men (23), but CFE did not influence body fat in overweight women (24). In both studies, the sampling pool was not large enough. Further investigation is needed to validate the effects of CFE/forskolin in humans.

It is reported that use of herbal weight-loss supple-
ments was associated with adverse events including hepatic injury or death (19, 25). CFE also caused adverse events, especially diarrhea (26). To further investigate the mechanism of diarrhea caused by CFE, we examined the effect of excess amount of CFE feeding on a rodent model. However, these rodents did not show any symptoms of diarrhea. Instead, CFE induced hepatotoxicity and fatty liver in a mouse model (27). Additionally, we also reported that CFE affects drug metabolizing enzymes. CFE markedly induced major hepatic cytochrome P450 (CYP) subtypes mRNA expression and its activities (28). When mice were fed with CFE for 1 wk, the anticoagulant effects of warfarin were found to be attenuated through the induction of hepatic CYP2C activity (29). This effect might have been caused by unidentified compounds in CFE, as warfarin and CYP2C enzyme interaction was inhibited by CFE, but not by forskolin.

In the present study, we examined the influence of CFE during diet-treatment and exercise to improve NASH.

**MATERIALS AND METHODS**

**Materials.** Powdered CFE standardized to 10% (w/w) forskolin was prepared as follows. Dried roots of *C. forskohlii*, obtained from Bangalore in southern India, were crushed and supercritically extracted with CO2 gas. The forskolin-rich extract (20–30%) was mixed with dextrin to give a forskolin concentration of 10%. These processes were outsourced to Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). The CFE comprised: 5.6% water, 0.3% protein, 22.7% lipids, 2.2% ash, and 69.2% carbohydrates. The HPLC chromatographic profile has been reported (30), and the analyzed contents of forskolin in the CFE sample were 10.4%. The quality of the standardized CFE was confirmed by HPLC analysis with an evaporative light scattering detector (30) using for- skolin (purity>99%: Biomol, CN-100; Lot. 3-H2325a, Plymouth Meeting, PA, USA) as a standard. Control diet (A06071314M) and NASH diet (A06071318M) (Table 1), which is a high-fat, low-methionine and choline-deficient diet that could induce NASH in mice by 12-wk feeding (31), were purchased from Research Diet Inc. (New Brunswick, NJ). All other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

**Animals and experimental design.** Male C57BL/6J mice (9-wk-old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). After 1 wk of acclimation, mice were fed a NASH diet for 12 wk to induce NASH (n=52). A 12 wk-feeding of this NASH diet induced NASH, which characterized with hepatic TG accumulation, inflamma-

| Ingredient (g)         | Control | NASH          |
|-----------------------|---------|---------------|
| L-Cystine             | 4.2     | 4.2           |
| L-Asparagine          | 7.6     | 7.6           |
| L-Alanine             | 15.8    | 15.8          |
| L-Lysine              | 13.2    | 13.2          |
| L-Methionine          | 5.1     | 1.7           |
| L-Phenylalanine       | 8.4     | 8.4           |
| L-Threonine           | 7.2     | 7.2           |
| L-Tryptophan          | 2.1     | 2.1           |
| L-Valine              | 9.3     | 9.3           |
| L-Histidine           | 4.6     | 4.6           |
| L-Alanine             | 5.1     | 5.1           |
| L-Arginine            | 6       | 6             |
| L-Aspartic acid       | 12.1    | 12.1          |
| L-Glutamic acid       | 38.2    | 38.2          |
| Glycine               | 3       | 3             |
| L-Proline             | 17.8    | 17.8          |
| L-Serine              | 10      | 10            |
| L-Tyrosine            | 9.2     | 9.2           |
| Corn starch           | 502     | 76.2          |
| Maltodextrin 10       | 130.1   | 100           |
| Sucrose               | 68.8    | 172.8         |
| Cellulose, BW200      | 50      | 50            |
| Soybean oil           | 25      | 25            |
| Lard                  | 20      | 177.5         |
| Mineral mix S10026    | 10      | 10            |
| Dicalcium phosphate   | 13      | 13            |
| Calcium carbonate     | 5.5     | 5.5           |
| Potassium citrate     | 16.5    | 16.5          |
| Sodium bicarbonate    | 7.5     | 7.5           |
| Vitamin mix V10001    | 10      | 10            |
| Choline bitartrate    | 2       | 0             |
| Total                 | 1,039.35| 839.55        |

| Ingredient (w/w)      | Control | NASH          |
|-----------------------|---------|---------------|
| Protein               | 17      | 21            |
| Carbohydrate          | 68      | 43            |
| Fat                   | 4       | 24            |
| kcal%                 | 18      | 18            |
| Carbohydrate          | 72      | 36            |
| Fat                   | 10      | 46            |

Table 1. Composition of experimental diets used in this study.
Table 2. Primer sequences (5′–3′).

| GenBank     | bp   | Forward                                      | Reverse                                      |
|-------------|------|----------------------------------------------|----------------------------------------------|
| Cyp1a1      | X01681 | 169 AGCTTGCCCTGGATTACGTG                   | AACCCCATCAACCCACATTAG                      |
| Cyp1a2      | NM_009993 | 181 ACATCACAGTGCTCCGGCTCAAGC              | ATCTTCCTGCTAGCAGTGGCAT                     |
| Cyp2c29     | BC019908 | 194 AGCTACTCTGTCATATGGCAGGGGT              | CATGCCCCAAATTTGCAGGGGTAC                   |
| Cyp3a11     | BC010528 | 109 AGGCCAAGTCCAAAGAAGGCGAAG                | TGAAGGAAATTCACGTTACCTC                  |
| IL-1β       | M15131 | 180 TGGAGATGTGGATCCCAAGAACAT              | TGCCCTGGACACGTTTGTTCCTC                  |
| TNF-α       | M13049 | 94 TCTCAGACCTACCATCAAGGCT                | TGACACTCTCCCTTGGAGAAG                        |
| F4/80       | X93328 | 120 TCAATTGAGTACCATGAGGCTC              | TGCACTGCTGTGGCATTGCTG                   |
| SAA         | M11131 | 112 AGAGGACATGAGGACCCATTGCT            | AAGAAGCTGATGGTATTTTGCAGGC                  |
| GAPDH       | M17701 | 152 TGAATCTGGTGCTGGATAGTGTG             | TCTCTGRTGTTACACCCATCAACAA                |

5 times/wk, started at 9 AM) for 8 wk (32). We also investigated the effects of different exercise conditions on NASH. C57BL/6J mice were induced NASH, then treadmill exercise was conducted at 5 d/wk for 3 wk under following conditions: Ex1) 10 m/min for 30 min, Ex2) 10 m/min for 90 min, Ex3) 20 m/min for 45 min, Ex4) 30 m/min for 30 min. As a result, Ex2–4 suppressed liver weights and hepatic TG levels, and Ex3 showed the most effective on hepatic TG contents. Therefore, exercise was performed under the conditions of Ex3 (20 m/min for 45 min, slope 0′), 5 d/wk in this study.

Plasma chemistry. After the experimental period, mice were fasted overnight (from 17:00 to 9:00) and were sacrificed humanely under 2% isoflurane anesthesia. Blood samples were obtained from the abdominal aorta, and heparinized blood was centrifuged at 4°C, and the supernatant was collected. The supernatant was recentrifuged at 105,000 g for 15 min to obtain plasma sample. Plasma levels of total cholesterol (TC), triglycerides (TG), non-esterified fatty acids (NEFA), and liver functional markers (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)) were measured using enzymatic methods (FUJIFILM Wako Pure Chemical Corporation).

Measurement of hepatic lipid contents. Hepatic lipids were extracted by a Folch method (33). The liver (approx. 100 mg) was homogenized in 6 mL of chloroform/methanol (2:1) with a polytron homogenizer. Lipids were extracted by overnight incubation, filtered to remove insoluble residues, and adjusted to 10 mL with chloroform/methanol. One milliliter of each sample was transferred to a new tube and the organic solvent was allowed to evaporate. The residue was dissolved in isopropanol (containing 10% Triton-X). The hepatic lipid contents were determined using the same enzymatic methods used for the plasma analyses.

Quantitative RT-PCR. Total RNA was extracted from the liver using the TRIzol Plus RNA Purification System (Life Technologies, Curlsbnd, CA), and reverse transcribed with the PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan). Quantitative RT-PCR was performed using the SYBR Green PCR Master Mix and mouse-specific primers as listed in Table 2 on a Thermal Cycler Dice Real Time System Single (Takara Bio Inc.). Copy numbers for each gene were determined by the absolute quantitation method using serially diluted standards of known concentration. Results are expressed as the copy number ratio of the target mRNA to GAPDH mRNA.

Preparation of liver microsomes. Livers were homogenized in 50 mM Tris-HCl buffer containing 0.25 M sucrose (pH 7.4) with a polytron homogenizer. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C, and the supernatant was collected. The supernatant was recentrifuged at 105,000 × g for 60 min at 4°C and the supernatant was discarded. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4), and used as the liver microsomal fraction. The protein concentration of the microsomal fraction was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA).

Measurement of CYP activity. The activity of each CYP subtype in liver microsomal fraction was measured by a luminescent method using the P450-GloTM CYP1A1 System (Luciferin-CEE) Assay, CYP1A2 System (Luciferin-1A2) Assay, CYP2C9 System (Luciferin-H) Assay, CYP3A4 System (Luciferin-PPX) Assay, and NADPH Regeneration System with GloMax-Multi+Detection System (Promega Co., Madison, WI). CYP activity was adjusted according to the protein concentration. The results obtained were represented as a percentage of the control.

Statistical analysis. Data are presented as means±SE. Comparison of data between multiple groups were performed by one-way ANOVA with a Tukey-Kramer test (SPSS 22.0J for Windows; IBM Co., Armonk, NY). A p value <0.05 was considered to be significant.

RESULTS

Diet-treatment and/or exercise with/without CFE on NASH

After establishment of NASH, C57BL/6J mice were subjected to diet-treatment and/or exercise with/without 0.5% CFE for 3 wk. After the treatment period, no significant difference was observed in total body weight and the weight of total fat obtained from three different sites among all treatment groups (Table 3). However, in each treatment group, the total body weight and the weight of total fat were found to be lower in the CFE fed mice compared to the mice not exposed to CFE. Food intake (kcal/d) was significantly lower in the Dt and the Dt + Ex groups. Decreased body weight and the weight of adipose tissues might be caused decreased food intake.
Table 3. Body weight, each tissue weight and plasma biomarkers.

|                          | Cont.          | NASH          | NASH+CFE      | Dt            | Dt+CFE        | Ex            | Ex+CFE       | Dt+Ex        | Dt+Ex+CFE    |
|--------------------------|----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Body weight (g)          | 31.3±1.2       | 29.5±1.5      | 26.9±0.9      | 30.5±1.0      | 27.6±0.8      | 27.8±1.2      | 25.3±0.5      | 28.7±0.6      | 26.0±0.7      |
| Liver (g)                | 1.14±0.05*     | 1.44±0.10     | 1.52±0.07     | 1.29±0.04     | 1.53±0.07     | 1.26±0.06     | 1.46±0.04     | 1.17±0.04*    | 1.48±0.05*    |
| % Liver (%)              | 3.7±0.1***#### | 4.8±0.1       | 5.6±0.1***####| 4.2±0.1**     | 5.6±0.1***####| 4.5±0.1      | 5.8±0.1***####| 4.1±0.1***    | 5.7±0.1***####|
| Kidney (g)               | 0.41±0.02      | 0.37±0.01     | 0.34±0.01     | 0.39±0.01     | 0.35±0.01     | 0.36±0.01     | 0.34±0.00     | 0.37±0.01     | 0.34±0.01     |
| % Kidney (%)             | 1.3±0.0        | 1.3±0.0       | 1.3±0.0       | 1.3±0.0       | 1.3±0.0       | 1.3±0.0       | 1.3±0.0       | 1.3±0.0       | 1.3±0.0       |
| Spleen (g)               | 0.070±0.0002** | 0.082±0.003   | 0.075±0.003   | 0.081±0.004   | 0.074±0.005   | 0.070±0.004   | 0.066±0.001*  | 0.072±0.004   | 0.063±0.003** |
| Total fat (g)            | 1.9±0.35       | 1.91±0.41     | 0.87±0.23     | 1.78±0.27     | 1.11±0.22     | 1.40±0.36     | 0.74±0.09     | 1.54±0.12     | 0.79±0.16     |
| % Total fat (%)          | 6.0±1.0        | 6.2±1.0       | 3.1±0.7       | 5.7±0.7       | 3.9±0.7       | 4.9±1.0       | 2.9±0.3       | 5.3±0.3       | 3.0±0.5       |
| Epididymal fat (g)       | 1.02±0.18      | 1.18±0.24     | 0.61±0.15     | 1.01±0.14     | 0.73±0.15     | 0.90±0.21     | 0.54±0.06     | 0.90±0.07     | 0.52±0.09     |
| Perirenal fat (g)        | 0.49±0.12      | 0.44±0.10     | 0.15±0.05     | 0.40±0.08     | 0.19±0.05     | 0.30±0.09     | 0.11±0.03*    | 0.32±0.03     | 0.13±0.03     |
| Mesenteric fat (g)       | 0.41±0.07      | 0.30±0.08     | 0.11±0.03     | 0.38±0.05     | 0.19±0.03     | 0.20±0.08     | 0.08±0.01     | 0.32±0.02     | 0.14±0.04     |
| Food intake (kcal/d)     | 11.9±0.2       | 11.3±0.5      | 11.3±0.3      | 9.0±0.1***    | 9.3±0.2***    | 11.5±0.2      | 11.2±0.4      | 9.1±0.1***    | 9.1±0.4***    |
| Body weight gain (g/d)   | 0.04±0.02      | -0.07±0.03    | -0.17±0.03    | -0.03±0.06    | -0.15±0.02    | -0.14±0.02    | -0.27±0.04**  | -0.11±0.03    | -0.22±0.03    |
| Energy efficiency (%)    | 0.3±0.2        | -0.6±0.3      | -1.5±0.2      | -0.3±0.7      | -1.6±0.2      | -1.2±0.2      | -2.4±0.4*     | -1.2±0.4      | -2.4±0.3*     |

Plasma biomarkers

|                          | Cont.          | NASH          | NASH+CFE      | Dt            | Dt+CFE        | Ex            | Ex+CFE       | Dt+Ex        | Dt+Ex+CFE    |
|--------------------------|----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| TC (mg/dL)               | 99.1±7.2       | 84.3±5.5      | 68.2±3.9      | 103.4±5.9     | 73.5±7.7###   | 86.7±4.5      | 80.0±3.1      | 108.8±3.8*    | 73.5±4.0*     |
| TG (mg/dL)               | 89.0±10.4      | 85.5±6.0      | 64.7±5.6      | 102.7±8.1     | 70.9±12.1     | 70.9±6.3      | 61.9±3.8      | 73.5±4.0      | 49.4±5.6*     |
| NEFA (meq/L)             | 1.43±0.09      | 1.29±0.04     | 1.13±0.04     | 1.49±0.06     | 1.05±0.11###  | 0.98±0.06*    | 0.91±0.04**   | 1.04±0.04     | 0.77±0.04###  |
| AST (IU/L)               | 26.4±2.8       | 47.4±5.3      | 39.7±1.5      | 30.6±2.0      | 43.6±8.8      | 45.2±3.5      | 54.3±7.2      | 32.6±2.7      | 38.2±4.5      |
| ALT (IU/L)               | 6.8±0.7        | 27.8±5.6      | 26.1±2.3      | 9.4±2.0       | 25.5±6.6      | 26.7±4.4      | 41.9±7.0      | 13.4±1.3      | 23.6±5.3      |
| ALP (IU/L)               | 43.6±2.3       | 50.9±1.9      | 59.0±1.5      | 52.9±2.6      | 83.5±4.6###   | 48.3±3.0      | 63.0±3.1      | 47.4±1.1      | 82.2±4.5###   |

C57BL/6j male mice were fed a NASH diet for 12 wk to establish NASH. Then, mice were fed NASH diet (NASH), diet-treatment (Dt), exercise (Ex), diet-treatment with exercise (Dt+Ex) with/without 0.5% CFE for 3 wk. Control group (Cont.) fed a control diet whole experimental period (15 wk). After overnight fasting, body weight and tissue weights were measured. Energy efficiency (%) was calculated as [body weight gain (g)/food intake (kcal)×100] during treatment period. Data are represented as the mean±SE. n=6 or 7 in each treatment. *p<0.05, **p<0.01, ***p<0.001 vs. NASH without CFE, *p<0.05, **p<0.01, ***p<0.001 vs. without CFE in each treatment by a one-way ANOVA with Tukey-Kramer test.
in the Dt and the Dt+Ex groups and increased energy expenditure in Ex group. Energy efficiency was lower in CFE groups compared to each treatment without CFE. Liver weights were also lower in the Dt and the Dt+Ex groups compared to the NASH group. This effect of reduced liver weight was attenuated by CFE in each treatment group. Plasma lipid levels were not affected by diet-treatment and/or exercise, but CFE slightly decreased TG levels in each treatment group. On the other hand, plasma AST and ALT levels were decreased in the Dt and the Dt+Ex groups compared to the NASH group, but CFE suppressed this improvement in all the treatment groups. Moreover, CFE significantly increased ALP in all treatment groups.

Hepatic TC and TG contents were significantly decreased in the Dt and the Dt+Ex groups when compared to the NASH group without CFE condition. CFE slightly suppressed TG contents in the NASH, the Ex, and the Dt+Ex groups compared to each treatment without CFE. However, CFE suppressed TG lowering effect in the Dt group (Fig. 1).

**CFE attenuated improvement of inflammatory cytokine gene expression in diet-treatment**

There was no significant difference in IL-1β, TNF-α, F4/80 (macrophage marker) and serum amyloid A (SAA) (inflammatory protein) mRNA expression levels among all treatment groups (Fig. 2). However, IL-1β, TNF-α, F4/80, and SAA mRNA expression levels were low in the Dt group compared to the NASH group, even though the reduction was not statistically significant. CFE suppressed this improvement in IL-1β, TNF-α, and SAA in each treatment.

**CFE induced major Cyp subtype gene expression and activities in each treatment**

Cyp1a1 mRNA expression level was high in the Dt and the Dt+Ex groups compared to the NASH group. CFE induced Cyp1a1 mRNA expression level in the NASH group, but not in the Dt, the Ex, and the Dt+Ex groups (Fig. 3a). Cyp1a2 mRNA expression level did not change in any treatment of the Dt, the Ex, and the Dt+Ex groups. CFE significantly induced Cyp1a2 mRNA expression level in only the Dt+Ex group (Fig. 3b). Cyp2r29 mRNA expression was slightly high in the Dt, the Ex, and the Dt+Ex groups compared to the NASH group. CFE significantly induced Cyp2r29 mRNA expression in all treatments of Dt, Ex, and Dt+Ex, and this effect was greater in the Dt and the Dt+Ex groups (Fig. 3c). Cyp3a11 mRNA expression was slightly high in the Dt, the Ex, and the Dt+Ex groups compared to the NASH group. CFE significantly induced Cyp3a11 mRNA expression in all treatments of Dt, Ex, and Dt+Ex (Fig. 3d).

There was no significant difference in CYP activities among all groups without CFE exposure. However, CFE significantly induced all CYP1A1, CYP1A2, CYP2C, and CYP3A activities and this effect was greater in the Dt and the Dt+Ex groups (Fig. 3e-h).

**DISCUSSION**

In this study, we examined the effects of CFE on the diet-treatment and/or exercise of NASH in mice model. Diet-treatment improved NASH, but CFE attenuated this improvement in addition. CFE induced major CYP subtype activities during improvement of NASH by diet-treatment and/or exercise. One important finding in this study is that CFE enhanced body weight loss by diet-treatment and exercise. In addition, diet-treatment, but not exercise, improved lipid accumulation in the liver, but CFE attenuated this improvement. Previously, our laboratory showed that CFE induced fatty liver in the rodent model.
However, hepatic lipid accumulation induced by CFE was dependent on the composition of the diet. CFE in high-starch diet increased lipid accumulation, but in high-fat diet did not promote much lipid accumulation (34). One possible mechanism of different observed effects on hepatic lipid accumulation is that CFE might induce fatty acid synthesis from glucose in high-starch diet. Forskolin did not affect hepatic lipid accumulation (34). In addition to lipid accumulation, inflammation is closely linked to the onset of NASH as a second hit (7, 8). Diet-treatment, but not exercise, slightly suppressed inflammatory cytokine gene expression in the liver, even though it did not reach statistical significance. CFE also attenuated this improvement. These results suggest that diet-treatment improved NASH in our mouse model, but CFE influences not only lipid accumulation, but also inflammation which associate with NASH condition.

Diet-treatment and exercise-regimen are the first choice to treat NASH, because no effective medication for NASH is currently available. However, NASH patients often suffer from dyslipidemia, insulin resistance, or diabetes mellitus, therefore medication is prescribed to treat these diseases along with diet-treatment and exercise in NASH patients. Previously, our group reported that mice fed a 0.5% CFE in control diet for 3 wk, which is the same condition in this experiment, showed markedly induced hepatic CYP2B, CYP2C, and CYP3A activities (28). In addition, we also reported that CYP1A1, 1A2, and 3A activities were higher in HFHC-diet induced NASH mice than in control, whereas CYP2C activity was significantly lower in HFHC-diet induced NASH mice compared to control mice (36). So, we evaluated whether CFE affect CYP activity during diet-treatment and/or exercise in NASH. In our experimental condition, diet-treatment and/or exercise did not affect hepatic CYP activities. In this condition, CFE induced major CYP subtype activities under diet-treatment and/or exercise in NASH and this induction was greater in the group under diet-treatment, which fed on a control diet, compared to the exercise group which fed on a high-fat based NASH-diet. Previously, we also reported that the intensity of CYP induction by CFE was different based on the macronutrient composition of diet. Induction of CYP2B activity by CFE was higher in control diet (7% soybean oil) compared to high fat diet (29.9% soybean oil) (34). However, protein composition (7%, 20%, and 33%) did not affect induction of CYP2B activity by CFE. In addition, inductions of CYP2C and CYP3A activities by CFE were not affected in macronutrient composition in diet. However, it is reported that nutrients and other dietary materials in food itself influenced CYP activities (37). In this study, CFE induced all CYP1A1, 1A2, 2C, and 3A activities, but these effects were greater in Dt and Dt+Ex groups fed a control diet (P : F : C = 17 : 4 : 68).
compared to NASH and Ex groups fed a NASH diet (P : F : C=21 : 24 : 43). This suggests that the difference of composition of diet, especially fat, might influence induction of CYP activity by CFE.

Again, CFE induced major CYP subtype activities and this effect was different in different diet conditions. So, we should be concerned about the influence of CFE on medication. NASH patients with diabetes mellitus may be prescribed rosiglitazone, pioglitazone and repaglinide which metabolized primarily by CYP2C8, tolbutamide, which metabolized primarily by CYP2C9 in humans. In other cases, patients with hyperlipidemia may be prescribed simvastatin, lovastatin, or atorvastatin, which are metabolized by CYP3A4, cerivastatin is metabolized by CYP2C8 and CYP3A4, fluvastatin is metabolized by CYP2C9. Even though our findings were in mice, we cannot deny the possibility of its occurrence in humans. Patients who take these medicines should avoid concomitant use of CFE.

Forskolin activated adenylate cyclase, increased the production of cAMP, enhanced lipolysis in fat cells, and induced weight loss in human studies. We also reported both forskolin and CFE suppressed body weight gain in mice model.
reports suggest that anti-obese effects of CFE are derived by forskolin. On the other hand, our group also reported that CFE induced liver damages, steatosis, and major CYP activations (27, 28), but forskolin did not. At this time, we do not know that which ingrediants in CFE are associated to these adverse effects. These results indicated that forskolin has beneficial effects, but other ingredients in CFE cause adverse effects. However, it is also reported that consumers experienced diarrhea at high rate by CFE supplement use (26, 42). The possible mechanism is that increased levels of cAMP by folskolin lead to the secretion of water in the intestine, and cause diarrhea in human. In this regard, not only CFE, but also forskolin have both merits and demerits of being used in humans.

In this study, we demonstrated that diet-treatment was more effective in NASH treatment compared to exercise, and CFE suppressed the improvement of NASH by diet-treatment. Furthermore, CFE induced major CYP subtypes mRNA expression and activities. These results suggest that CFE not only suppress the benefit of diet-treatment, but also influences the effect of medication on NASH. Further investigation is needed to clarify the influence of CFE and its active ingredient, forskolin, on NASH in human.

Disclosure of state of COI
The authors declare no conflict of interest.

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REFERENCES
1) Jou J, Choi SS, Diehl AM. 2008. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* **28**: 370–379.

2) Anstee QM, Targher G, Day CP. 2013. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nat Rev Gastroenterol Hepatol* **10**: 330–344.

3) Ludwig J, Viggiano TR, McGill DB, Oh BJ. 1980. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* **55**: 434–438.

4) Sheth SG, Gordon FD, Chopra S. 1997. Nonalcoholic steatohepatitis. *Ann Intern Med* **126**: 137–145.

5) Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, Natale S, Vanni E, Villanova N, Melchionda N, Rizzetto M. 2003. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* **37**: 917–923.

6) Rector RS, Tbyfault JP, Wei Y, Ibdah JA. 2008. Non-alcoholic fatty liver disease and the metabolic syndrome: an update. *World J Gastroenterol* **14**: 185–192.

7) Te Sligte K, Bourass I, Sels JP, Driessen A, Stockbrugger RW, Koek GH. 2004. Non-alcoholic steatohepatitis: review of a growing medical problem. *Eur J Intern Med* **15**: 10–21.

8) van Hoek B. 2004. Non-alcoholic fatty liver disease: a brief review. *Scand J Gastroenterol Suppl* **241**: 56–59.

9) Nishijima C, Kobayashi E, Sato Y, Chiba T. 2019. A nationwide survey of the attitudes toward the use of dietary supplements among Japanese high-school students. *Nutrients* **11**: pii: E1469.

10) Kobayashi E, Sato Y, Umegaki K, Chiba T. 2017. The prevalence of dietary supplement use among college students: a nationwide survey in Japan. *Nutrients* **9**: pii: E1250.

11) Kobayashi E, Nishijima C, Sato Y, Umegaki K, Chiba T. 2018. The prevalence of dietary supplement use among elementary, junior high, and high school students: A nationwide survey in Japan. *Nutrients* **10**: pii: E1176.

12) Chiba T, Sato Y, Nakanishi T, Yokotani K, Suzuki S, Umegaki K. 2014. Inappropriate usage of dietary supplements in patients by miscommunication with physicians in Japan. *Nutrients* **6**: 5392–5404.

13) Kantor ED, Rehm CD, Du M, White E, Giovannucci EL. 2016. Trends in dietary supplement use among US adults from 1999–2012. *JAMA* **316**: 1464–1474.

14) Rashrash M, Schommer JC, Brown LM. 2017. Prevalence and predictors of herbal medicine use among adults in the United States. *J Patient Exp* **4**: 108–113.

15) Lentjes MA, Welch AA, Keogh RH, Luben RN, Khaw KT. 2015. Opposites don’t attract: high spouse concordance for dietary supplement use in the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) cohort study. *Public Health Nutr* **18**: 1060–1066.

16) Metzger H, Lindner E. 1981. The positive inotropic-acting forskolin, a potent adenylate cyclase activator. *Arzneimittel Forschung* **31**: 1248–1250.

17) Seamon KB, Duly JW. 1981. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. *J Cyclic Nucleotide Res* **7**: 201–224.

18) Ammon HP, Muller AB. 1985. Forskolin: from an ayurvedic remedy to a modern agent. *Planta Med* **51**: 473–477.

19) Nishijima C, Chiba T, Sato Y, Yamada H, Umegaki K. 2018. Nationwide online survey method to estimate ongoing adverse events caused by supplement use: Application to diarrhea. *Shokuhin Eiseigaku Zasshi (J Food Hygienic Soc Jpn)* **59**: 106–113.

20) Allen DO, Ahmed B, Naseer K. 1986. Relationships between cyclic AMP levels and lipolysis in fat cells after isoproterenol and forskolin stimulation. *J Pharmacol Exp Ther* **238**: 659–664.

21) Okuda H, Morimoto C, Tsujiita T. 1992. Relationship between cyclic AMP production and lipolysis induced by forskolin in rat fat cells. *J Lipid Res* **33**: 225–231.

22) Han LK, Morimoto C, Yu RH, Okuda H. 2005. Effects of Coleus forskohlii on fat storage in ovariectomized rats. *Yakugaku Zasshi (Jpn J Pharmacol)* **125**: 449–453.

23) Godard MP, Johnson BA, Richmond SR. 2005. Body composition and hormonal adaptations associated with forskolin consumption in overweight and obese men. *Obes Res* **13**: 1335–1343.

24) Henderson S, Magu B, Rasmussen C, Lancaster S, Kerkovich C, Smith P, Melton C, Cowan P, Greenwood M, Earnest C, Almada A, Milnor P, Magrans T, Bowden R, Onnpraseth S, Thomas A, Kreider RB. 2005. Effects of Coleus forskohlii supplementation on body composition and hematological profiles in mildly overweight women. *J Int Soc Sports Nutr* **2**: 54–62.

25) Pittler MH, Schmidt K, Ernst E. 2005. Adverse events of herbal food supplements for body weight reduction: systematic review. *Obes Rev* **6**: 93–111.

26) Nishijima C, Chiba T, Sato Y, Umegaki K. 2019. Nationwide online survey enables the reevaluation of the safety
of Coleus forskohlii extract intake based on the adverse event frequencies. Nutrients 11: pii: E866.
27) Virgona N, Taki Y, Yamada S, Umegaki K. 2013. Dietary Coleus forskohlii extract generates dose-related hepatotoxicity in mice. J Appl Toxicol 33: 924–932.
28) Virgona N, Yokotani K, Yamaizaki Y, Shimura F, Chiba T, Taki Y, Yamada S, Shinozuka K, Murata M, Umegaki K. 2012. Coleus forskohlii extract induces hepatic cytochrome P450 enzymes in mice. Food Chem Toxicol 50: 750–755.
29) Yokotani K, Chiba T, Sato Y, Taki Y, Yamada S, Shinozuka K, Murata M, Umegaki K. 2012. Hepatic cytochrome P450 mediates interaction between warfarin and Coleus forskohlii extract in vivo and in vitro. J Pharm Pharmacol 64: 1793–1801.
30) Virgona N, Taki Y, Umegaki K. 2010. A rapid HPLC with evaporative light scattering method for quantification of forskolin in multi-herbal weight-loss solid oral dosage forms. Pharmazie 65: 322–326.
31) Chiba T, Suzuki S, Sato Y, Itoh T, Umegaki K. 2016. Evaluation of methionine content in a high-fat and choline-deficient diet on body weight gain and the development of non-alcoholic steatohepatitis in mice. PLoS One 11: e0164191.
32) Cho J, Lee I, Kim D, Koh Y, Kong J, Lee S, Kang H. 2014. Effect of aerobic exercise training on non-alcoholic fatty liver disease induced by a high fat diet in C57BL/6 mice. J Exerc Nutr Biochem 18: 339–346.
33) Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226: 497–509.
34) Yokotani K, Chiba T, Sato Y, Nakamishi T, Murata M, Umegaki K. 2013. Influence of dietary macronutrients on induction of hepatic drug metabolizing enzymes by Coleus forskohlii extract in mice. J Nutr Sci Vitaminol 59: 37–44.
35) Umegaki K, Yamazaki Y, Yokotani K, Chiba T, Sato Y, Shimura F. 2014. Induction of fatty liver by Coleus forskohlii extract through enhancement of de novo triglyceride synthesis in mice. Toxicol Rep 1: 787–794.
36) Suzuki S, Sato Y, Umegaki K, Chiba T. 2015. The major cytochrome P450 subtype activities in diet-induced non-alcoholic steatohepatitis mouse model. Endocrinol Metab Syndrome 4: 1000190.
37) Guengerich FP. 1995. Influence of nutrients and other dietary materials on cytochrome P-450 enzymes. Am J Clin Nutr 61: 651s–658s.
38) Daily EB, Aquilante CL. 2009. Cytochrome P450 2C8 pharmacogenetics: a review of clinical studies. Pharmacogenomics 10: 1489–1510.
39) Komatsu K, Ito K, Nakajima Y, Kanamitsu S, Imaoka S, Funae Y, Green CE, Tyson CA, Shimada N, Sugiyama Y. 2000. Prediction of in vivo drug-drug interactions between tolbutamide and various sulfonamides in humans based on in vitro experiments. Drug Metab Dispos 28: 475–481.
40) Neuvonen PJ, Niemi M, Backman JT. 2006. Drug interactions with lipid-lowering drugs: mechanisms and clinical relevance. Clin Pharmacol Ther 80: 565–581.
41) Jacobsen W, Kuhn B, Soldner A, Kirchner G, Sewing KF, Kollman PA, Benet LZ, Christians U. 2000. Lactonization is the critical first step in the disposition of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor atorvastatin. Drug Metab Dispos 28: 1369–1378.
42) Kamohara S, Terasaki Y, Horikoshi I, Sunayama S. 2015. Safety of a Coleus forskohlii formulation in healthy volunteers. Personalized Medicine Universe 4: 63–65.