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Components of Boiogito Suppress the Progression of Hypercholesterolemia and Fatty Liver Induced by High-Cholesterol Diet in Rats

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ABSTRACT

Background Ogi, one main component of boiogito (BOT), is reported to have an effect on hypercholesterolemia and NAFLD. In this experiment, we examined effects of ogi on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats and compared with the effects of ogi combined with ginger or hesperidin.

Methods Hypercholesterolemia and fatty liver were induced by a high cholesterol diet in rats. Extract of ogi, ogi with hesperidin, and ogi with ginger were added to the high-cholesterol diet, respectively. Ezetimibe was also added to the high-cholesterol diet as a positive control. After 6 and 12 weeks, body, liver and adipose tissue weights, blood chemistry, lipid-related and inflammatory-related factors were examined.

Result The high cholesterol diet increased body, liver and adipose tissue weights, and serum cholesterol concentrations. Ogi, ogi with hesperidin or ginger and ezetimibe improved them. In the histological examinations, we observed a significant improvement after treatment. The lipid-related factors (RBP4, HFABP and CFABP) were improved by treatment. Biomarkers of cholesterol synthesis (lathosterol) and absorption (campesterol, beta-sitosterol) were lower in the treatment groups. Inflammatory-related factors (MCP1, CCR2 and TNF-alpha) and ICAM-1 were ameliorated after treatment, especially by ogi with ginger.

Conclusion Ogi, ogi with hesperidin or ginger have a similar effect of BOT and ezetimibe on hypercholesterolemia and fatty liver. Ogi with ginger reveals a stronger additive effect with no significant difference. However, as for the anti-inflammatory (MCP1, CCR2 and TNF-alpha) and anti-arteriosclerotic (ICAM-1) effects, additive effects of ogi with ginger are more potent than that of ogi alone or ezetimibe.

Key words fatty liver; ginger; hesperidin; hypercholesterolemia; ogi

The increased prevalence of hypercholesterolemia and fatty liver diseases has provided an increasingly negative connotation toward lipids and cardiovascular diseases (CVD), such as atherosclerosis and hypertension.[1, 2] Non-alcoholic fatty liver disease (NAFLD) occurs more frequently in patients with hypercholesterolemia.[3] During the past few years, increasing evidence shows that NAFLD has also become a multisystem disease that affects many extra-hepatic organ systems, including the heart and the vascular system.[4] Much experimental, clinical and epidemiological evidence supports a strong association between NAFLD and CVD, especially atherosclerosis.[5, 6]

A lot of lipid metabolism-related molecules and inflammatory cytokines are associated with the progression of hypercholesterolemia and NAFLD. Retinol binding protein 4(RBP4) is a 21 kDa secreted protein that is the transport protein for vitamin A (retinol) in blood.[7] It takes part in the control of metabolic and proliferative cell functions,[8] including steatogenesis.[9] The RBP4 gene has been linked to increasing fasting glucose levels[10] and related to hypercholesterolemia and NAFLD.[11] Heart-type fatty acid binding protein (HFABP) and cutaneous fatty acid-binding protein (CFABP) belong to the fatty acid binding protein (FABP) super-family, which is as-

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; BOT, boiogito; BS, blood sugar C, control group; CFABP, cutaneous fatty acid-binding protein; Cr, creatinine; CVD, cardiovascular disease; FABP, fatty acid binding protein; H, high-cholesterol diet group; HO, high-cholesterol diet with ogi group; HOH, high-cholesterol diet with ogi and hesperidin group; HOG, high-cholesterol diet with ogi and ginger group; HDL-C, high-density lipoprotein cholesterol; HE, high-cholesterol diet with ezetimibe group; HFABP, heart fatty acid-binding protein; ICAM-1, intercellular adhesion molecule-1; LDH, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; MCP1, monocyte chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; RBP4, retinol-binding protein 4; RT-PCR, real-time polymerase chain reaction; TBil, total bilirubin; TC, total cholesterol; TG, triglyceride; VEC, vascular endothelial cells

signed to transport fatty acids towards the mitochondria for beta-oxidation and energy expenditure.[12, 13] Recent findings indicate that monocyte chemoattractant protein 1 (MCP1) and its receptor C-C chemokine receptor type 2 (CCR2) play a key role in hypercholesterolemia[14] and CVD.[15] Intercellular adhesion molecule-1 (ICAM-1) is over expressed in conditions with evidence of endothelial inflammation such as atherosclerosis.[16] TNF-alpha plays a similar role with ICAM-1 in atherosclerosis.[17] Therefore, in this study, we have monitored the expression of RBP4, HFABP, CFABP, MCP1, CCR2, ICAM-1 and TNF-alpha to detect the mechanisms of hypercholesterolemia and atherosclerosis.

Based on evidence from our previous study, we proved that boiogito (BOT) has a protective effect on the progression of hypercholesterolemia and fatty liver. And the anti-inflammatory (MCP1, CCR2) and anti-arteriosclerotic (ICAM-1) effects of BOT are more potent than ezetimibe.[18] Our results are consistent with another previous study.[19] Ogi (Astragalus membranaceus Bunge), one main component of BOT, has been reported to have an effect on hypercholesterolemia and NAFLD. Ogi showed statistically significant beneficial effects on alanine aminotransferase (ALT) and glutamyltransferase activity,[26] and significantly lowered plasma total cholesterol by 45.8%, triglycerides by 30%, and low-density lipoprotein-cholesterol (LDL-C) by 47.4%, comparable to simvastatin.[27] We have also proven that ginger (Zingiber officinale Roscoe) and hesperidin could improve hypercholesterolemia and NAFLD by inhibiting both the synthesis and absorption of cholesterol and regulating the expression of mRNA for RBP4, HFABP and CFABP.[11, 20] We have also proven that hesperidin combined with ginger has a stronger effect than hesperidin alone on hypercholesterolemia.[21] However, the inhibitory effect of the key components of BOT (ogi and ginger) on hypercholesterolemia and NAFLD has not yet been proven. Moreover, the synergistic action of ogi with hesperidin is unclear. In this experiment, we examined the effects of ogi on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats and compared with the effects of ogi combined with ginger or hesperidin. Furthermore, we have studied the mechanism of these kampo components on hypercholesterolemia and the protective effect on atherosclerosis.

MATERIALS AND METHODS

Rats and feeding method

Sixty 8-week-age male Wistar rats (purchased from Charles River Laboratories Japan) were acclimated in an air-conditioned room at 25 °C with 55% humidity and given standard chow for 3 days. Then the rats were divided into six groups: control group (C, $n = 10$), high-cholesterol diet group (H, $n = 10$), high-cholesterol diet with ogi group (HO, $n = 10$), high-cholesterol diet with ogi and hesperidin group (HOH, $n = 10$), high-cholesterol diet with ogi and ginger group (HOG, $n = 10$), high-cholesterol diet with ezetimibe group (HE, $n = 10$). The rats in each group were numbered from 1 to 10. Group C was fed a standard rat diet (moisture 8.83%, protein 25.13%, fat 4.92 %, fiber 4.42% and crude ash 6.86%, CE-2; Japan Clea, Tokyo, Japan). A high-cholesterol diet[11, 20] was supplied for Group H; it was made by adding 2% cholesterol and 0.5% cholic acid to the standard diet. The high-cholesterol diet with ogi was made by adding 1% ogi extract to the high-cholesterol diet. The high-cholesterol diet with ogi and hesperidin was made by adding 1% ogi extract and 0.08% hesperidin[11] (Wako Pure Chemical Industries, Osaka, Japan). The high-cholesterol diet with ogi and ginger was made by adding 1% ogi extract and 1% ginger extract. Ogi and ginger extracts were purchased from Tsumura & CO. (Tokyo). The high-cholesterol diet with ezetimibe was made by adding 0.0006% of ezetimibe (LKT laboratories, St Paul, MN). The concentrations of the extracts administered were calculated based on body surface area. Adult patients take ogi, ginger extract, hesperidin and ezetimibe 15 g, 15 g, 1.2 g and 8 mg per day, respectively. The body surface area conversion coefficient from humans to rats is 0.018. The amount of feed for each rat was regulated to 25 g/day and water was supplied ad libitum. Body weights, systolic and diastolic blood pressure and heart rate were measured every week. Blood pressure and heart rate were measured by a noninvasive computerized tail-cuff method (BP-98A; Softron, Tokyo).

Sample collection

On days 42 and 84, 5 rats of each group in the order of how they were numbered were sacrificed by collecting blood from the heart under pentobarbital anesthesia after fasting for 12 h. Liver tissue, adipose tissue around the left kidney, and abdominal aorta were removed for specimen, and then portions of the samples were stored in a 10% formalin solution for hematoxylin and eosin (HE) and oil red O staining.[22] The remaining samples were immediately transferred into EP tubes containing 500 µL of RNA later (Ambion, Austin, TX), quickly frozen in liquid nitrogen, and stored at –80 °C. Blood chemistry was analyzed for rats using an auto analyzer at an accredited clinical laboratory (SRL, Tokyo).

ICAM-1 and RBP4 enzyme-linked immunosorbent assay (ELISA)

Serum samples were applied for an ELISA of ICAM-1 (R&D, Minneapolis, MN) and RBP4 (Aviscera Bioscience), according to the manufacturer's instructions.

RT-PCR

Total RNA from the liver and adipose tissue around the left kidney was extracted using TRIzol reagent according to the manufacturer's instructions (Promega, Carlsbad, CA). A semiquantitative real-time polymerase chain reaction (RT-PCR) was performed using Line-Gene (Toyobo, Tokyo) and SYBR Green I (Roche, Basel, Switzerland). The detection was executed at the extension reaction stage in each cycle. The primer sets for RBP4, HFABP, CFABP, MCP1, CCR2 and beta-actin mRNA were all synthesized by Hokkaido System Science (Sapporo, Japan). The sequence of each primer is listed in Table1. Using the $2^{-\Delta CT}$ method, mRNA expression was semi-quantitatively measured as a relative amount of each target RNA to a known housekeeping gene (beta-actin) expression level.

Table 1. The sequence of each PCR primer

Gene	Primer
RBP4	Forward 5′-gacaaggctcgtttctctgg-3′
	Reverse 5′-gactcgtcccttggctgtag-3′
HFABP	Forward 5′-ctagcatgagggaagcaagg-3′
	Reverse 5′-tgcttcatccagacaagtgg-3′
CFABP	Forward 5′-gggctggctcttaggaagat-3′
	Reverse 5′-aaaacacggtcgtcttcacc-3′
MCP1	Forward 5′-ctgtagcatccacgtgctgt-3′
	Reverse 5′-tgctgctggtgattctcttg-3′
CCR2	Forward 5′-gatcctgcccctacttgtca-3′
	Reverse 5′-agatgagcctcacagcccta-3′
Beta-actin	Forward 5′-gtagccatccaggctgtgtt-3′
	Reverse 5′-ccctcagatgggcacagt-3′

CCR2, C-C chemokine receptor type 2; CFABP, cutaneous fatty acid-binding protein; HFABP, heart fatty acid-binding protein; MCP1, monocyte chemoattractant protein-1; PCR, polymerase chain reaction; RBP4, retinol-binding protein 4.

Western blotting

Proteins from the liver were extracted using CelLytic MT according to the manufacturer's instructions (Sigma, St. Louis, MO). We performed western blotting with 20 mg/mL of proteins and the i-Blot gel transfer system (Invitrogen, Tokyo). Anti- TNF-alpha (1:500 dilutions, Sigma) and beta-actin (MBL, Nagoya, Japan) antibodies were used in the study according to the manufacturer's instructions. Chemiluminescent signals were detected within 1 min with LAS-4000 (Fujifilm, Tokyo). The gels were run under the same experimental conditions. The expression of TNF-alpha was normalized by beta-actin. The Gel-Pro Analyzer 4.0 software (Media Cybernetics, Rockville, MD) was used to analyze the integrating optical density of target band.

Immunohistochemical studies

During the immunohistochemical analyses, 4% paraformaldehyde-fixed aorta tissue specimens were processed. Anti-ICAM-1 (Abcam, Cambridge, United Kingdom) was used. As a negative control, tissues were stained without the primary antibody. The optical densities were measured by Image-Pro Plus v 6.0 software (Media Cybernetics, Rockville, MD).

The condition of LC/MS/MS analysis

Lathosterol, campesterol and beta-sitosterol were analyzed using a HPLC Prominence (Shimadzu, Kyoto, Japan) coupled with a mass spectrometer (AB SCIEX QTRAP 5500 LC/MS/MS System, SCIEX, Tokyo). In brief, separations of lathosterol, campesterol and beta-sitosterol were carried out using a Poroshell 120 EC-C18 column 2.1 × 150 mm, 2.7 μm particle size (Agilent Technologies, CA). The elution (150 μL/min) was completed at 40 °C in 25 min with linear gradient from 20% eluent B (ethanol) to 80% of eluent B (acetonitrile).The injection volume was 10 μL. The LC–MS instrument was operated in the positive atmospheric pressure chemical ionization (APCI) mode with corona current of 3 μA, and capillary temperature of 500 °C. Declustering and entrance potential were set at 60 and 10 V, respectively. MS/MS spectra at m/z 315 were obtained, and parameters for the specific MRM transition were optimized. The collision energy was 30 V. MRM transition of 315.0 → 176.0 was found to be the optimal transitions.

Statistical analyses

The data are expressed as the means ± standard deviation. Statistical comparisons were made using the two independent samples *t*-test by SPSS 11.0J (IBM, Armonk, NY). $P < 0.05$ was considered statistically significant.

RESULTS

Histological examination of liver tissues

From hematoxylin-eosin staining we can found that the fatty degeneration (steatosis) of liver was observed in high-cholesterol diet-fed Groups, but not in Group C. The tiny and large vacuoles as well as pleomorphic nuclei were more conspicuous in treatment groups than in Group H (Fig. 1A). Oil red O staining revealed the same changes that the livers in all high-cholesterol diet supplemented groups were filled with microvesicular or macrovesicular fat deposits; they were depicted as reddish deposits (Fig. 1B). Groups HOG and HE seems to show better effect (× 400).

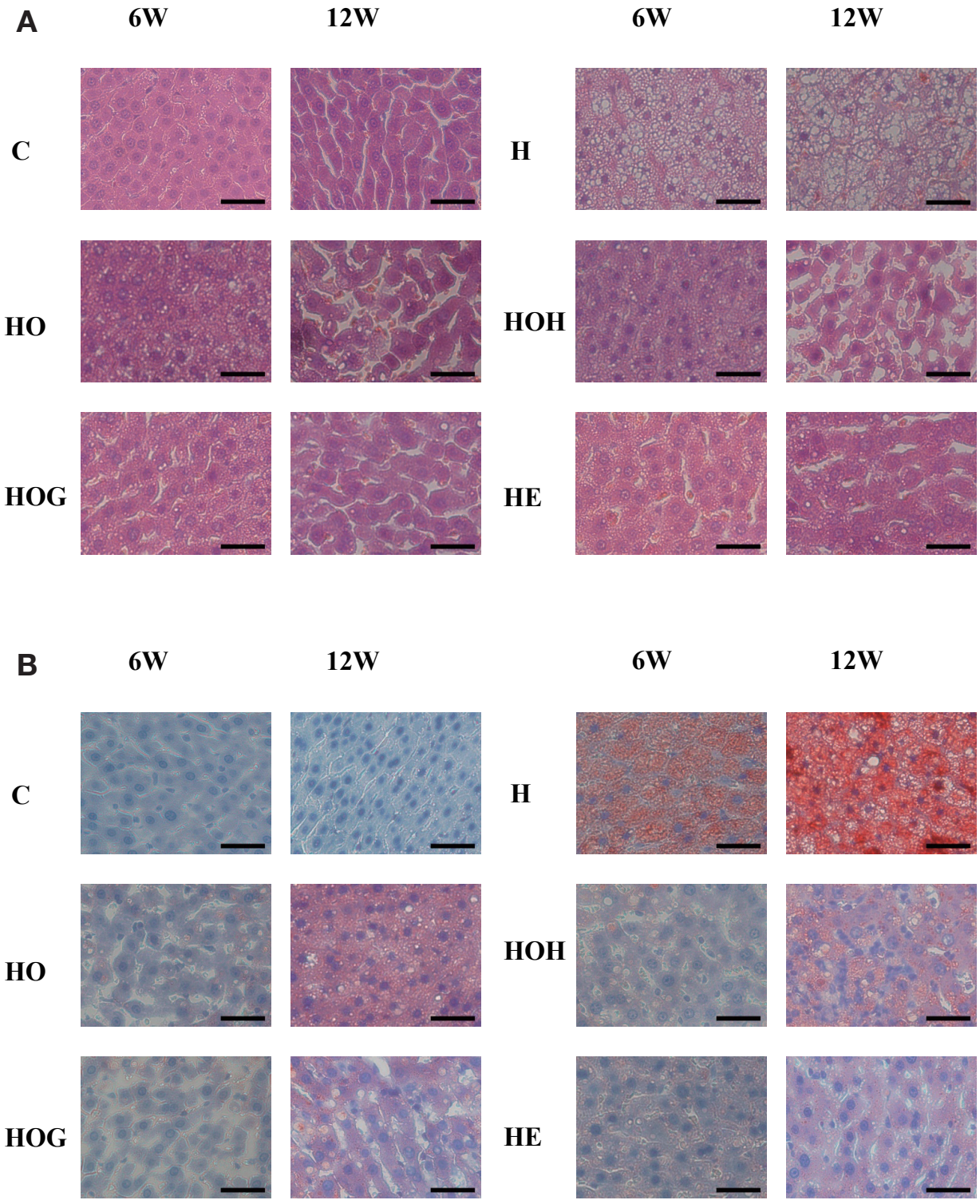

Fig. 1. Histopathological examination of liver.
A: Fatty degeneration (steatosis) of the liver is observed in the high-cholesterol diet–fed groups (H, HO, HOH, HOG and HE), but not in Group C in hematoxylin-eosin stained tissues. Fatty changes increased with time to a greater extent in Group H than Groups HO, HOH, HOG and HE. **B**: Oil red O staining revealed more lipid droplets (stained red) to be accumulated in vacuoles in Group H than other groups. 6W: 6 weeks, 12W: 12 weeks. C: control, standard diet for 6 and 12 weeks ($n = 5$); H: high-cholesterol diet for 6 and 12 weeks ($n = 5$); HO: high-cholesterol diet with ogi for 6 and 12 weeks ($n = 5$); HOH: high-cholesterol diet with ogi and hesperidin for 6 and 12 weeks ($n = 5$); HOG: high-cholesterol diet with ogi and ginger for 6 and 12 weeks ($n = 5$); HE: high-cholesterol diet with ezetimibe for 6 and 12 weeks ($n = 5$). Bars express 25 μm.

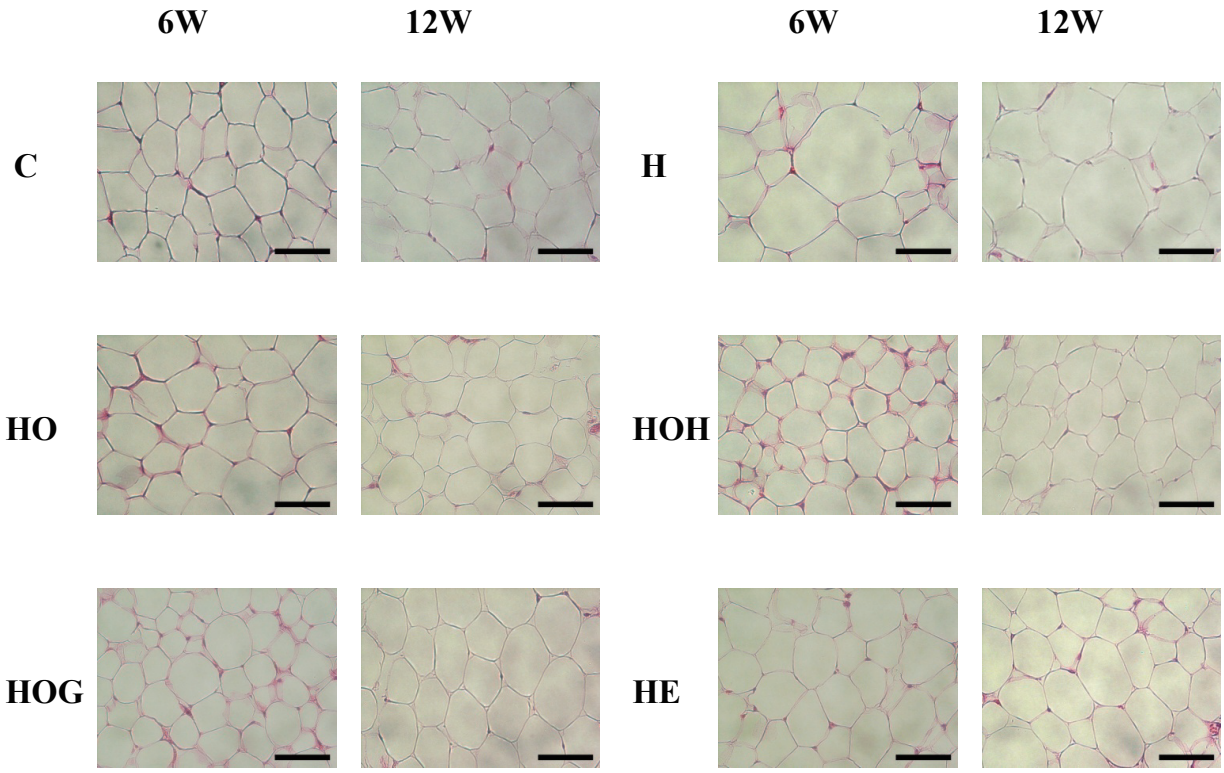

Fig. 2. Histopathological examination of adipose tissue.
The larger fat cells were observed in high-cholesterol diet-fed groups (H, HO, HOH, HOG and HE), but not in Group C (55.16 ± 7.21 μm and 55.78 ± 6.58 μm after 6 and 12 weeks respectively) in hematoxylin-eosin stained tissues. Fat cells enlarged with time to a greater extent in Group H (90.91 ± 12.73 and 94.03 ± 8.54 μm in diameter after 6 and 12 weeks respectively) than Groups HO, HOH, HOG and HE (54.94 ± 9.22 μm and 59.16 ± 8.93 μm, 53.28 ± 8.40 μm and 56.88 ± 7.99 μm, 46.09 ± 7.66 μm and 54.54 ± 8.91 μm, 58.91 ± 8.25 μm and 59.06 ± 7.00 μm in diameter after 6 and 12 weeks respectively). 6W: 6 weeks, 12W: 12 weeks. C: control, standard diet for 6 and 12 weeks (n = 5); H: high-cholesterol diet for 6 and 12 weeks (n = 5); HO: high-cholesterol diet with ogi for 6 and 12 weeks (n = 5); HOH: high-cholesterol diet with ogi and hesperidin for 6 and 12 weeks (n = 5); HOG: high-cholesterol diet with ogi and ginger for 6 and 12 weeks (n = 5); HE: high-cholesterol diet with ezetimibe for 6 and 12 weeks (n = 5). Bars express 50 μm.

Histological examination of adipose tissue

The larger fat cells were observed in high-cholesterol diet-fed groups (H, HO, HOH, HOG and HE), but not in Group C. It was more notable in Group H than Groups HO, HOH, HOG and HE (Fig. 2, × 400).

Body, liver, and adipose tissue weights

During the whole experiment, the amount of feed for each rat was regulated to 25g/day and there was no significant difference in each group. As Table 2 shows, there were no significant differences in the baseline of body weights at the beginning of the experiment. High-cholesterol diet increased body, liver and adipose tissue weights in Group H compared with Group C both after 6 and 12 weeks ($P < 0.05$). Body, liver and adipose tissue weights were significantly lower in Groups HO, HOH, HOG, HE than Group H after 6 and 12 weeks ($P < 0.05$). Liver weights in Group HE were lower than that in Groups HO, HOH and HOG after 6 weeks ($P < 0.05$).

Blood chemistry and cholesterol concentrations

As Table 3 shows, the high-cholesterol diet increase the concentrations of LDL-C, TC, ALT, ALP, LDH and reduce the concentrations of HDL-C in Group H compared with Group C after 6 and 12 weeks in serum ($P < 0.05$). Compared with Group H, the concentrations of LDL-C and TC decreased in Groups HOH, HOG, HE after 6 weeks and Groups HO, HOH, HOG, HE after 12 weeks ($P < 0.05$), while the concentration of HDL-C has a opposite trends ($P < 0.05$). After 12 weeks, the activities of ALT, ALP, LDH reduced in different degree in Groups HO, HOH, HOG, HE compared with Group H ($P < 0.05$). The BS, TBil, TG and Cr have no significant differences during the experiment.

Lathosterol, Campesterol and beta-Sitosterol concentrations determined by LC/MS/MS

Concentrations of serum lathosterol decreased in the Group HO, HOH, HOG and HE compared with Group H (Table 4, $P < 0.05$). The same trend was found in the concentrations of serum campesterol and beta-sitosterol, which were lower in the treatment groups (HO, HOH, HOG and HE) than Group H ($P < 0.05$).

ICAM-1 and RBP4 in serum

ICAM-1 concentrations were increased in Group H compared with Groups C, HO, HOH, HOG and HE group

Table 2. Body, liver and Lipid weights

		C	H	HO	HOH	HOG	HE
Body weights (g)	0W	308.68 ± 6.72	315.86 ± 8.56	310.47 ± 9.23	310.67 ± 8.77	311.73 ± 7.96	311.57 ± 7.59
	6W	453.03 ± 16.69	503.12 ± 17.27*	456.91 ± 17.68†	456.20 ± 22.70†	453.88 ± 20.55†	451.48 ± 18.23†
	12W	507.97 ± 24.12	576.09 ± 20.54*	497.23 ± 20.29†	503.52 ± 29.72†	506.20 ± 15.24†	510.63 ± 8.27†
Liver weights (g)	6W	11.66 ± 1.09	19.62 ± 1.90*	16.39 ± 1.03†	17.21 ± 1.09†	16.79 ± 2.15†	13.24 ± 1.76†‡§‖
	12W	13.26 ± 1.48	28.99 ± 4.42*	19.90 ± 5.03†	20.44 ± 3.06†	20.22 ± 3.32†	16.79 ± 3.40†
Adipose tissue weights (g)	6W	2.59 ± 0.67	3.74 ± 0.40*	2.35 ± 0.70†	2.78 ± 0.89†	2.09 ± 0.69†	2.58 ± 0.48†
	12W	3.53 ± 0.55	5.88 ± 0.50*	2.09 ± 0.50†	2.99 ± 0.74†	3.35 ± 0.30†	2.20 ± 0.90†‖

C: control, standard diet for 6 and 12 weeks ($n = 5$); H: high-cholesterol diet for 6 and 12 weeks ($n = 5$); HO: high-cholesterol diet with ogi for 6 and 12 weeks ($n = 5$); HOH: high-cholesterol diet with ogi and hesperidin for 6 and 12 weeks ($n = 5$); HOG: high-cholesterol diet with ogi and ginger for 6 and 12 weeks ($n = 5$); HE: high-cholesterol diet with ezetimibe for 6 and 12 weeks ($n = 5$). *$P < 0.05$ vs. Group C, †$P < 0.05$ vs. Group H, ‡$P < 0.05$ vs. Group HO, §$P < 0.05$ vs. Group HOH, ‖$P < 0.05$ vs. Group HOG. Data are expressed as the means ± standard deviation. 0W: 0 week, 6W: 6 weeks, 12W: 12 weeks.

Table 3. Blood chemistry and cholesterol concentrations

		C	H	HO	HOH	HOG	HE
LDL-C (mg/dL)	6W	9.20 ± 3.19	53.00 ± 17.90*	54.75 ± 18.91	35.75 ± 6.24†‡	28.33 ± 3.06†‡	9.00 ± 1.22†‡§‖
	12W	9.20 ± 1.30	65.67 ± 15.95*	29.60 ± 10.11†	41.00 ± 9.49†‡	31.75 ± 9.84†	28.20 ± 2.17†§
HDL-C (mg/dL)	6W	17.20 ± 4.02	6.40 ± 1.67*	6.60 ± 1.14	12.00 ± 2.74†‡	13.80 ± 4.15†‡	16.80 ± 2.28†‡§
	12W	19.00 ± 2.35	13.00 ± 1.63*	17.25 ± 0.96†	18.00 ± 1.00†	16.00 ± 1.00†	17.75 ± 2.06†
TC (mg/dL)	6W	56.80 ± 15.01	150.25 ± 34.06*	124.00 ± 33.09	106.00 ± 20.83†	90.00 ± 13.53†‡	54.60 ± 7.16†‡§‖
	12W	55.60 ± 8.20	160.75 ± 48.75*	83.80 ± 13.05†	115.60 ± 23.54†	120.00 ± 40.28†	94.00 ± 9.43†
TG (mg/dL)	6W	20.75 ± 5.50	18.60 ± 8.11	19.75 ± 4.57	21.20 ± 4.92	15.25 ± 3.40	13.00 ± 5.57
	12W	26.80 ± 8.81	31.80 ± 2.59	23.00 ± 6.96	28.50 ± 8.76	39.00 ± 10.10	17.40 ± 11.59
ALT (U/L)	6W	30.00 ± 3.67	50.80 ± 11.30*	70.00 ± 2.00	52.00 ± 3.39‡	46.33 ± 9.29‡	91.33 ± 20.53†‡§‖
	12W	30.40 ± 4.16	168.00 ± 53.84*	35.67 ± 3.79†	70.60 ± 19.19†	55.00 ± 14.14†	36.50 ± 10.47†
ALP (U/L)	6W	192.00 ± 25.50	372.75 ± 32.47*	363.67 ± 8.14	327.00 ± 29.46†	314.33 ± 28.94†‡	390.33 ± 20.74§‖
	12W	202.00 ± 25.29	515.67 ± 36.09*	258.80 ± 29.18†	289.33 ± 27.75†	264.33 ± 38.89†	265.25 ± 39.66†
TBil (mg/dL)	6W	0.05 ± 0.01	0.03 ± 0.01	0.041 ± 0.02	0.04 ± 0.01	0.03 ± 0.02	0.06 ± 0.00
	12W	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.01
Cr (mg/dL)	6W	0.34 ± 0.06	0.31 ± 0.03	0.42 ± 0.10	0.32 ± 0.05	0.41 ± 0.02	0.36 ± 0.04
	12W	0.37 ± 0.05	0.41 ± 0.09	0.40 ± 0.10	0.33 ± 0.03	0.32 ± 0.07	0.35 ± 0.07
LDH (U/L)	6W	156.00 ± 18.46	516.67 ± 32.75*	262.00 ± 32.08†	277.33 ± 25.48†	356.33 ± 33.56†‡§	342.67 ± 35.84†‡§
	12W	177.50 ± 64.57	599.00 ± 19.80*	192.00 ± 48.08†	214.00 ± 16.46†	318.00 ± 87.68†	282.33 ± 82.71†
BS (mg/dL)	6W	103.40 ± 7.54	92.80 ± 6.14	89.60 ± 10.41	108.33 ± 4.51	104.80 ± 7.56	93.67 ± 6.11
	12W	95.80 ± 10.66	101.80 ± 6.14	78.00 ± 15.07	82.20 ± 13.74	75.00 ± 13.17	103.20 ± 18.90

C: control, standard diet for 6 and 12 weeks ($n = 5$); H: high-cholesterol diet for 6 and 12 weeks ($n = 5$); HO: high-cholesterol diet with ogi for 6 and 12 weeks ($n = 5$); HOH: high-cholesterol diet with ogi and hesperidin for 6 and 12 weeks ($n = 5$); HOG: high-cholesterol diet with ogi and ginger for 6 and 12 weeks ($n = 5$); HE: high-cholesterol diet with ezetimibe for 6 and 12 weeks ($n = 5$). *$P < 0.05$ vs. Group C, †$P < 0.05$ vs. Group H, ‡$P < 0.05$ vs. Group HO, §$P < 0.05$ vs. Group HOH, ‖$P < 0.05$ vs. Group HOG. Data are expressed as the means ± standard deviation. 6W: 6 weeks, 12W: 12 weeks.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; BS, blood sugar; Cr, creatinine; HDL-C, high-density lipoprotein cholesterol; LDH, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; TBil, total bilirubin; TC, total cholesterol; TG, triglyceride.

Table 4. Lathosterol, campesterol, and beta-sitosterol concentrations in serum determined by LC/MS/MS

	C	H	HO	HOH	HOG	HE
Lathosterol (10^{-2} μg/mL)	1.81 ± 0.29	3.84 ± 0.43*	2.53 ± 0.88†	2.55 ± 0.37†	2.40 ± 0.41†	2.09 ± 0.79†
Campesterol (μg/mL)	1.19 ± 0.46	2.26 ± 0.18*	1.36 ± 0.36†	1.50 ± 0.33†	1.40 ± 0.84†	1.29 ± 0.04†
beta-Sitosterol (μg/mL)	0.77 ± 0.27	2.51 ± 0.35*	1.46 ± 0.39†	1.47 ± 0.11†	0.92 ± 0.24†‡§	0.95 ± 0.51†‡§

C: control, standard diet for 6 and 12 weeks ($n = 5$); H: high-cholesterol diet for 12 weeks ($n = 5$); HO: high-cholesterol diet with ogi for 12 weeks ($n = 5$); HOH: high-cholesterol diet with ogi and hesperidin for 12 weeks ($n = 5$); HOG: high-cholesterol diet with ogi and ginger for 12 weeks ($n = 5$); HE: high-cholesterol diet with ezetimibe for 12 weeks ($n = 5$). *$P < 0.05$ vs. Group C, †$P < 0.05$ vs. Group H, ‡$P < 0.05$ vs. Group HO, §$P < 0.05$ vs. Group HOH, ‖$P < 0.05$ vs. Group HOG. Data are expressed as the means ± standard deviation.

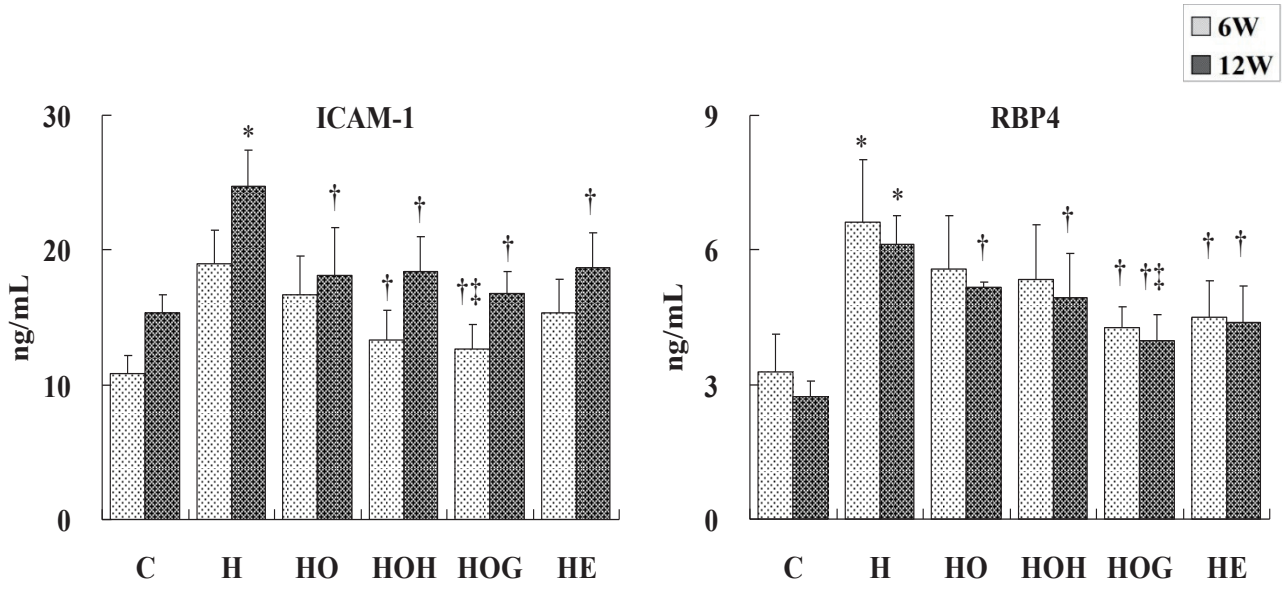

Fig. 3. ICAM-1 and RBP4 concentrations in serum.
ICAM-1 and RBP4 concentrations in standard diet group (C, $n = 5$), high-cholesterol diet group (H, $n = 5$), high-cholesterol diet with ogi group (HO, $n = 5$), high-cholesterol diet with ogi and hesperidin group (HOH, $n = 5$), high-cholesterol diet with ogi and ginger group (HOG, $n = 5$) and high-cholesterol diet with ezetimibe group (HE, $n = 5$). Dotted and shaded columns represent 6 and 12 weeks, respectively. *$P < 0.05$ vs. Group C, †$P < 0.05$ vs. Group H, ‡$P < 0.05$ vs. Group HO. 6W: 6 weeks, 12W: 12 weeks.
ICAM-1, intercellular adhesion molecule-1; RBP4, retinol-binding protein 4.

after 12 weeks (Fig. 3, $P < 0.05$). Compared with other treatment groups, Group HOG seemed to have the best effect. RBP4 concentrations were increased in Group H compared with the other groups, including Groups HOG and HE after 6 weeks ($P < 0.05$) and Groups HO, HOH, HOG and HE after 12 weeks ($P < 0.05$). It has less concentration in Group HOG than Group HO after 12 weeks ($P < 0.05$).

mRNA expression in liver and adipose tissue

As Fig. 4 shows, RBP4 mRNA expression in liver in Group H was significantly increased compared to that in other groups ($P < 0.05$). HFABP mRNA expression in Group H was significantly increased compared to that in Groups C, HOH, HOG and HE after 6 weeks ($P < 0.05$), and then continued to increase after 12 weeks compared to all the other 5 groups ($P < 0.05$). Compared with the other groups, the mRNA expression of CFABP in liver in Group H were higher than that in the other groups after 12 weeks ($P < 0.05$), whereas they were not obvious after 6 weeks. Compared with the other groups, the mRNA expression of MCP1 in liver in Group H remained high during the entire experiment, especially after 12 weeks. And Group HOG revealed a better effect than Groups HO and HE at 12 weeks ($P < 0.05$). The expressions of CCR2 in Groups HO, HOH and HOG were lower than that in Group H ($P < 0.05$) after 6 and 12 weeks, but in Group HE it was not significantly reduced than that in Group H after 6 and 12 weeks.

The same trend was found in adipose tissue around

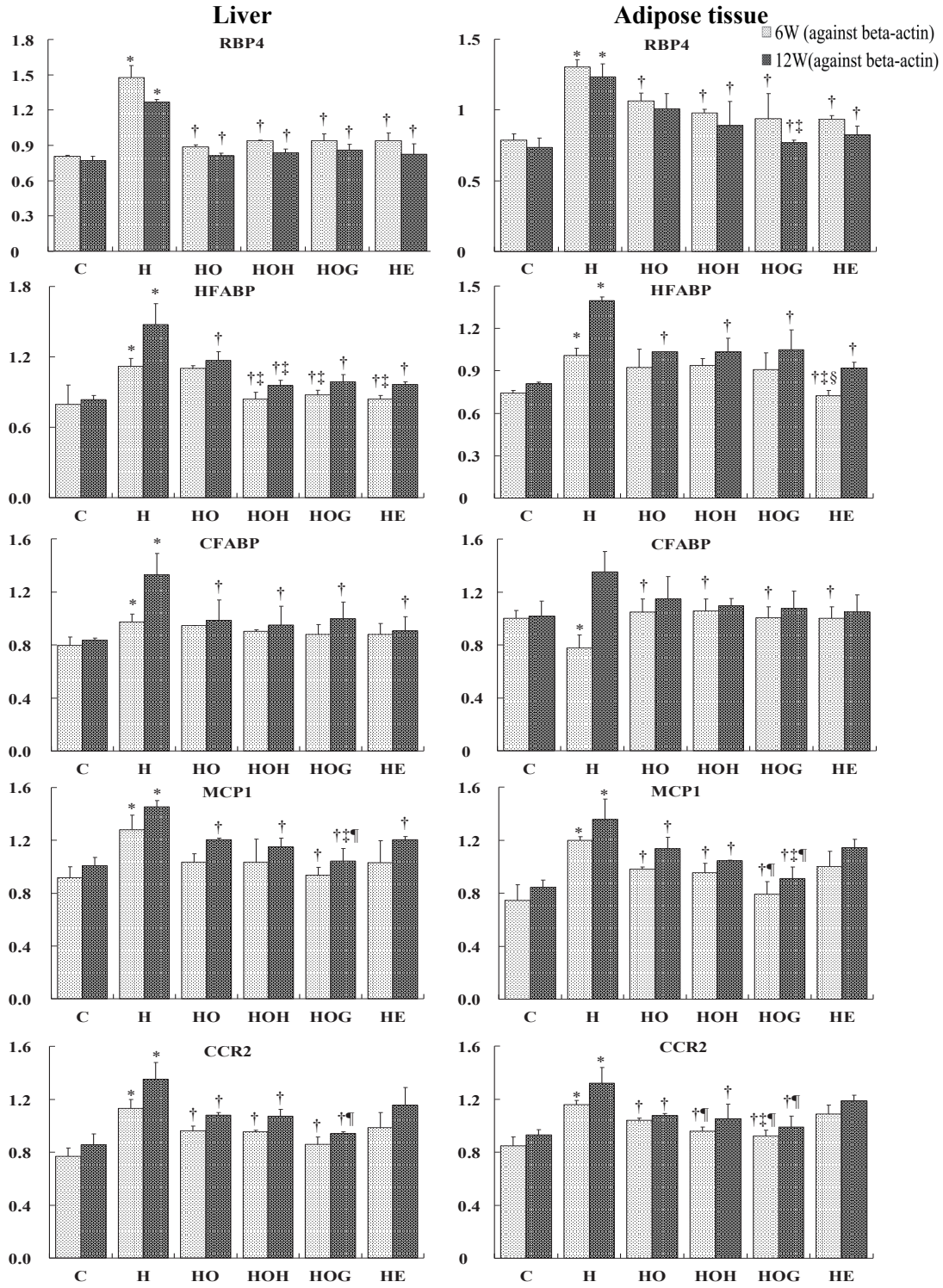

Fig. 4. Changes of mRNA expression with time in liver and adipose tissue.
Levels of RBP4, HFABP, CFABP, MCP1 and CCR2 against beta-actin mRNA expression are shown as above histogram. The left-side graphs show the expression in liver. The right-side graphs show the expression in adipose tissue around the left kidney. C: control, standard diet for 6 and 12 weeks ($n = 5$); H: high-cholesterol diet for 6 and 12 weeks ($n = 5$); HO: high-cholesterol diet with ogi for 6 and 12 weeks ($n = 5$); HOH: high-cholesterol diet with ogi and hesperidin for 6 and 12 weeks ($n = 5$); HOG: high-cholesterol diet with ogi and ginger for 6 and 12 weeks ($n = 5$); HE: high-cholesterol diet with ezetimibe for 6 and 12 weeks ($n = 5$). Dotted and shaded columns represent 6 and 12 weeks, respectively. *$P < 0.05$ vs. Group C, †$P < 0.05$ vs. Group H, ‡$P < 0.05$ vs. Group HO, §$P < 0.05$ vs. Group HOH, ¶$P < 0.05$ vs. Group HE. 6W: 6 weeks, 12W: 12 weeks.
CCR2, C-C chemokine receptor type 2; CFABP, cutaneous fatty acid-binding protein; HFABP, heart fatty acid-binding protein; MCP1, monocyte chemoattractant protein-1; RBP4, retinol-binding protein 4.

the left kidney. The up-regulation of RBP4, HFABP and CFABP mRNA expression were found in Group H. After treatment, they were decreased. MCP1 and CCR2 mRNA expression was not observed differences between Group H and Group HE. However Group HOG showed significant differences compared with other treatment groups ($P < 0.05$).

ICAM-1immunostaining expression in aorta

As shown in Fig. 5A, after 12 weeks, seldom ICAM-1immunostaining (Red-brown deposits indicate positive staining) was found on the whole layers of thoracic aortas in Group C. Significantly positive ICAM-1 immunostaining was observed in Group H and mainly localized on the endothelial layers. Positive ICAM-1 immunostaining was also observed in Groups HO, HOH, HOG and HE, but less than Group H (× 400). Mean optical density values of ICAM-1 are showed in Fig. 5B. The photographs generated were quantitatively analyzed the optical density of ICAM-1 by Image - Pro Plus. After treatment, the expressions in Groups HO, HOH, HOG and HE were decreased than Group H. And Group HOG

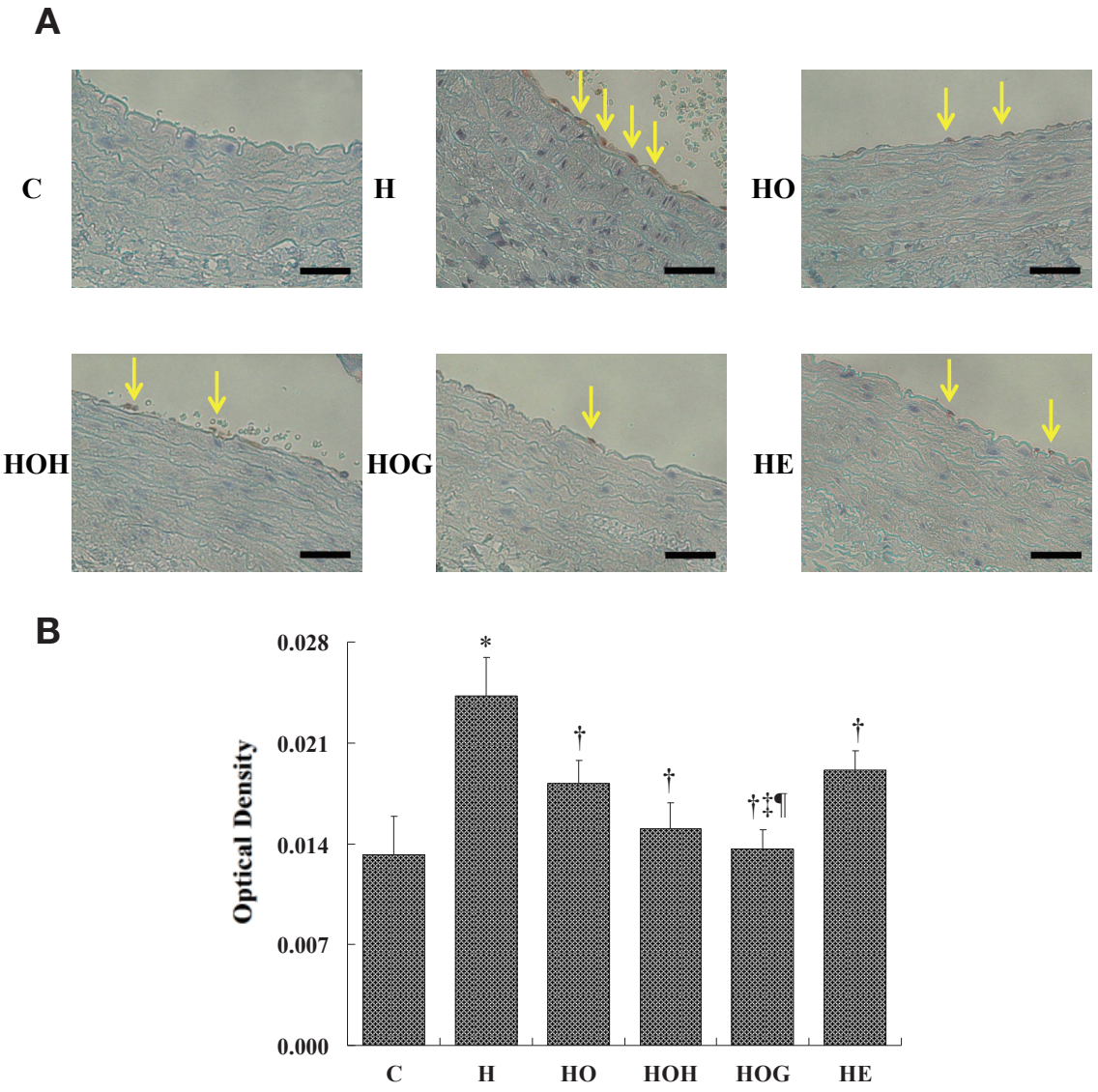

Fig. 5. ICAM-1 expression in aorta after 12 weeks.
A: Immunohistochemistry analyses of ICAM-1 in aorta (The arrows indicate positive expressions). **B**: Mean optical density values of ICAM-1. The optical density of ICAM-1 were quantitatively analyzed with Image - Pro Plus after 12 weeks. C: control, standard diet for 12 weeks ($n = 5$); H: high-cholesterol diet for 12 weeks ($n = 5$); HO: high-cholesterol diet with ogi for 12 weeks ($n = 5$); HOH: high-cholesterol diet with ogi and hesperidin for 12 weeks ($n = 5$); HOG: high-cholesterol diet with ogi and ginger for 12 weeks ($n = 5$); HE: high-cholesterol diet with ezetimibe for 12 weeks ($n = 5$). *$P < 0.05$ vs. Group C, †$P < 0.05$ vs. Group H, ‡$P < 0.05$ vs. Group HO, ¶$P < 0.05$ vs. Group HE. Bars express 25 μm.

was significantly lower compared with Groups HO and HE ($P < 0.05$).

TNF-alpha western blotting expression in liver

After 12weeks, the TNF-alpha protein levels in liver of Groups HO, HOH, HOG and HE were significantly inhibited compared with that of Group H (Fig. 6A, $P < 0.05$). It has less expression of TNF-alpha protein in Group HOG than Group HO and HE (Fig. 6B, $P < 0.05$).

Blood pressure and heart rate

No significant changes in systolic or diastolic blood pressure and heart rates were observed during the experiment (data not shown).

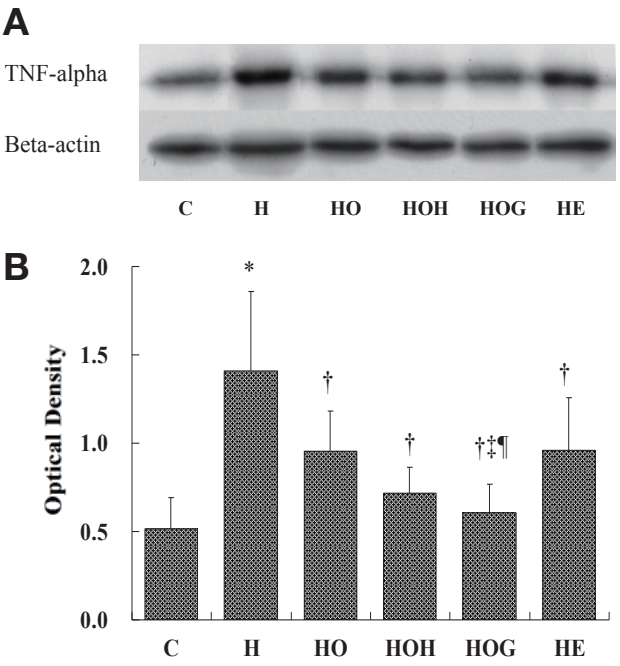

Fig. 6. TNF-alpha expression in liver.
A: Western blot analysis of TNF-alpha expression in liver. **B**: The TNF-alpha expression levels were quantitatively analyzed with Gel-Pro Analyzer 4. C: control, standard diet for 12 weeks ($n = 5$); H: high-cholesterol diet for 12 weeks ($n = 5$); HO: high-cholesterol diet with ogi for 12 weeks ($n = 5$); HOH: high-cholesterol diet with ogi and hesperidin for 12 weeks ($n = 5$); HOG: high-cholesterol diet with ogi and ginger for 12 weeks ($n = 5$); HE: high-cholesterol diet with ezetimibe for 12 weeks ($n = 5$). *$P < 0.05$ vs. Group C, †$P < 0.05$ vs. Group H, ‡$P < 0.05$ vs. Group HO, ¶$P < 0.05$ vs. Group HE.

DISCUSSION

A high cholesterol diet can lead to high lipid levels and establish fatty liver model in rats. Del Moral ML et al administered a high cholesterol diet in rats, and evaluated the serum lipid and lipoprotein levels as well as the steatotic process by biochemical and histological methods, respectively.[23] The results showed that fatty liver was well developed with long-term high cholesterol diets, and hepatocytes were filled with many lipid droplets. This process was more evident in the portal zones, where fat hepatocytes were more numerous. It has reported that the abnormal serum levels of HDL-C and LDL-C are associated with NAFLD and CVD.[24] Statins, hydroxyl-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are one kind of lipid-lowering drugs, which can inhibit cholesterol synthesis. They can well decrease LDL-C and increase HDL-C. However, they sometimes caused rhabdomyolysis and hepatitis. Kampo medicines are considered as a safe way to harmonize the abnormal serum levels of HDL-C and LDL-C and to minimize the side effects. We have already proved that BOT can improve blood chemistry and cholesterol concentrations.[18] In this experiment, we found that ogi, ogi with hesperidin, ogi with ginger and ezetimibe could decrease the concentrations of LDL-C and TC and increase HDL-C. ALT and ALP activities (after 6 weeks) in Group HE were significantly elevated than kampo groups. The reason may be kampo medicines have fewer side effects than ezetimibe. The marked elevation of ALT and ALP activities in Group H were consistent with our previous study.[18] However, the ALT and ALP activities in Group HE were decreased after 12 weeks, which was probably due to the individual differences in the rats.

Lipid-soluble molecules share several aspects of physiology due to common adaptations that allow them to function in a hydrophilic environment. RBP4, the transport protein for vitamin A (retinol) in blood, plays the role during hypercholesterolemia through binding to specific nuclear receptors like the peroxisome proliferator-activated receptor (PPAR) and the retinoid X receptor (RXR).[25] PPAR could directly regulate RBP4 expression, at least in certain tissues.[26] On the other hand, the increase of RBP4 levels may potentially enhance retinoid availability for RXR-PPAR action, by promoting retinol transport to the periphery of the body.[27] RBP4 has also been identified as an adipokine, which is involved in the modulation of glucose metabolism.[28] Graham et al[29] reported that increased levels of RBP4 were associated with obesity and hypercholesterolemia. Thus, the measurement of serum or plasma RBP4 levels is a helpful way for revealing metabolic disorders. In our experiment, we also found the increased trend of RBP4 in serum as well as mRNA expression of liver and adipose with high cholesterol diet rats. Ogi, ogi with hesperidin, ogi with ginger and ezetimibe could decrease the expres-

sions of the up-regulated RBP4, suggesting their specific effects in alleviating hypercholesterolemia to be partly induced through a lowering of the RBP4 expressions.

HFABP is a 15 kDa small protein consisting of 132 amino acids.[33] It is rich in the myocardium[34] and produced by skeletal muscle,[35] cardiomyocytes, kidney distal tubular cells,[36] and specific parts of the brain.[37] And CFABP is abundant in the psoriatic skin.[38] HFABP and CFABP are thought to play key roles in fatty acid metabolism, like fatty acid storage and transport.[38] FABPs are also expressed in mononuclear cells (predominantly monocytes and macrophages).[30] FABPs mRNA expressions have been reported to increase in abdominal white adipose tissue in rats fed a high cholesterol diet for 30 days.[39] They were reported to be up-regulated in liver and adipose tissue of rats fed high-cholesterol diet for 12 weeks. Our previous study proved that HFABP and CFABP maintained high expression levels for 12 weeks.[11, 18] Here, this phenomenon was also demonstrated. HFABP and CFABP mRNA expressions in the liver and adipose tissue of high-cholesterol diet group were increased compared to Group C while decreased after treatment.

Intestinal cholesterol absorption is a kind of active and selective process, which mediated by several transporter proteins located at the intestinal brush border membrane. Cholesterol and plant sterols are taken up by the enterocyte through the Niemann-Pick C1 Like 1 transporter.[40] Moreover, the ATP-binding cassette (ABC) transporters G5 and G8 actively induced plant sterols efflux and, to lesser extent cholesterol, back into the intestinal lumen. In addition, ABCG5 and ABCG8 are located at the canalicular membranes of hepatocytes, where they facilitate efflux of cholesterol and plant sterols into bile.[41] Animal cell membranes normally contain only a single major class of sterol, cholesterol.[42] However, plant cell membranes typically contain a complex mixture of sterols, such as lathosterol, campesterol and beta-sitosterol,[42, 43] which have similar structure with cholesterol. The serum lathosterol concentration is an indicator of whole-body cholesterol synthesis and can reveal the expression of cholesterol synthesis.[44] However, campesterol and beta-sitosterol are known to reduce blood cholesterol levels, and indicated cholesterol absorption.[45–47] It is reported that high cholesterol absorption and low cholesterol synthesis are associated with increased severity of coronary artery disease.[48] However, in an experiment, which takes plasma non-cholesterol sterols as a diagnostic tool, Noto et al also found increases in both cholesterol synthesis and absorption in pediatric hypercholesterolemia as we found in the high-cholesterol diet group.[49] In this experiment, concentrations of these three biomarkers were increased in Group H, while decreased after treatment, consistent with our previous research, in which concentrations of lathosterol, campesterol and beta-sitosterol were simultaneously increased in high cholesterol diet rats.[11] Brahma Naidu P et al also proved that synthesis of cholesterol was increased in high fat diet rats, which was probably due to the increase HMG CoA reductase in liver.[50] So, we have demonstrated that ogi, ogi with hesperidin, and ogi with ginger have a similar effect with BOT and ezetimibe on hypercholesterolemia and fatty liver. These kampo components can efficiently reduce cholesterol concentrations by inhibiting both absorption and synthesis by adjusting mRNA or enzymes associated with cholesterol absorption or synthesis.

A lot of evidences showed that inflammatory factors could take part in the occurrence and development of hypercholesterolemia, NAFLD and atherosclerosis.[6] MCP1 and its receptor CCR2 are two valuable inflammation related cytokines associated with NAFLD and atherosclerosis.[51] They can be engendered by vascular endothelial cells, monocytes and vascular smooth muscle cells.[52] It was reported that they would increase by hypercholesterolemia and obesity.[53, 54] TNF-alpha, participated in many inflammatory diseases, was also up-regulated on atherosclerosis.[55] In our experiment, kampo treatment groups could down-regulate the MCP1 and CCR2 mRNA expression in both liver and adipose tissue. Ezetimibe showed a reduced trend however not significantly different. Ogi showed a stronger anti-inflammatory effect when combined with ginger than other treatment groups. A similar effect was revealed on the protein expression of TNF-alpha in liver. Group HOG had advantage on anti-inflammatory effect during the whole experiment.

ICAM-1, one of the markers of endothelial cell activation, is expressed in endothelial cells, which has participated in neutrophil as well as monocyte migration, migration and adhesion of endothelial cells.[56] Some studies proved that the concentration of serum ICAM-1 correlated with the expression of ICAM-1 in the media and the vasa vasorum.[57] Therefore, it is a reliable measure using the concentration of serum ICAM-1 to detect the expression of the molecule in the tissue of the aortic wall. It was reported that ICAM-1 was up-regulated after vascular injury in neointimal and medial smooth muscle cells.[58] And experiment evidences also showed that the expressions of both serum ICAM-1 and tissue ICAM-1 were increased in the early stages of atherosclerosis rats.[57] In our experiment, both the concentration of serum ICAM-1 and expression of ICAM-1 in aorta were increased in Group H. Kampo components and ezetimibe could down-regulate the high expression with

high-cholesterol diet. Ogi combined with ginger still showed advantage both in serum and aorta. We speculate that down-regulated ICAM-1 may be one of the mechanisms to prevent atherosclerosis of ogi, ogi with hesperidin and ezetimibe, especially ogi combined with ginger.

Our previous study proved that BOT has a protective effect on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats. BOT is composed of ogi (Astragalus membranaceus Bunge), boi (Sinomenium acutum Rehder et Wilson), sojyutsu (Atractylodes lancea De Candolle), ginger (Zingiber officinale Roscoe), taiso (Zizyphus jujuba Miller var. inermis Rehder) and kanzo (Glycyrrhiza uralensis Fischer). Ogi and ginger are the key components of BOT. Wang D et al. reported that ogi could significantly decrease concentrations of TC and LDL-C and improved the atherosclerosis profile.[59] Matsuda A et al. also found that ginger tended to reduce RBP4 mRNA expression levels in the liver and visceral fat in hyperlipidemia.[20] Wang X et al. revealed that hesperidin could improve hypercholesterolemia and fatty liver by inhibiting both the synthesis and absorption of cholesterol.[11] However few studies have assessed the mechanism of ogi on hypercholesterolemia atherosclerosis as well as the synergistic action between ogi and hesperidin or ginger.

In the present findings, three kampo component groups showed a similar effect with BOT and ezetimibe on hypercholesterolemia and fatty liver. These kampo components could efficiently reduce cholesterol concentrations by inhibiting both absorption and synthesis. The synergistic action of ogi with ginger showed stronger effect but had no significant difference. On the other hand, ogi combined with ginger revealed the most powerful anti-arteriosclerotic effect than other kampo components groups as well as ezetimibe group. According to ogi and ginger are the key components of BOT and the results above, we speculate that ogi has the similar effects with BOT both on the progression of hypercholesterolemia and arteriosclerosis, while ginger maybe helpful for other components of BOT especially on anti-inflammation and anti-arteriosclerosis.

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The authors declare no conflict of interest.

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