

## Effects of Kampo Formulas on the Progression of Hypercholesterolemia and Fatty Liver Induced by High-Cholesterol Diet in Rats

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### ABSTRACT

**Background** Bofutsushosan is a well known Kampo, traditional Japanese medicine, based on ancient Chinese medicine mainly used in the treatment of hypercholesterolemia in Japan. We selected two Kampo formulas, Boiogito and Keishibukuryogan mainly used in the treatment of hypercholesterolemia in China to compare with Bofutsushosan and cholesterol absorption inhibitor ezetimibe.

**Methods** Hypercholesterolemia and fatty liver were induced by high cholesterol (containing 2% cholesterol and 0.5% cholic acid) diet in male Wistar rats for 6 and 12 weeks. Kampo formulas Boiogito, Bofutsushosan, Keishibukuryogan and ezetimibe were added to the high-cholesterol diet, respectively. After 6 and 12 weeks, body and liver weights, blood chemistry, cholesterol concentrations, fat-related and inflammatory-related factors were examined.

**Results** High-cholesterol diet increased body and liver weights, and serum cholesterol concentrations. Boiogito and ezetimibe improved them. Serum ICAM-1 and RBP4 were increased in the high cholesterol diet group. Boiogito and ezetimibe improved them too. In the histological examinations of liver and adipose tissues, we observed a significant improvement after treatment. Immunostaining expression of ICAM-1 in aorta was improved by Boiogito, Bofutsushosan, Keishibukuryogan and ezetimibe. The mRNA expression of RBP4,

HFABP, CFABP, MCP1 and CCR2 in liver and adipose tissue were decreased by Boiogito and ezetimibe.

**Conclusion** Boiogito has a protective effect on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats and more effective than Bofutsushosan and Keishibukuryogan. The lipid-lowering effect of Boiogito is not stronger than ezetimibe. But the anti-inflammatory (MCP1, CCR2) and anti-arteriosclerotic (ICAM-1) effects of Boiogito are more potent than ezetimibe.

**Key words** Bofutsushosan; Boiogito; Keishibukuryogan; fatty liver; hypercholesterolemia

Hypercholesterolemia is one of the major risk factors for many cardiovascular diseases, such as atherosclerosis, hypertension and myocardial infarction.<sup>1, 2</sup> Non-alcoholic fatty liver disease (NAFLD) is common in the general population, and it occurs even more frequently in patients with hypercholesterolemia.<sup>3, 4</sup> Patients with NAFLD have a high risk of cardiovascular disease (CVD).<sup>5</sup> In addition, NAFLD is often associated with atherosclerotic signs including the presence of carotid plaques<sup>6</sup> and coronary arterial calcification.<sup>7, 8</sup>

It is generally believed that the occurrence and development of hypercholesterolemia have significantly correlation to lipid metabolism-related genes, such as retinol-binding protein 4 (RBP4), heart fatty acid-binding protein (HFABP), cutaneous fatty acid-binding protein (CFABP).<sup>9, 10</sup> RBP4, a protein secreted by hepatocytes and adipose tissue, is closely related to hypercholesterolemia and NAFLD.<sup>11</sup> HFABP and CFABP belongs to FABPs family which may play a broad role in cellular fatty acid metabolism.<sup>12</sup> In addition to lipid metabolism-related genes, inflammatory cytokines are associated with hypercholesterolemia. Engström et al. have supported the view that inflammation could be a risk factor for developing hypercholesterolemia.<sup>13</sup> Monocyte chemoattractant protein-1 (MCP-1) and its receptor CC chemokine receptor 2 (CCR2) are important inflammatory chemokines linked with hypercholesterolemia.<sup>14, 15</sup> Intercellular adhesion molecule-1 (ICAM-1) can promote the development and progression of atherosclerosis.<sup>16</sup>

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; BOT, Boiogito; BTS, Bofutsushosan; C, control group; CFABP, cutaneous fatty acid-binding protein; CVD, cardiovascular disease; H, high-cholesterol diet group; HA, high-cholesterol diet with Boiogito group; HB, high-cholesterol diet with Bofutsushosan group; HC, high-cholesterol diet with Keishibukuryogan 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; KBG, Keishibukuryogan; LDL-C, low-density lipoprotein cholesterol; MCP1, monocyte chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; RBP4, retinol-binding protein 4; TC, total cholesterol; VEC, vascular endothelial cells

Therefore, it is a simple and convenient method to detect hypercholesterolemia by monitoring RBP4, C-FABP, H-FABP, MCP1, CCR2, ICAM-1 expression.

Bofutsushosan (BTS), Boiogito (BOT) and Keishibukuryogan (KBG) are well-known Japanese Kampo and Chinese traditional herbal medicines which are used to improve obesity.<sup>17, 18</sup> BTS has been reported to inhibit atherosclerosis,<sup>19</sup> obesity,<sup>17, 18, 20</sup> hypertension<sup>17</sup> and hyperglycemia.<sup>21</sup> BOT and KBG are also Kampo preparations which have been used in patients with obesity.<sup>22, 23</sup> However, pharmacological evidence for the effects of treatment of NAFLD of BOT and KBG still remains obscure. Using the well-established experimental model of high-cholesterol diet in rats, we compared the pharmacological efficacies of BOT, BTS and KBG.

## MATERIALS AND METHODS

### Rats and feeding method

Sixty male Wistar rats aged 8-weeks (purchased from Shimizu Laboratory Supplies, Kyoto, Japan) were kept in an air-conditioned room at 25 °C with 55% humidity and given standard chow. After 3 days of acclimation, the rats were divided into 6 groups: control group (C,  $n = 10$ ), high-cholesterol diet group (H,  $n = 10$ ), high-cholesterol diet with Kampo formula BOT group (HA,  $n = 10$ ), high-cholesterol diet with Kampo formula BTS group (HB,  $n = 10$ ), high-cholesterol diet with Kampo formula KBG group (HC,  $n = 10$ ), 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 (CE-2; Japan Clea, Tokyo, Japan). A high-cholesterol diet<sup>11, 24</sup> was supplied for Group H; it was prepared by adding 2% cholesterol and 0.5% cholic acid to the standard diet. The high-cholesterol diets with BOT, BTS or KBG were made by adding 1% extract formulations of BOT, BTS or KBG to the high-cholesterol diet, respectively. These Kampo extract formulations were generous gift from Tsumura (Tokyo, Japan). The high-cholesterol diet with ezetimibe was made by adding 0.0006% of ezetimibe (LKT laboratories, St Paul, MN). 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 weekly. 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, and then portions of the samples were stored in a 10% formalin solution for hematoxylin-eosin staining and oil red O staining.<sup>25</sup> The remaining samples were immediately transferred into EP tubes containing 500  $\mu$ L of RNA later (Ambion, Austin, TX), quickly frozen in liquid nitrogen, and stored at  $-80$  °C. Serum levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride, blood sugar, creatinine, total bilirubin, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were analyzed for rats using an auto analyzer at an accredited clinical laboratory (SRL, Tokyo).

### ICAM-1 and RBP4

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

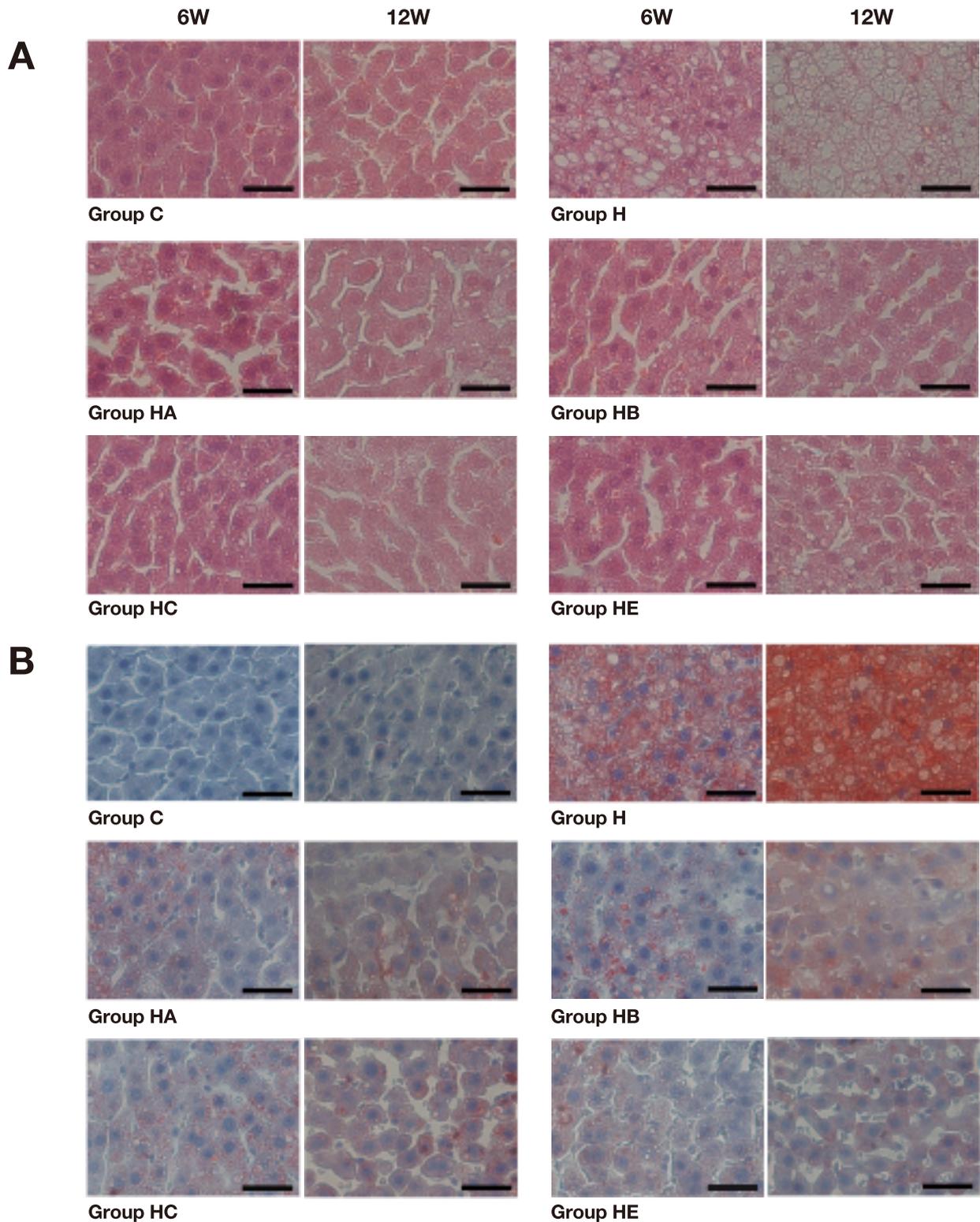
### RT-PCR

Total RNA was extracted from the liver and adipose tissue around the left kidney using TRIzol reagent according to the manufacturer's instructions (Promega, Carlsbad, CA). A semiquantitative real-time PCR (RT-PCR) was performed with 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 Table 1. Using the  $2^{-\Delta\Delta CT}$  method, mRNA expression was semi-quantitatively measured as a relative amount of each target RNA to a known housekeeping gene (beta-actin) expression level.<sup>26, 27</sup>

**Table 1. The sequence of each PCR primer**

Gene	Primer
RBP	Forward 5'-gacaaggctcgtttctctg-3'
	Reverse 5'-gactcgtcccttggtgtag-3'
H-FABP	Forward 5'-ctagcatgaggaagcaagg-3'
	Reverse 5'-tgcttcatccagacaagtgg-3'
C-FABP	Forward 5'-gggctggctcttaggaagat-3'
	Reverse 5'-aaaacacggctcgtcttcacc-3'
MCP1	Forward 5'-ctgtagcatccacgtgctgt-3'
	Reverse 5'-tgctgctggtgattctcttg-3'
CCR2	Forward 5'-gatcctgcccacttctgtca-3'
	Reverse 5'-agatgagcctcacagcccta-3'
Beta-actin	Forward 5'-gtagccatccaggctgtgtt-3'
	Reverse 5'-ccctcagatgggacagt-3'

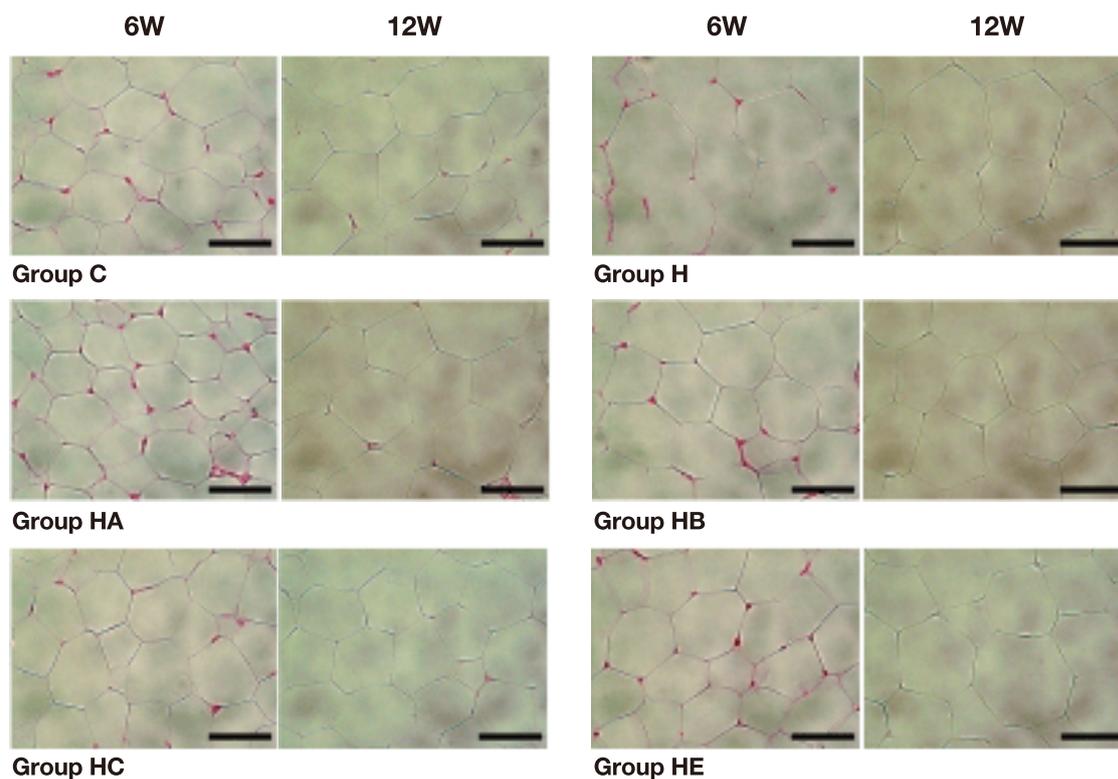


**Fig. 1.** Histopathological examination of liver.

**A:** Fatty degeneration (steatosis) of the liver is observed in the high-cholesterol diet-fed (H, HA, HB, HC and HE) groups, but not in Group C in hematoxylin-eosin stained tissues. Fatty changes increase with time to a greater extent in Group H than Groups HA, HB, HC and HE.

**B:** Oil red O staining reveals 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 ( $n = 5$ ). HA: high-cholesterol diet with Boiogito for 6 and 12 weeks ( $n = 5$ ). HB: high-cholesterol diet with Bofutsushosan for 6 and 12 weeks ( $n = 5$ ). HC: high-cholesterol diet with Keishibukuryogan for 6 and 12 weeks ( $n = 5$ ). HE: high-cholesterol diet with ezetimibe for 6 and 12 weeks ( $n = 5$ ). Bars express 25  $\mu$ m.



**Fig. 2.** Histopathological examination of adipose tissue.

The larger fat cells are observed in the high-cholesterol diet-fed (H, HA, HB, HC and HE) groups, but not in Group C ( $56.13 \pm 11.51$  and  $64.88 \pm 13.96$   $\mu\text{m}$  after 6 and 12 weeks, respectively) in hematoxylin-eosin stained tissues. Fat cells change with time to a greater extent in Group H ( $97.00 \pm 20.14$  and  $104.63 \pm 22.56$   $\mu\text{m}$  after 6 and 12 weeks, respectively) than Groups HA, HB, HC and HE ( $55.63 \pm 5.85$  and  $78.88 \pm 16.34$   $\mu\text{m}$ ,  $75.63 \pm 11.53$  and  $78.13 \pm 16.47$   $\mu\text{m}$ ,  $74.63 \pm 13.94$  and  $88.13 \pm 8.58$   $\mu\text{m}$ ,  $74.38 \pm 13.21$  and  $83.13 \pm 16.63$   $\mu\text{m}$  after 6 and 12 weeks, respectively). Bars express 50  $\mu\text{m}$ .

### Immunohistochemical studies

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

### Statistical analyses

The data are expressed as the mean  $\pm$  SEM. For the continuous variables, differences in responses among groups were compared using Mann-Whitney's *U* test for non-parametric variables by SPSS 11.0 J (SPSS Japan, Tokyo).  $P < 0.05$  was considered statistically significant.

## RESULTS

### Histological examination of liver tissues

As Fig. 1 shows, the fatty degeneration (steatosis) of liver was observed in the high-cholesterol diet-fed (H, HA, HB, HC and HE) groups, but not in Group C. These

changes comprising tiny and large vacuoles as well as pleomorphic nuclei were more prominent in Groups HA, HB, HC and HE than in Group H (Fig. 1A). Oil red O staining revealed 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). Overall, fatty liver changes were more prominent in Group H than Groups HA, HB, HC and HE ( $\times 400$ ).

### Histological examination of adipose tissue

As Fig. 2 shows, the larger fat cells were observed in the high-cholesterol diet-fed (H, HA, HB, HC and HE) groups, but not in Group C. It was more prominent in Group H than Groups HA, HB, HC and HE ( $\times 400$ ).

### Body and liver weights

As Table 2 shows, there were no significant differences in the baseline of body weights at the beginning of the experiment. The body weights had no significant differences during the experiment too. Liver weights were significantly lower in Group HE than Group H after 6 and 12 weeks ( $P < 0.05$ ).

**Table 2. Body and liver weights**

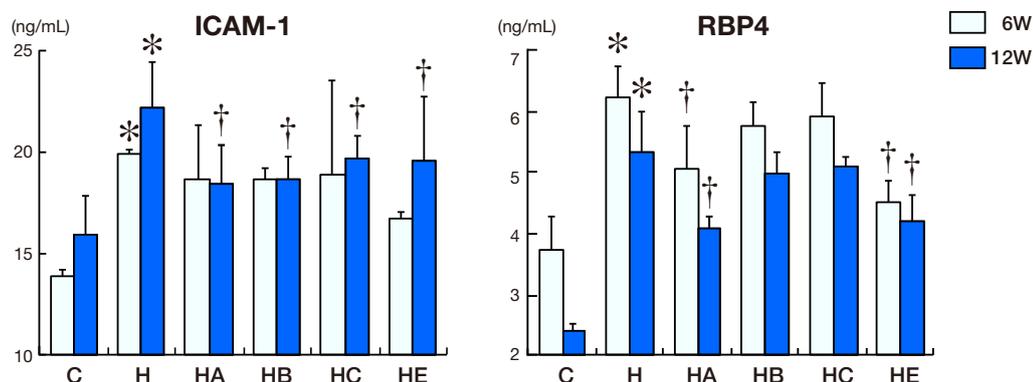
	Group C	Group H	Group HA	Group HB	Group HC	Group HE
Body weight (g)						
0W	233.1 ± 6.6	233.5 ± 6.6	235.9 ± 6.7	237.2 ± 9.4	237.1 ± 7.8	236.1 ± 6.7
6W	402.8 ± 15.9	398.5 ± 23.2	390.0 ± 21.9	392.4 ± 8.6	395.9 ± 23.9	385.8 ± 24.6
12W	448.6 ± 14.0	477.1 ± 15.5*	461.6 ± 26.1	457.6 ± 32.7	470.2 ± 32.9	453.3 ± 29.2
Liver weight (g)						
6W	10.1 ± 0.9	15.0 ± 0.8*	15.5 ± 2.6	15.8 ± 1.4	14.9 ± 1.1	10.8 ± 1.0†
12W	12.5 ± 3.5	19.0 ± 3.1*	18.9 ± 1.5	19.9 ± 2.4	19.9 ± 2.2	12.1 ± 1.1†

0W, 0 weeks; 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). HA: high-cholesterol diet with Boiogito for 6 and 12 weeks (*n* = 5). HB: high-cholesterol diet with Bofutsushosan for 6 and 12 weeks (*n* = 5). HC: high-cholesterol diet with Keishibukuryogan for 6 and 12 weeks (*n* = 5). HE: high-cholesterol diet with ezetimibe for 6 and 12 weeks (*n* = 5). \**P* < 0.05 versus Group C. †*P* < 0.05 versus Group H. Data are expressed as the mean ± SEM.

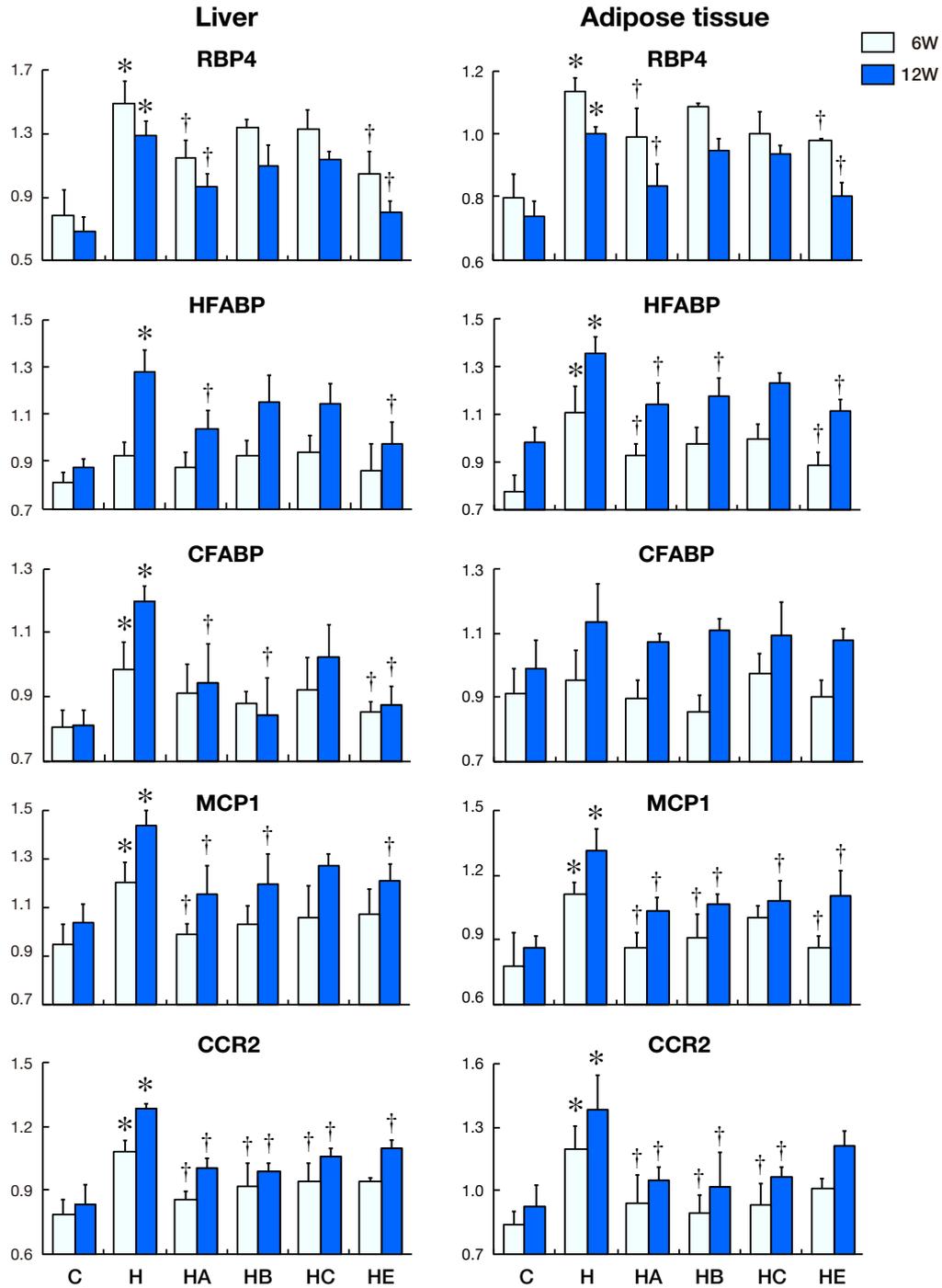
**Table 3. Blood chemistry and cholesterol concentrations**

		Group C	Group H	Group HA	Group HB	Group HC	Group HE
TC (mg/dL)	6W	55.5 ± 15.2	68.2 ± 6.8	67.4 ± 13.7	65.4 ± 9.8	68.6 ± 13.8	78.5 ± 12.4
	12W	56.6 ± 13.0	78.8 ± 5.4*	73.7 ± 10.4	75.2 ± 12.0	70.5 ± 12.0	73.2 ± 3.5
HDL-C (mg/dL)	6W	18.0 ± 2.6	21.0 ± 2.2	20.4 ± 4.5	19.4 ± 2.9	20.0 ± 3.2	17.5 ± 2.7
	12W	16.0 ± 2.6	22.2 ± 0.8*	22.7 ± 3.2	23.8 ± 3.1	20.0 ± 3.4	19.4 ± 1.1
LDL-C (mg/dL)	6W	5.6 ± 1.1	8.4 ± 1.7*	8.6 ± 2.3	8.0 ± 1.9	8.8 ± 2.6	8.8 ± 2.2
	12W	6.0 ± 1.4	11.8 ± 0.8*	9.0 ± 1.7†	9.8 ± 2.7	10.5 ± 4.4	8.0 ± 1.2†
TG (mg/dL)	6W	31.5 ± 7.5	34.4 ± 11.2	28.0 ± 17.1	18.2 ± 3.9†	21.8 ± 8.8	25.0 ± 8.0
	12W	29.2 ± 9.2	26.4 ± 4.7	22.7 ± 6.9	24.0 ± 7.9	19.8 ± 5.4	16.4 ± 7.0†
BS (mg/dL)	6W	109.4 ± 7.7	99.6 ± 7.2	112.8 ± 8.8†	105.4 ± 6.6	110.8 ± 12.0	112.6 ± 10.5
	12W	102.8 ± 6.1	112.0 ± 11.0	111.3 ± 20.0	114.8 ± 17.8	99.2 ± 7.6	101.2 ± 9.2
Cr (mg/dL)	6W	0.30 ± 0.03	0.30 ± 0.04	0.35 ± 0.06	0.35 ± 0.05	0.34 ± 0.04	0.34 ± 0.07
	12W	0.32 ± 0.04	0.35 ± 0.03	0.34 ± 0.02	0.36 ± 0.11	0.34 ± 0.03	0.32 ± 0.03
TBil (µmol/L)	6W	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
	12W	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
ALT (U/L)	6W	60.0 ± 11.7	72.2 ± 11.4*	71.8 ± 18.2	61.3 ± 10.2	93.6 ± 17.1	111.0 ± 11.1†
	12W	53.4 ± 3.0	148.7 ± 25.7*	79.0 ± 17.4†	104.3 ± 26.1†	79.2 ± 18.5†	109.7 ± 25.8
ALP (U/L)	6W	413.0 ± 22.6	497.8 ± 24.8	377.5 ± 31.2†	399.0 ± 20.1†	450.0 ± 35.6	519.0 ± 34.9
	12W	331.0 ± 28.5	432.7 ± 31.7*	334.3 ± 32.1†	337.0 ± 25.5†	283.3 ± 27.1†	514.8 ± 17.8†

6W, 6 weeks; 12W, 12 weeks. ALP, alkaline phosphatase; ALT, alanine aminotransferase; BS, blood sugar; Cr, creatinine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TBil, total bilirubin; TC, total cholesterol; TG, triglyceride. \**P* < 0.05 versus Group C. †*P* < 0.05 versus Group H. Data are expressed as the mean ± SEM.



**Fig. 3.** Intercellular adhesion molecule-1 (ICAM-1) and retinol-binding protein 4 (RBP4) concentration in serum. ICAM-1 and RBP4 concentrations in control, standard diet group (C, *n* = 5), high-cholesterol diet group (H, *n* = 5), high-cholesterol diet with BOT group (HA, *n* = 5), high-cholesterol diet with BTS group (HB, *n* = 5), high-cholesterol diet with KBG group (HC, *n* = 5) and high-cholesterol diet with ezetimibe group (HE, *n* = 5) for 6 and 12 weeks. \**P* < 0.05 versus Group C. †*P* < 0.05 versus Group H.

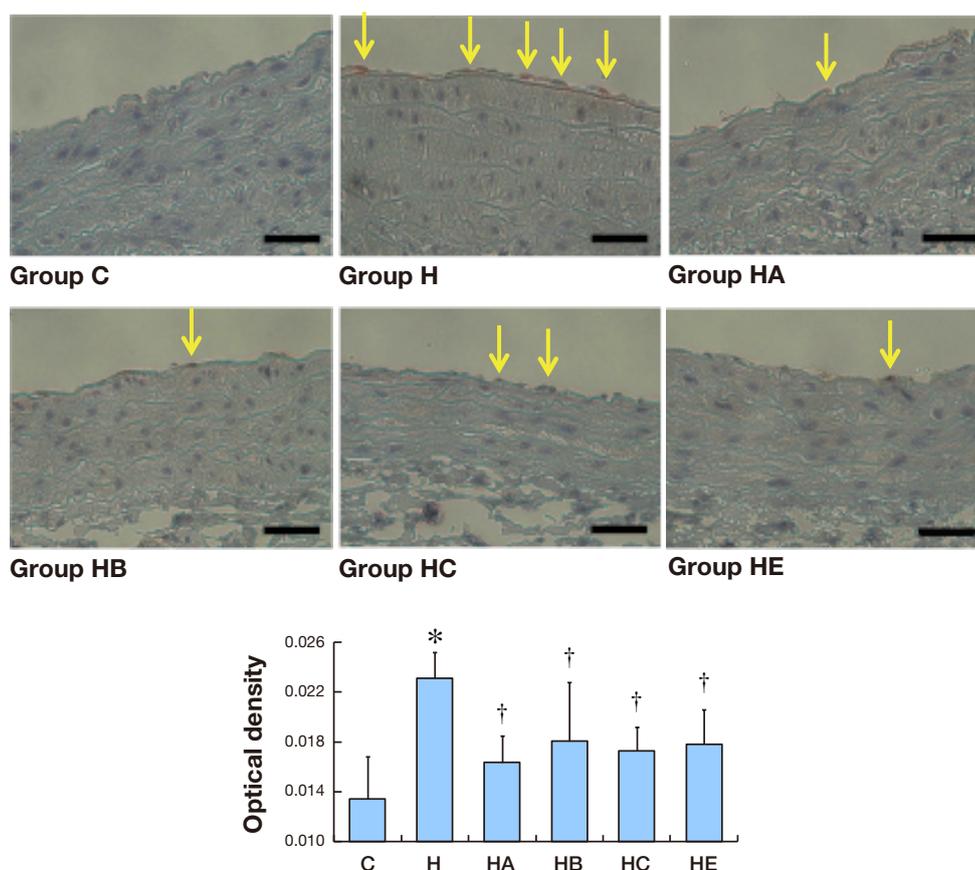


**Fig. 4.** Changes in mRNA expression with time in liver and adipose tissue. Levels of RBP4, HFABP, CFABP, MCP1 and CCR2 against beta-actin mRNA expression are shown in the above histograms. The left-side graphs show the expression in liver. The right-side graphs show the expression in adipose tissue around the left kidney.

**Blood chemistry and cholesterol concentrations**

After 6 weeks of the experiment, the concentration of LDL-C in Group H ( $8.4 \pm 1.7$  mg/dL) was significantly elevated compared to the concentration in Group C ( $5.6 \pm 1.1$  mg/dL) ( $P < 0.05$ ). After 12 weeks, the concentration of TC in Group H ( $78.8 \pm 5.4$  mg/dL) was significantly elevated compared with it in Group C ( $56.6 \pm$

$13.0$  mg/dL) ( $P < 0.05$ ). The concentration of HDL-C in Group H ( $22.2 \pm 0.8$  mg/dL) was significantly elevated compared with it in Group C ( $16.0 \pm 2.6$  mg/dL) ( $P < 0.05$ ). The concentration of LDL-C elevated to a greater extent in Group H ( $11.8 \pm 0.8$  mg/dL) than that in Groups HA and HE ( $9.0 \pm 1.7$  and  $8.0 \pm 1.2$  mg/dL, respectively) ( $P < 0.05$ ). The concentration of ALT in Group H ( $148.7 \pm$



**Fig. 5.** Immunohistochemistry expression of ICAM-1 in aorta.

**A:** Seldom ICAM-1 immunostaining (red-brown deposits indicate positive staining) is found on the whole layers of abdominal aortas in Group C. Bars express 25  $\mu$ m.

**B:** Mean optical density values of ICAM-1. The photographs generated were quantitatively analyzed the optical density of ICAM-1 with Image-Pro Plus version 6.0 software. \* $P < 0.05$  versus Group C. † $P < 0.05$  versus Group H.

25.7 U/L) was significantly elevated compared with it in Group C ( $53.4 \pm 3.0$  U/L) ( $P < 0.05$ ). It was decreased in Groups HA, HB and HC ( $79.0 \pm 17.4$ ,  $104.3 \pm 26.1$ ,  $79.2 \pm 18.5$  U/L, respectively) than in Group H ( $P < 0.05$ ). The concentration of ALP in Group H ( $432.7 \pm 31.7$  U/L) was significantly elevated compared with it in Group C ( $331.0 \pm 28.5$  U/L) ( $P < 0.05$ ). It was lower in Groups HA, HB and HC ( $334.3 \pm 32.1$ ,  $337.0 \pm 25.5$ ,  $283.3 \pm 27.1$  U/L, respectively) than in Group H ( $P < 0.05$ ) (Table 3).

#### ICAM-1 and RBP4 in serum

ICAM-1 concentrations were increased in Group H compared to Groups C, HA, HB and HE after 12 weeks (Fig. 4,  $P < 0.05$ ). RBP4 concentrations were increased in Group H compared to the other groups including Groups C, H, HA and HE after 6 and 12 weeks (Fig. 3,  $P < 0.05$ ).

#### mRNA expression in liver and adipose tissue

Figure 4 demonstrates the changes of mRNA expression in liver and adipose tissue round the left kidney of rats

for 6 and 12 weeks.

RBP4 mRNA expression in liver in Group H was significantly increased compared to that in Groups C, HA and HE after 6 and 12 weeks ( $P < 0.05$ ). HFABP mRNA expression in Group H was significantly increased compared to that in Groups C, HA and HE after 12 weeks ( $P < 0.05$ ). Compared with the other groups, the mRNA expression of CFABP in liver in Group H were higher than that in Groups C, HA, HB and HE at 12 weeks ( $P < 0.05$ ), whereas they were not obvious at 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 ( $P < 0.05$ ). The expression of CCR2 in liver in Groups HA, HB, HC (at 6 and 12 weeks) and HE (at 12 weeks) were lower than that in Group H ( $P < 0.05$ ).

A down-regulation of RBP4 mRNA expression in Group H appeared in adipose tissue around the left kidney after 12 weeks; even it was higher in Group H than that in Groups C, HA and HE ( $P < 0.05$ ). The mRNA expression of HFABP was higher in Group H than that

in Groups C, HA, HE (at 6 and 12 weeks) and HB (at 12 weeks) ( $P < 0.05$ ). CFABP mRNA expression had no significant difference during the whole experiment among all the groups. The expression of MCP1 in adipose tissue in Groups HA, HB, HE (at 6 and 12 weeks) and HC (at 12 weeks) was lower than that in Group H ( $P < 0.05$ ). The CCR2 mRNA expression in Group H was higher than that in Groups HA, HB and HC ( $P < 0.05$ ).

#### ICAM-1 immunostaining expression in aorta

As shown in Fig. 5, after 12 weeks, little ICAM-1 immunostaining (red-brown deposits indicate positive staining) was found on the whole layers of abdominal 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 HA, HB, HC and HE, but less than Group H. While ICAM-1 immunoreactivities were very weak in Groups HA, HB, HC and HE, the positive area and the strength of ICAM-1 stainings was markedly lower than those in Group H ( $\times 400$ ).

#### Blood pressure and heart rate

No significant changes in systolic or diastolic blood pressure and heart rates were observed during the experiment.

#### DISCUSSION

Hypercholesterolemia is one of major risk factors contributing to cardiovascular diseases.<sup>3, 4</sup> Patients with NAFLD have a high risk of CVD, too.<sup>5</sup> Elevated concentrations of plasma TC and LDL-C as well as reduced concentration of HDL-C are negative risk factors for CVD is well documented.<sup>28-30</sup> Lipid-lowering drugs, such as statins, hydroxyl-methylglutaryl-coenzyme A reductase inhibitors can well reduce LDL-C and increase HDL-C. Adverse reactions such as rhabdomyolysis and hepatitis were sometimes caused by them. Therefore, minimizing the side effects of lipid-lowering drugs is also very important. The mechanism of Kampo formula is to restore the balance of the body. It is a safe way to achieve the balance between LDL-C and HDL-C concentrations. In this study, we observed the effect of Kampo formulas (BOT, BTS, KBG) on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats. LDL-C, ALT and ALP concentrations in the serum of rats fed high-cholesterol diet with BOT decreased. ALT (at 6 weeks) and ALP (at 12 weeks) concentrations in Group HE was significantly elevated compared with that in Group H. The reason is still unknown. The steatosis

of liver and hypertrophy of fat cells caused by high-cholesterol diet could be alleviated by BOT, BTS and KBG as shown by histopathological examinations.

Retinol-binding protein 4 (RBP4), a protein secreted by hepatocytes (80%) and adipose tissue (20%), is a 21-kDa protein that facilitates the transport of retinol through the circulation to peripheral tissues.<sup>11</sup> It plays a key role in the control of metabolic and proliferative cell functions,<sup>31</sup> including steatogenesis.<sup>32</sup> Recently, the role of adipokines, specifically RBP4 in the pathogenesis of obesity-related diseases is widely being discussed.<sup>33-36</sup> Many studies have reported the relationship between RBP4 and obesity as well as its related complications.<sup>35-37</sup> Recent studies demonstrated that RBP4 levels were increased in obese and insulin-resistant humans and mouse models.<sup>38-41</sup> Stefan et al. found a direct relation between hepatic fat content and blood levels of RBP4 in healthy subjects, too.<sup>42</sup> It has been reported that RBP4 mRNA expression can be up-regulated in liver and adipose tissue of rats with high cholesterol diet for 12 weeks.<sup>11</sup> In our experiment, RBP4 expression in liver and adipose tissue of rats fed a high-cholesterol diet as well as the expression in serum were up-regulated. BOT and ezetimibe shows greater effect to decrease the level of RBP4 mRNA expression in liver and adipose tissue, the serum expression of RBP4 can also be decreased by BOT and ezetimibe.

HFABP is a member of a family of binding proteins with distinct tissue distributions and diverse roles in fatty acid metabolism, trafficking, and signaling.<sup>43</sup> It is a low molecular-weight cytoplasmic protein that is abundant in the myocardium<sup>44</sup> and produced by skeletal muscle,<sup>45</sup> cardiomyocytes, kidney distal tubular cells<sup>46</sup> and specific parts of the brain.<sup>47, 48</sup> HFABP mRNA expression has been reported to be up-regulated in livers of rats fed high-cholesterol diet for 28 days.<sup>11</sup> In our study, this phenomenon was also demonstrated. HFABP mRNA expression in the liver and adipose tissue of high-cholesterol diet groups was significantly increased compared to the control group after 12 weeks.

CFABP was originally identified as being over-expressed in the psoriatic skin.<sup>49, 50</sup> It is typical of the FABP family and binds long-chain fatty acids with high affinity<sup>49, 51</sup> and were thought to play a important role in the storage and transport of fatty acids.<sup>50, 52, 53</sup> CFABP was reported to be up-regulated after 12 weeks in liver and adipose tissue of high-cholesterol diet rats.<sup>11</sup> Here, the mRNA expression of CFABP in liver of high-cholesterol diet rats were higher after 12 weeks while there were no significant differences be-

tween groups in adipose tissue. BOT, BTS and ezetimibe can down-regulate the CFABP mRNA expression in liver.

BOT, BTS and KBG are all used as anti-obesity medications in Japan.<sup>54</sup> BOT is used for people with a solid build and thick abdominal subcutaneous fat.<sup>54</sup> It could decrease the mouse body weight, fat accumulation, TC and triglyceride level, but cannot influence fasting blood glucose levels or insulin levels.<sup>22</sup> BTS is used for patients with flabby constitution and proneness to fatigue.<sup>54</sup> It can decrease food intake, body weight, blood pressure, white adipose tissue weight and ameliorate the adipocytokine dysregulation in white adipose tissue.<sup>55</sup> KBG is commonly used for women with sudden weight gain in menopause and could lead to a reduction in blood cholesterol.<sup>54, 56</sup> According to these reports, even BOT, BTS and KBG are all used in the treatment of obesity, they are suitable for different situations. In our study, BOT has a better effect on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats. Ougi and Ginger are the key components of BOT, and it has been reported that Ougi and Ginger of BOT contribute greatly to the beneficial effects on abnormal lipid metabolism.<sup>22</sup> Matsuda et al. also found that Ginger tends to improve lipid metabolism.<sup>24</sup> Wang et al. reported that Ougi could significantly reduce plasma levels of TC and LDL-C and improved the atherosclerosis profile.<sup>57</sup> These results partly explain the present findings. We speculate that inhibition of lipid absorption might contribute to the effect of BOT.<sup>22</sup> However, the mechanisms of BTS and KBG are still unknown.

Inflammatory factors play important roles in NAFLD and atherosclerosis progression.<sup>8</sup> MCP1 is an important inflammatory chemokine that can be produced by a variety of cells including vascular endothelial cells, vascular smooth muscle cells, monocytes and other cells. It is a member of the CC chemokine family.<sup>58, 59</sup> Plasma concentration of MCP1 increases with obesity.<sup>60, 61</sup> CCR2 is a receptor of MCP1 and can help MCP1 to accomplish its effect.<sup>62</sup> Dietary cholesterol can induce the MCP1 gene expression,<sup>14</sup> and CCR2 expression was increased in hypercholesterolemic patients compared with normocholesterolemic controls.<sup>15, 63</sup> In our experiment, compared with the other groups, the mRNA expression of MCP1 and CCR2 in both liver and adipose tissue in Group H remained high level during the entire experiment, especially after 12 weeks. BOT, BTS, KBG and ezetimibe can down-regulate the MCP1 and CCR2 expression. This effect of BOT, BTS and KBG is direct or indirect through inhibition of cholesterol metabolism is still

unknown.

ICAM-1, a member of the immunoglobulin superfamily, is one of the markers of endothelial cell activation. It plays an important role in neutrophil migration and adhesion of endothelial cells<sup>64</sup> and is partly involved in the whole process of monocyte adhesion, migration and transformation.<sup>16</sup> This migration is one of the earliest events in the atherosclerotic process.<sup>65</sup> ICAM-1 was up-regulated in neointimal and medial smooth muscle cells after vascular injury.<sup>66</sup> Sekiya et al. reported that KBG could prevent the progression of atheromatous plaque,<sup>67</sup> but no literature could prove the effect of BOT and BTS on preventing atherosclerosis. In our experiment, serum ICAM-1 concentration and immunostaining expression were increased in Group H after 12 weeks. BOT, BTS, KBG and ezetimibe can down-regulate it. We speculate that down-regulated ICAM-1 may be one of the mechanisms of BOT, BTS and KBG to prevent atherosclerosis.

Overall, BOT has a protective effect on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats and more effective than BTS and KBG. Ezetimibe has the similar effect on lipid related factors, such as LDL-C, RBP4, HFABP and CFABP. However, the anti-inflammatory (MCP1, CCR2) and anti-arteriosclerotic (ICAM-1) effects of BOT are more potent than ezetimibe. This may be a guide on clinical use. The mechanism is still uncertain and further studies should be designed.

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