

Evidence-Based Efficacy of Kampo Formulas in a Model of Non Alcoholic Fatty Liver

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Data on the efficacy of herbal compounds are often burdened by the lack of appropriate controls or a limited statistical power. Treatments to prevent the progression of non alcoholic fatty liver disease (NAFLD) to steatohepatitis (NASH) remain unsatisfactory. A total of 56 rabbits were arrayed into 7 groups fed with standard rabbit chow (SRC), SRC with 1% cholesterol, or each of the five experimental treatments (Kampo formulas 1% keishibukuryogan [KBG], 1% orengedokuto [OGT], and 1% shosaikoto [SST]; vitamin E [VE]; or pioglitazone [PG]) in a 1% cholesterol SRC. We analyzed changes after 12 weeks in plasma and liver lipid profiles, glucose metabolism, adipocytokines, oxidative stress, and liver fibrosis. Data demonstrated that all five treatments were associated with significant amelioration of lipid profiles, oxidative stress, and liver fibrosis compared to no supplementation. KBG was superior to VE and PG in the reduction of liver total cholesterol ($P < 0.01$) and lipid peroxidase

levels ($P < 0.05$), urinary 8-hydroxy-2'-deoxyguanosine ($P < 0.05$), hepatic α -smooth muscle actin positive areas ($P < 0.01$) and activated stellate cells ($P < 0.01$). In conclusion, there was a statistically significant benefit of Kampo formulas (KBG in particular) on a dietary model of NAFLD/NASH. Future studies need to be directed at the mechanisms in the treatment of NASH. *Exp Biol Med* 233:328–337, 2008

Key words: nonalcoholic steatohepatitis; alternative medicine; hepatic stellate cells; adiponectin

Introduction

Traditional Japanese and Chinese herbal medicine in the treatment of chronic liver diseases has been supported by limited scientific evidence (1). In fact, available data addressing their efficacy in human conditions are commonly burdened by small study populations, inadequate controls, or insufficient endpoints. We were intrigued by the observation that keishibukuryogan (KBG), orengedokuto (OGT), and shosaikoto (SST), belonging to the family of Kampo formulas, appear to exert anti-oxidant and anti-inflammatory activities in models of liver fibrosis (2), rheumatoid arthritis (3), carcinogenesis (4), and atherosclerosis (5), in some cases superior to other anti-oxidants (6).

Obesity currently affects approximately 30% of the United States population (7) and clusters with several abnormalities related to insulin resistance, including non-alcoholic fatty liver disease (NAFLD). NAFLD represents a broad spectrum of diseases ranging from simple fatty

The study was supported by a Grant-in-Aid for the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Received July 30, 2007.
Accepted November 27, 2007.

DOI: 10.3181/0707-RM-207
1535-3702/08/2333-0328\$15.00
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changes to severe steatohepatitis with marked fibrosis, i.e. nonalcoholic steatohepatitis (NASH), that may progress to cirrhosis and hepatocellular carcinoma (8). What causes the transition from NAFLD to NASH remains enigmatic, and a two-hit theory has been proposed (9), with oxidative stress currently considered to provide the “second hit” on a fatty liver background (10). Based on experimental observations and prompted by the growing impact of NASH, treatments with vitamin E (VE) (11) and pioglitazone (PG) (12) have been proposed.

We thus hypothesized that the mentioned Kampo formulas might prove beneficial in a recently established cholesterol-fed non obese rabbit model of NAFLD/NASH (13). Our readouts included (i) markers of liver function, lipid and glucose metabolism; (ii) liver fibrosis and lipid contents; (iii) markers of oxidative stress; and (iv) plasma mediators of inflammation and fibrosis. Data indicate that the proposed formulas exert a protective effect at different levels, with KBG being equal or superior to currently proposed treatments.

Materials and Methods

Compounds. VE was purchased from CLEA Japan, Inc. (Tokyo, Japan); PG was kindly provided by Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). KBG (TJ-25), OGT (TJ-15), and SST (TJ-9) were obtained from Tsumura & Co. (Tokyo, Japan). Kampo formula powdered extracts were derived from a total of 16 medical plants (all registered in the Japanese Pharmacopoeia) as illustrated in Table 1.

Animal Models and Sample Collection. Fifty-six male white rabbits (baseline weight 2 kg) were purchased at 10 weeks of age from Nippon SLC Co. (Hamamatsu, Japan) and kept in individual cages at room temperature ($23 \pm 1^\circ\text{C}$) under a 12-hour dark-light cycle. They were allowed an adaptation period of 2 weeks and were subsequently randomly allocated to 7 groups (8 rabbits/group) for 12 additional weeks. All groups were fed with 100g/day of standard rabbit chow (CR-3; CLEA Japan, Inc.), and in 6 groups 1% cholesterol was added. Among the latter groups, group C was not given any additional supplement or compound. The other 5 groups received 1% KBG (group KBG), 1% OGT (group OGT), 1% SST (group SST), 0.045% vitamin E (group VE), or 300 ppm PG (group PG). Treatment regimens and compounds were chosen based on previous studies from our group (6) and other laboratories (14, 15).

As mentioned above, one group (N) was fed with CR-3 only. Following the 12-week period and an overnight fasting, all rabbits were weighted and euthanized under anesthesia and tissues were collected. These included urine, blood (from the inferior vena cava), and liver (perfused with physiological saline and dissected). All procedures complied with the current guidelines for the care and use of laboratory animals, and the protocol was approved by the

Committee on Animal Experimentation of the University of Toyama.

Plasma Markers for Lipid and Glucose Metabolism and Liver Injury. Plasma total cholesterol (T-chol), high-density lipoprotein cholesterol (HDL-chol), low-density lipoprotein cholesterol (LDL-chol), triglyceride (TG), free fatty acid (FFA), serum aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured in all samples using routine methods. Hyaluronic acid (HA) levels were also tested as markers of fibrosis following methods described elsewhere (16). Plasma insulin and glucose levels were determined by Insulin ELISA Kit, Ultra Sensitive (Morinaga Institute of Biological Science, Inc., Yokohama, Japan) and Antisense III (Horiba, Ltd., Kyoto, Japan), respectively.

Plasma Adipocytokines and TGF- β 1. Adipocytokines (i.e. leptin, adiponectin) play an important role in the liver and peripheral glucose and lipid metabolism (17) as well as in liver fibrosis (18), and they have been suggested as critical factors in NASH development (19). Plasma samples were analyzed for leptin and adiponectin using commercially available kits (Multi-Species Leptin RIA Kit, Linco Research Inc., Saint Charles, MO; Adiponectin ELISA Kit, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Transforming growth factor- β 1 (TGF- β 1) is an established mediator for fibrosis development, and its levels have been correlated with NASH onset (11). All plasma samples were analyzed for TGF- β 1 following activation of the latent form to an immunoreactive one using 2.5 M acetic acid and 10 M urea followed by 2.7 N and 1 M HEPES. Activated samples were then diluted and analyzed using a quantitative sandwich enzyme immunoassay technique with recombinant human TGF- β 1 soluble receptor type II onto a microplate (R&D System Inc., Minneapolis, MN). Optical densities were then measured at 450 nm and the concentration of TGF- β 1 determined on a standard curve.

Markers of Oxidative Stress. The degree of oxidative stress was evaluated by measuring levels of plasma and hepatic lipid peroxide (LPO) and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG). Liver samples were first weighed and minced in ice-cold PBS (pH 7.4), then homogenized at a ratio of 1:10 (w:v). Following centrifugation at 3,000 g for 10 minutes at 4°C , supernatants were collected and used for the analysis. LPO levels were determined using a lipid peroxidation assay kit (Determinant LPO: Kyowa Medex Co., Tokyo, Japan). Urine samples were centrifuged at 10,000 g for 15 minutes and the supernatants used for 8-OHdG measurement by competitive ELISA (Japan Institute for the Control of Aging, Fukuroi, Japan). Urinary creatinine was also determined using standard methods to allow the appropriate corrections of 8-OHdG levels.

Liver Lipid Content and Fibrosis. For lipid content determination, liver samples were homogenized and lipids extracted using a mixture of chloroform and methanol (2:1, v:v) to subsequently analyze the amount of

Table 1. Medicinal Plants and Weight (g) Used to Obtain Keishibukuryogan (KBG, TJ-25), Orenge dokuto (OGT, TJ-15) and Shosaikoto (SST, TJ-9)

		Weight (g)
KBG		
Cinnamomi cortex	<i>Cinnamomum cassia</i> Blume	3.0
Paeoniae radix	<i>Paeonia lactiflora</i> Pallas	3.0
Persicae semen	<i>Prunus persica</i> Batsch	3.0
Hoelen	<i>Poria cocos</i> Wolf	3.0
Moutan cortex	<i>Paeonia suffruticosa</i> Andrews	3.0
OGT		
Scutellariae radix	<i>Scutellaria baicalensis</i> Georgi	3.0
Coptidis rhizoma	<i>Coptis japonica</i> Makino	2.0
Gardeniae fructus	<i>Gardenia jasminoides</i> Ellis	2.0
Phellodendri cortex	<i>Phellodendron amurense</i> Ruprecht	1.5
SST		
Bupleuri radix	<i>Bupleurum falcatum</i> Linné	7.0
Pinelliae tuber	<i>Pinellia ternata</i> Breitenbach	5.0
Scutellariae radix	<i>Scutellaria baicalensis</i> Georgi	3.0
Zizyphi fructus	<i>Zizyphus jujuba</i> Miller	3.0
Ginseng radix	<i>Panax ginseng</i> C.A. Meyer	3.0
Glycyrrhizae radix	<i>Glycyrrhiza uralensis</i> Fischer	2.0
Zingiberis rhizoma	<i>Zingiber officinale</i> Roscoe	1.0

T-chol and free cholesterol, TG and FFA using commercially available kits (Triglyceride, Cholesterol, Free cholesterol, and NEFA tests, all from Wako Pure Chemical Ind., Ltd., Osaka, Japan). Sections (5- μ m-thick) of formalin-fixed and paraffin-embedded liver tissues were obtained from each experimental animal. The degree of fibrosis was quantitated through the tissue density of activated hepatic stellate cells (HSCs) and the expression of α -smooth muscle actin (α -SMA). HSCs were stained using a mouse monoclonal anti-macrophage antibody (DAKO Cytomation Co., Glostrup, Denmark) and guinea pig polyclonal anti adipocyte differentiation-related protein (ADRP) antibody (Progen Biotechnik, Heidelberg, Germany) as previously described (13), and cells were counted in three microscopic fields ($\times 400$). For the study of α -SMA expression, a specific murine monoclonal antibody was purchased from DAKO Cytomation Co. and used for immunohistochemistry. Areas of α -SMA positive lesions were evaluated in 6 microscopic fields ($\times 40$) per specimen and expressed as the percentage of the total area of the fields. For both HSC count and the α -SMA expression study, two independent pathologists blindly evaluated all samples.

Statistical Analysis. All continuous variables are expressed as mean $\pm$ standard error of measurement (SEM). To compare the efficacy of Kampo formulas with other treatments, pairwise comparisons between groups C, VE, and PG and each of the other three groups were performed by establishing one-way analysis of variance models for multiple comparison tests (Bonferroni). All analyses were two-tailed, and P values < 0.05 were considered as statistically significant. Importantly, all comparisons were performed blindly as to the treatments corresponding to each group. Statistical analyses were performed using Intercooled Stata 8.0 (Stata Corp, College Station, TX).

Results

Body Weight and Liver/Body Weight Ratio. To ascertain if the used supplementations had an effect on lipid adsorption and on body and liver weight, these variables were compared among groups at the end of the treatment (Table 2). Cholesterol-fed groups had similar body weight, in all cases significantly lower than group N. Similarly, all cholesterol-fed groups showed higher liver/body weight ratios compared to group N.

Plasma Markers for Lipid and Glucose Metabolism and Liver Injury. Table 2 illustrates the plasma lipid profiles at the end of the study in each treatment group. Importantly, T-chol levels in group C were significantly higher compared to each of the other supplemented groups ($P < 0.01$ for all comparisons), and the KBG group had the lowest T-chol levels with the expected exception of the group receiving neither cholesterol nor treatments. Data on LDL-chol and FFA also manifested the same degree of significance, while levels of HDL-chol and TG failed to reach statistically significant differences. As for glucose metabolism and biochemical signs of liver injury (Table 3), no significant differences were observed between treatment groups in fasting glucose or insulin levels and transaminase (AST, ALT) activities.

Serum HA, Plasma Adipocytokines, and TGF- $\beta 1$. Serum levels of HA were significantly lower in groups KBG and N compared to group C, while other differences were not statistically significant (Table 3). Plasma levels of TGF- $\beta 1$ following PG treatment were significantly lower compared to group C, while no significant differences were observed among groups for leptin levels (Table 3). When adiponectin levels were compared, KBG was the only supplement associated with significantly higher levels compared to group C (Table 3).

Table 2. Body Weight, Liver/Body Weight Ratio, and Lipid Metabolism in the 7 Experimental Groups^a

Parameters	N	C	KBG	OGT	SST	VE	PG
Body weight (kg)	3.42 ± 0.06 ^{b,c}	2.90 ± 0.05	2.97 ± 0.08	2.95 ± 0.03	2.81 ± 0.05	3.02 ± 0.09	3.09 ± 0.05
% Liver/body wt	2.8 ± 0.1 ^b	4.1 ± 0.2	3.7 ± 0.2	3.7 ± 0.1	5.3 ± 0.2 ^{b,c,d}	4.2 ± 0.2	3.6 ± 0.1
T-chol (mg/dL)	19 ± 2 ^{b,c}	1999 ± 276	675 ± 159 ^b	960 ± 145 ^b	1227 ± 128 ^b	1213 ± 178 ^b	1293 ± 76 ^b
HDL-chol (mg/dL)	13.0 ± 2.0	16.4 ± 3.8	16.9 ± 2.2	14.6 ± 1.9	15.9 ± 1.4	16.5 ± 3.0	12.6 ± 2.0
LDL-chol (mg/dL)	5.0 ± 0.8 ^{b,c}	741.6 ± 69.5	307.1 ± 54.3 ^{b,d}	423.1 ± 46.4 ^b	494.0 ± 61.3 ^b	426.1 ± 50.4 ^b	568.8 ± 39.1
TG (mg/dL)	37.8 ± 10.8	69.1 ± 24.5	52.3 ± 17.7	45.9 ± 13.1	98.6 ± 23.4	58.8 ± 16.6	46.9 ± 13.6
FFA (mEq/L)	0.28 ± 0.02 ^b	0.64 ± 0.11	0.39 ± 0.03 ^b	0.35 ± 0.03 ^b	0.28 ± 0.03 ^b	0.32 ± 0.03 ^b	0.32 ± 0.02 ^b

^a T-chol, total cholesterol; HDL-chol, high-density lipoprotein cholesterol; LDL-chol, low-density lipoprotein cholesterol; TG, triglyceride; FFA, free fatty acid. Values are expressed as mean ± SEM. Groups: N and C, no supplement; KBG, keishibukuryogan; OGT, orengedokuto; SST, shosakoto; VE, vitamin E; PG, pioglitazone. All groups were fed with standard chow with 1% cholesterol except for group N.

^b $P < 0.01$ vs. group C.

^c $P < 0.01$ vs. group VE.

^d $P < 0.01$ vs. group PG.

Table 3. Profiles of Glucose Metabolism, Liver Injury, TGF-β1, Leptin, Hyaluronic Acid, and Adiponectin Levels in the 7 Experimental Groups^a

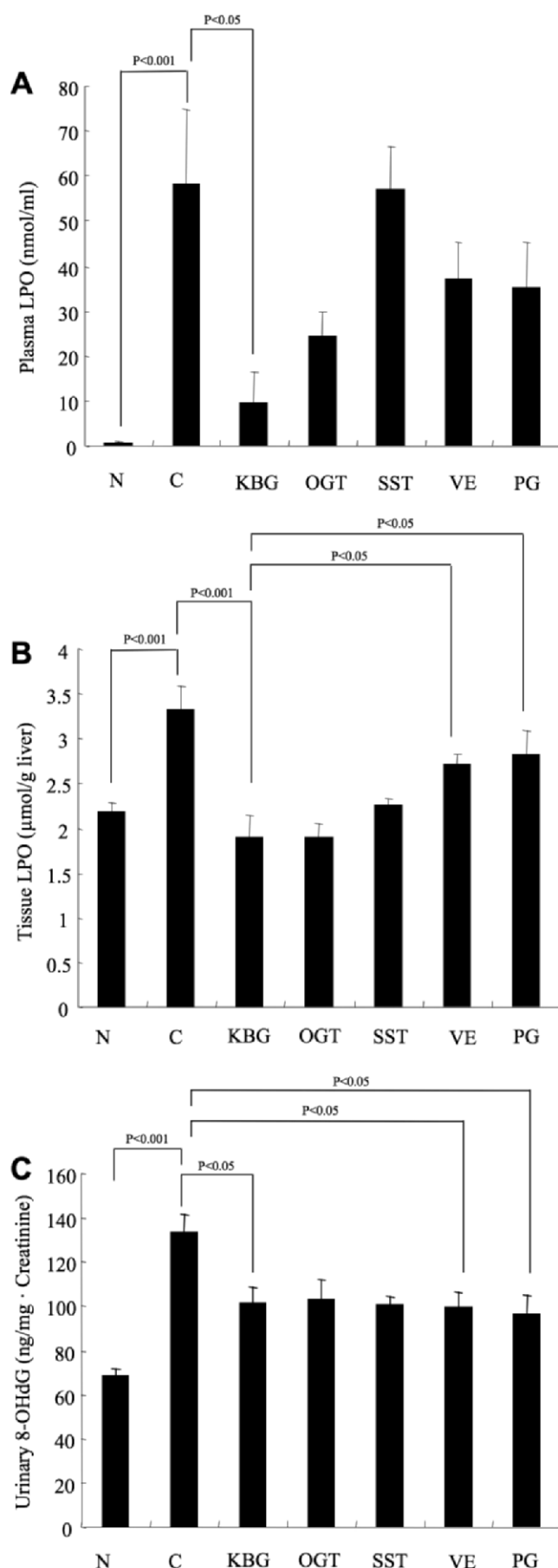
Parameters	N	C	KBG	OGT	SST	VE	PG
Fasting plasma glucose (mg/dL)	161.6 ± 36.4	120.4 ± 6.4	117.8 ± 7.7	138.1 ± 7.8	121.8 ± 3.5	151.9 ± 19.1	181.3 ± 17.0
Insulin (ng/mL)	244.0 ± 74.8	237.9 ± 71.4	183.1 ± 48.5	170.8 ± 56.4	329.9 ± 57.6	383.8 ± 151.9	209.9 ± 82.9
AST (IU/L)	50.0 ± 13.2	31.5 ± 3.1	55.4 ± 18.5	30.1 ± 3.0	37.6 ± 4.2	41.8 ± 6.8	37.5 ± 5.0
ALT (IU/L)	54.0 ± 11.5	58.6 ± 8.5	56.4 ± 11.8	45.8 ± 7.8	56.0 ± 14.9	49.3 ± 7.4	44.5 ± 4.7
TGF-β1 (pg/mL)	1.22 ± 0.17 ^b	1.71 ± 0.12	1.40 ± 0.12	1.29 ± 0.12	1.22 ± 0.06	1.37 ± 0.14	1.12 ± 0.10 ^b
Leptin (ng/mL)	2.30 ± 0.28	2.06 ± 0.18	1.66 ± 0.27	1.79 ± 0.29	2.57 ± 0.11	1.89 ± 0.25	1.93 ± 0.27
HA (pg/mL)	25.03 ± 5.77 ^d	214.91 ± 33.03	93.03 ± 15.56 ^c	115.31 ± 17.29	214.73 ± 32.51	117.11 ± 28.19	171.13 ± 28.91
Adiponectin (mg/mL)	266.88 ± 48.50	176.69 ± 17.94	415.50 ± 69.74 ^c	340.88 ± 49.70	220.75 ± 15.12	260.63 ± 45.62	272.13 ± 56.42

^a AST, aspartate aminotransferase; ALT, alanine aminotransferase; TGF-β1, transforming growth factor-β1; HA, hyaluronic acid. Values are expressed as mean ± SEM. Groups: N and C, no supplement; KBG, keishibukuryogan; OGT, orengedokuto; SST, shosakoto; VE, vitamin E; PG, pioglitazone. All groups were fed with standard chow with 1% cholesterol except for group N.

^b $P < 0.01$ vs. group C.

^c $P < 0.05$ vs. group C.

^d $P < 0.001$ vs. group C.



Markers of Oxidative Stress. With the exception of group N, group KBG was the only group with significantly lower plasma LPO levels compared to group C (Fig. 1A), while such significant difference was observed in liver tissue levels with KBG superior to PG or VE (Fig. 1B). Urinary amounts of 8-OHdG were significantly lower in the KBG, VE and PG supplement groups compared to group C (Fig. 1C).

Liver Lipid Content and Fibrosis. Lipid contents in the liver tissue from all experimental groups are shown in Table 4. KBG treatment was associated with the lowest levels of T-chol, free cholesterol, and TG. Importantly, KBG treatment was also associated with significantly lower T-chol and free cholesterol, FFA and TG compared to PG, while the former three variables also manifested significant decreases in KBG compared to VE. Figure 2 illustrates the distribution of α -SMA immuno-positive areas as an index of HSC activation in representative liver tissues from each of the 7 study groups. Such areas were virtually absent in group N (Fig. 2A) while being well represented in group C (Fig. 2B). Using semi-quantitative analysis, we observed that all groups had lower representation of α -SMA positive areas per microscopy field compared to group C, while KBG treatment led also to significantly lower cell prevalence compared to groups PG and VE (Fig. 2H). Furthermore, our double immunohistochemical staining for HSCs (Fig. 3A) demonstrated that these cells were less represented in liver tissues from groups KBG and OGT compared to group C. Importantly, groups KBG and OGT had significantly lower values of HSCs compared to both VE or PG supplementations, with the latter surprisingly higher than group C (Fig. 3B).

Discussion

We report herein for the first time a comprehensive set of rigorous data supporting that traditional Japanese Kampo formulas appear as effective preventive treatments for NASH development in a non obese rabbit model. Importantly, such results are consistent in all our study endpoints representative of the features of the disease and favorably compare to currently supported treatments (i.e. VE, PG). Among Kampo formulas, KBG induced the largest amelioration in nearly all analyzed features, and in several readouts KBG was superior to VE and PG.

The use of Japanese and Chinese traditional herbal medicine for human diseases is becoming more common (20) despite being often supported by scanty or poorly

Figure 1. Lipid peroxide (LPO) plasma (A) and liver tissue (B) levels and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels (C) at the end of the study period. Values are expressed as mean $\pm$ SEM. Groups: N and C, no supplement; KBG, keishibukuryogan; OGT, orangedokuto; SST, shosaikoto; VE, vitamin E; PG, pioglitazone. All groups were fed with standard chow with 1% cholesterol except for group N. Pairwise comparisons were performed between Kampo formulas and groups C, VE and PG, and *P* values were corrected for multiple testing.

Table 4. Liver Lipid Contents in the 7 Experimental Groups^a

Parameters	N	C	KBG	OGT	SST	VE	PG
T-choI (mg/g liver)	3.41 ± 0.08 ^b	21.65 ± 3.70	11.05 ± 2.10 ^{b,c,d}	14.58 ± 2.63	16.14 ± 1.32	21.07 ± 1.21	21.39 ± 1.67
TG (mg/g liver)	7.26 ± 0.90 ^d	9.95 ± 0.81	6.99 ± 0.60 ^d	8.45 ± 1.10	11.98 ± 0.46	10.11 ± 0.73	11.21 ± 0.53
Free cholesterol (mg/g liver)	4.36 ± 0.10 ^b	11.21 ± 1.72	4.82 ± 1.29 ^{b,c,d}	6.56 ± 1.44 ^{b,d}	10.14 ± 0.57	10.11 ± 0.73	13.59 ± 0.94
FFA (10 ⁻³ mEq/g liver)	4.00 ± 0.13	6.69 ± 0.54	5.28 ± 0.72 ^{c,d}	4.66 ± 0.67	8.03 ± 0.98	9.61 ± 0.90	10.85 ± 1.02 ^b

^a T-choI, total cholesterol; TG, triglyceride; FFA, free fatty acid. Values are expressed as mean ± SEM. Groups: N and C, no supplement; KBG, keishibukuryogan; OGT, orengedokuto; SST, shosaikoto; VE, vitamin E; PG, pioglitazone. All groups were fed with standard chow with 1% cholesterol except for group N.

^b $P < 0.01$ vs. group C.

^c $P < 0.01$ vs. VE.

^d $P < 0.01$ vs. PG.

reproducible scientific evidence. In fact, the conclusions of most available studies are limited by several methodological flaws (21). For these reasons, and based on preliminary *in vitro* data, we performed an extensive blind analysis of the effects of Kampo formulas in a liver disease animal model and report for the first time their efficacy in ameliorating NASH features.

Although what causes the transition from NAFLD to NASH remains largely unknown, a current concept involves a “two-hit” hypothesis in which an initial metabolic disturbance (represented by lipid and glucose dysregulation) induces the fatty liver onset; a second pathogenic stimulus leads to oxidative stress and ultimately NASH with inflammation and fibrosis (9). The transition might be mediated by HSC activation by reactive oxygen species, and our observation that all tested supplements led to lower levels of HSC activation in the areas of α -SMA positive lesions supports this thesis (Fig. 2). Similarly, all supplements except PG produced similar changes of the HSC phenotype compatible with an anti-fibrotic effect. The reasons for the PG discrepancy can currently be only hypothesized in the fact that its peroxisome proliferators-activated receptor γ (PPAR- γ) agonist activity might inhibit the activation of quiescent HSCs to be myofibroblast-like cells (22). The fact that PPAR- γ expression is a sign of the phenotypic switch from activated to quiescent HSCs (23) also strengthens this view. Further, our data cumulatively support the existence of different mechanism pathways for Kampo formulas and other proposed NAFLD treatments such as PG. It is of note that the α -SMA positive area in the group treated with KBG was significantly lower than in animals treated with VE or PG, thus indicating a stronger effect of this Kampo formula on preventing liver fibrosis. The anti-fibrotic effect of Kampo formulas had been previously suggested by Shimizu and colleagues reporting that SST inhibited lipid peroxidation in hepatocytes and HSCs in a rat liver fibrosis model (2), while others demonstrated that SST prevented fibrosis by inhibiting the activation of HSCs in a diet-induced rat NASH model (24). We also submit that additional pathology changes were observed in treated groups, including hepatocellular fat deposition, megamitochondria and hepatocellular ballooning with Mallory bodies, but fibrosis was the prominent observation and was thus used for comparisons.

One major issue raised by these data is based on the need to identify what components of Kampo formulas are ultimately effective and produce the observed changes. While we cannot provide an answer to this critical question, we can surmise some directions from previous reports. For example, the tannins found in berries as well as in KBG components (25, 26) are known to be potent anti-oxidants (27). Similarly, other substances (baicalein, baicalin, wogonin) or herbs (*Coptidis rhizoma* and *Phellodendri cortex*) found in OGT and SST also exert a scavenging activity (28, 29). We are also aware of the possibility that multiple Kampo formulas might produce a synergistic effect

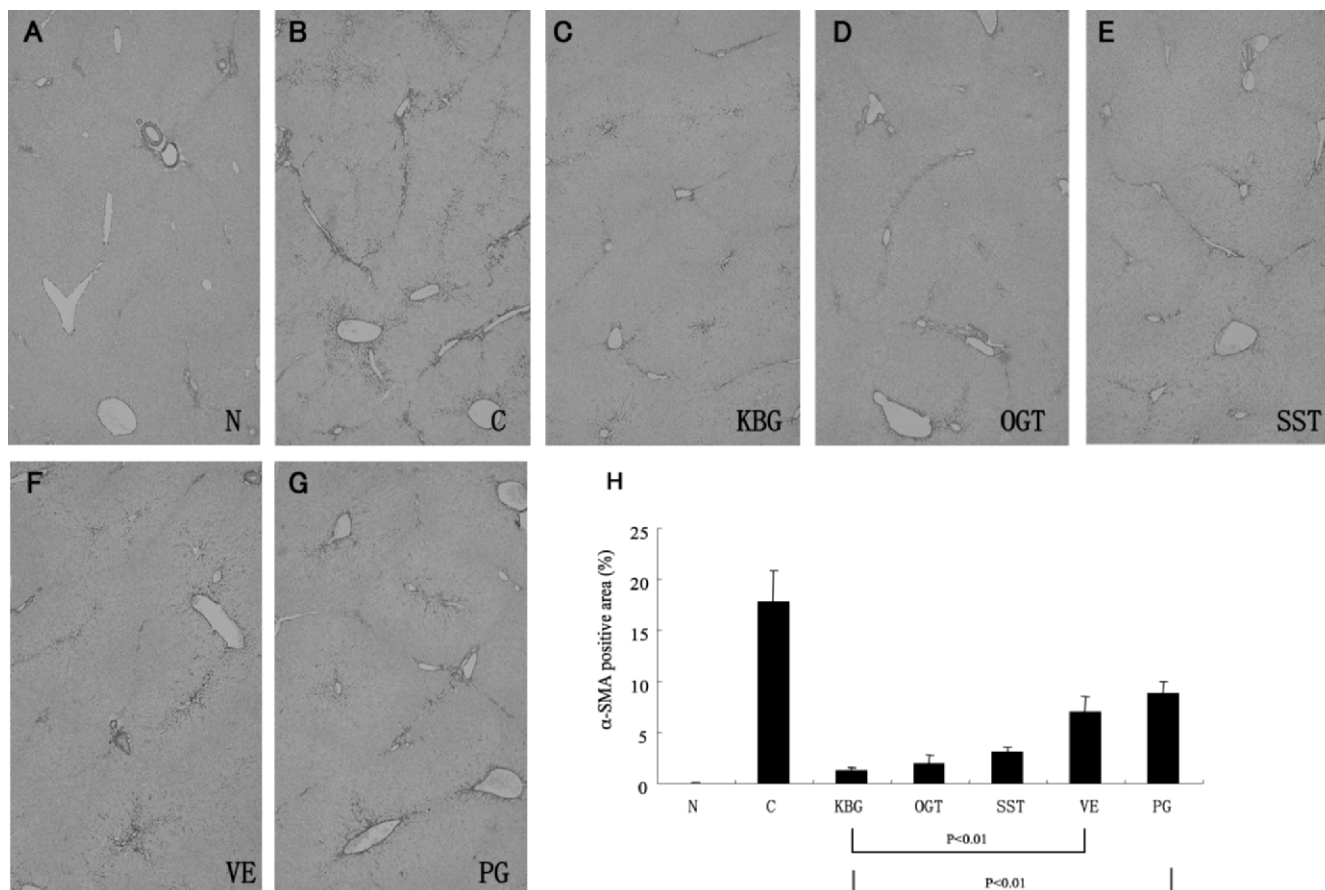


Figure 2. Alpha-smooth muscle actin (α -SMA) immunohistochemical findings in the liver tissue ($\times 40$) at the end of the study period. Groups: N (A) and C (B), no supplement; KBG, keishibukuryogan (C); OGT, orengedokuto (D); SST, shosaikoto (E); VE, vitamin E (F); PG, pioglitazone (G). All groups were fed with standard chow with 1% cholesterol except for group N. α -SMA immuno-positivity was not observed in the rabbits of group N. α -SMA immuno-positive areas are extended in the perivenular area in the rabbits of group C. α -SMA immuno-positive areas appear in the rabbits of groups KBG, OGT, SST, VE and PG, but they are milder than group C. H illustrates the percentage of α -SMA positive area evaluated by computer-assisted semi-quantitative analysis. Values are expressed as mean $\pm$ SEM. Pairwise comparisons were performed between Kampo formulas and C, VE and PG groups, and P values were corrected for multiple testing; for all comparisons of group C vs. other groups, $P < 0.001$ (not shown).

when supplemented or that a dose-dependent effect might apply, but we are convinced that, for scientific purposes, the priority in future studies will be dedicated to the search for the active compounds.

Notably, nearly all measured parameters manifested similar degrees of improvement using KBG, with the possible exception of the glucose metabolism that was characterized by non significant changes in all groups. On the other hand, KBG markedly influenced the lipid profiles that commonly characterize NAFLD, as mainly indicated by the reduced plasma T-chol levels. Previous reports had demonstrated a similar activity for KBG in a rat model of diabetic nephropathy (30) as well as its preventive effectiveness on atherosclerosis in cholesterol-fed rabbits (6). On a different level, the observed effect of Kampo formulas on adipocytokines is a novel observation since data on these molecules in NASH are limited to tumor necrosis factor α (TNF- α) expression (31) and leptin levels (32) as proinflammatory indicators. While we did not

measure TNF- α expression, we could not recapitulate the reported differences in leptin levels, possibly because of the lack in our model of obesity and hyperinsulinemia, two major determinants of leptin levels (33). On the other hand, adiponectin is a metabolic mediator produced by adipocytes, and its plasma levels were shown to be reduced in patients with NAFLD (34). Adiponectin in fact appears to play a role in liver injury development (35), possibly by protecting against oxidative stress (36) and inflammation (37). This is also supported by the activity of several pharmacological compounds (including PG) used in the metabolic syndrome that increase adiponectin plasma levels (38), thus suggesting that adiponectin might ultimately constitute an effective target for NASH therapies (39). In our experimental model, PG supplementation did not induce significant changes, possibly due to the lack of insulin resistance and obesity that mediate adiponectin levels (40). On the other hand, only KBG induced a significant increase in adiponectin levels, despite the similar body weights among groups. This effect

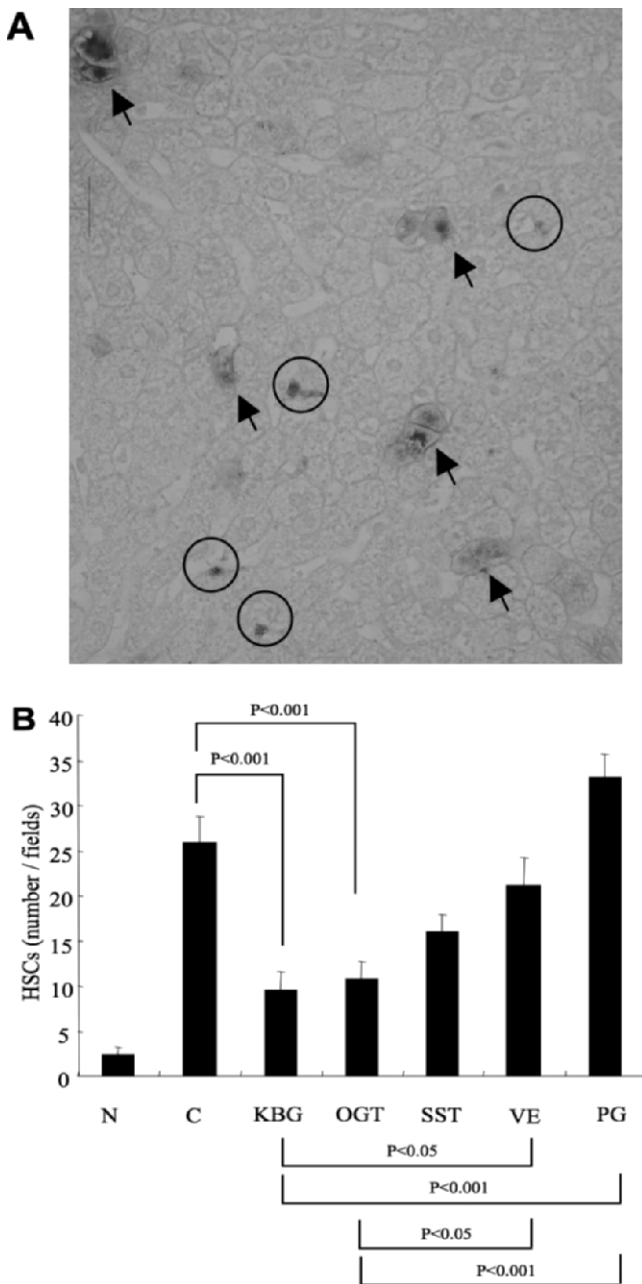


Figure 3. Double immunostaining (A, magnification $\times 400$) for hepatic stellate cells (HSCs) using adipocyte differentiation-related protein (ADRP, circles) brown and macrophage blue staining (arrows). Groups: N and C, no supplement; KBG, keishibukuryogan; OGT, orengedokuto; SST, shosaikoto; VE, vitamin E; PG, pioglitazone. The number of HSCs showing single ADRP staining in the liver tissue of all groups is illustrated in B. Values are expressed as mean $\pm$ SEM. Pairwise comparisons were performed between Kampo formulas and groups C, VE and PG, and *P* values were corrected for multiple testing.

might then be explained with the observed changes in the lipid contents in the examined liver tissues from model rabbits. Quite surprisingly, liver FFA content was significantly higher in the PG-treated group compared to group C, and the significance of this observation remains unclear. We can only hypothesize that increased blood glucose levels

and body fat mass might play a role (12, 41) or that new interactions between metabolic and inflammatory pathways should be identified, similar to what was reported in autoimmune diseases (42, 43).

One limitation of the present rabbit model for the metabolic syndrome is the absence of obesity, insulin resistance, and hypertransaminasemia. However, clinical data suggest that these conditions do not consistently associate with NASH, with frequently encountered lean patients (44) with no transaminase elevation (45). In contrast, liver pathology is the ultimate criterion for fatty liver diagnosis. To this extent, we submit that a major strength of this model is in the liver histology that includes fatty and inflammatory changes as well as extensive perivenular and pericellular fibrosis, resembling the unique features of human NASH. Finally, we hypothesize that longer observations might produce different features and reproduce other NASH features such as frank cirrhosis and possibly liver cancer.

The collected evidence appears to indicate that Kampo formulas in general and KBG in particular are effective in exerting a protective effect in the NAFLD establishment as well as the transition to NASH, that is, both “hits” of the disease pathogenesis. This hypothesis will warrant further studies in other animal models manifesting also obesity and insulin resistance before pilot human data are sought. In fact, data obtained in animal models, particularly for NASH, might prove non applicable to humans in the clinical practice, particularly in terms of efficacy, while the plethora of animal models (46) proposed thus far paradoxically contributes to make the picture less clear. Furthermore, researchers in other phenotypes of the metabolic syndrome are encouraged to investigate the effects of Kampo formulas on other outcomes (i.e. cardiovascular events). In fact, a growing fund of knowledge is recently stressing the importance of comorbidities in the resulting scenario, as it appears to be the case for intermittent hypoxia from obstructive sleep apnea (47) or new effector mechanisms (48). In conclusion, we acknowledge that a pharmacological approach to slowly progressing diseases such as NAFLD and NASH must be primarily preventive, and we submit that Kampo formulas are an excellent candidate for future studies, particularly considering the paucity of side effects observed in other clinical settings (49) and the need for prolonged treatments.

The authors are grateful to Tokimasa Kumada and Hideki Hatta for their technical assistance.

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