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Research Paper

Chinese prescription Kangen-karyu prevents dyslipidaemia and oxidative stress in mouse model of type 2 diabetes

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Abstract

Objectives We have investigated the effects of Kangen-karyu, a Chinese prescription, on the lipid metabolism and oxidative stress in a type 2 diabetes model.
Methods Male *db/db* mice were divided into three groups: control (vehicle), Kangen-karyu 100 or 200 mg/kg body weight/day orally administered mice. Age-matched non-diabetic *m/m* mice were used as a normal group.
Key findings The administration of Kangen-karyu reduced hyperglycaemia and hyperlipidaemia in *db/db* type 2 diabetic mice through a decline in the serum levels of glucose and lipids, and an improvement of lipoprotein profiles. The increased oxidative stress in *db/db* mice was attenuated by the administration of Kangen-karyu through inhibiting the generation of reactive oxygen species and lipid peroxidation. The enhanced hepatic triglyceride and total cholesterol levels of the *db/db* mice were significantly reduced by Kangen-karyu administration through down-regulation of sterol regulatory element-binding protein-1 and lipogenic enzymes in liver. Furthermore, the expressions of hepatic nuclear factor-kappa B (NF-κB) and cyclooxygenase-2 and inducible nitric oxide synthase protein levels were also augmented in *db/db* mice. However, Kangen-karyu reduced the expressions of these inflammatory proteins by inhibiting NF-κB activation in *db/db* type 2 diabetes.
Conclusions This study suggests that Kangen-karyu may improve oxidative stress via the regulation of dyslipidaemia in type 2 diabetes.
Keywords *db/db* mice; hyperlipidaemia; Kangen-karyu; oxidative stress; type 2 diabetes

Introduction

Type 2 diabetes, the predominant type of diabetes mellitus, is characterized by absolute or relative deficiencies in insulin secretion or insulin action that lead to chronic hyperglycaemia, with deleterious effects on β-cell function. In addition, individuals with type 2 diabetes almost invariably show a marked disruption of lipid dynamics, often reflected by elevated levels of circulating free fatty acids (FFA) and triglycerides (TG), together with excess fat deposition in various tissues including the liver. An abnormal accumulation of fat in the liver and muscle plays an important role in the etiology of insulin resistance, and possibly also in β-cell reduction in type 2 diabetes.[1,2] Hyperglycaemia and dyslipidaemia induce generation of free radicals, inflammatory responses and oxidative stress reactions accounts for the complications and mortality of obesity and type 2 diabetes. In the absence of an appropriate compensatory response from the endogenous antioxidant network against glucotoxicity and lipotoxicity caused by hyperglycaemia and hyperlipidaemia in diabetes, oxidative stress becomes marked, leading to the activation of stress-sensitive intracellular signalling pathways.[3,4] Therefore, the attenuation of oxidative stress and regulation of hyperlipidaemia have been considered as ways to alleviate diabetes and its complications.

Kangen-karyu (Guan-Yuan-Ke-Li), a crude drug developed from a traditional Chinese prescription consisting of six herbs (Paeoniae Radix, Cnidii Rhizoma, Carthami Flos, Cyperi Rhizoma, Saussureae Radix and Salviae Miltiorrhizae Radix), has been clinically used as a treatment for cardiovascular diseases, such as angina pectoris and cerebrovascular diseases. Kangen-karyu shows biological activity, such as an anti-aging effect, the inhibition of platelet aggregation, hypotensive effect and the recovery of learning and memory impairment induced by senescence.[5–8] In our previous studies, Kangen-karyu showed favorable ameliorative effects on signs of fructose-induced metabolic syndrome, such as hyperglycae-

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mia, hyperlipidaemia and hypertension, through the reduction of TG and cholesterol levels with the regulation of hepatic sterol regulatory element-binding protein (SREBP)-1 expression, and also exhibited protective effects against diet-induced hypercholesterolaemia in rats.[9,10] In addition, we also reported the beneficial effect of Kangen-karyu on hyperlipidaemia in streptozotocin-induced type 1 diabetic rats.[11] However, the effect of Kangen-karyu on dyslipidaemia and oxidative stress in type 2 diabetes has not yet been studied.

As an experimental model of obesity-associated type 2 diabetes mellitus, *db/db* mice are widely used and well-established.[12,13] C57BLKS/J *db/db* mice develop diabetes due to mutation of the mouse diabetes (*db*) gene that encodes a receptor for leptin. The lack of leptin-receptor signalling results in increased food intake in combination with a phenotype of reduced energy expenditure, reminiscent of the neuroendocrine starvation response.[14] Consequently, the homozygotes (*db/db*) after birth show unrepressed eating behaviour, become obese, and by 3–6 months after birth, develop severe insulin resistance associated with hyperinsulinaemia, hyperglycaemia and hypertriglyceridaemia. Therefore, in this study, we investigated whether Kangen-karyu ameliorates oxidative stress and metabolic disorders, including hyperlipidaemia as well as hyperglycaemia, to reduce the risk of type 2 diabetes, using well-established *db/db* type 2 diabetic mice.

Materials and Methods

Reagents

Protease inhibitor mixture dimethyl sulfoxide (DMSO) solution, 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid, TBA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka. Japan). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, USA). The Bio-Rad protein assay kit and pure nitrocellulose membrane were purchased from Bio-Rad Laboratories (Tokyo, Japan). β-Actin, *o*-phthalaldehyde, phenylmethylsulfonyl fluoride (PMSF) and *N*-ethylmaleimide were purchased from Sigma Chemical Co. (St Louis, USA). Rabbit polyclonal antibodies against peroxisome proliferator-activated receptor α (PPARα), SREBP-1, SREBP-2 and nuclear factor-kappa B (NF-κB)p65, and mouse monoclonal antibody against cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Goat anti-rabbit and goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). ECL Western Blotting Detection Reagents were purchased from GE Health Care (Piscataway, USA).

Preparation of Kangen-karyu extract

The composition of Kangen-karyu used in this study was: 2.25 g Paeoniae Radix (*Paeonia lactiflora* Pallas root), 2.25 g Cnidii Rhizoma (*Cnidium officinale* Makino rhizome), 2.25 g Carthami Flos (*Carthamus tinctrius* L. petal), 1.125 g Cyperi Rhizoma (*Cyperus rotundus* L. rhizome), 1.125 g Aucklandiae Radix (*Aucklandia lappa* Dcne. root) and 4.5 g Salviae Miltiorrhizae Radix (*Salvia miltiorrhiza* Bunge root). This prescription was extracted with 25 volumes of water at 100°C for 1 h. After filtration, the solution was evaporated under reduced pressure to give an extract at a yield of 44%, by weight, of the starting materials. A typical high-performance liquid chromatogram of Kangen-karyu is given in Figure 1. Each sample was dissolved in 50% aqueous ethanol with sonication, and filtered through a Cosmonice filter (PVDF, 0.45 μm; Nacalai Tesque, Inc., Kyoto, Japan). Reverse-phase high-performance liquid chromatography was performed using a Cosmosil $5C_{18}$-AR II column (250 × 4.6 mm i.d.; Nacalai Tesque, Inc.) with elution gradients of 4–30% (39 min) and 30–75% (15 min) CH_3CN in 50 mM H_3PO_4 at a flow rate of 0.8 ml/min. The UV absorbance from 200 to 400 nm was monitored with a Jasco MD-910 photodiode array detector (Jasco, Tokyo, Japan). Peak areas were quantified at 311 nm for lithospermic acid B and 331 nm for rosmarinic acid. A voucher specimen has been deposited in the herbarium of the University of Toyama.

Experimental animals and treatment

The 'Guidelines for Animal Experimentation' approved by the University of Toyama were followed (Registration No. S-2006 INM-22). Male, 5-week-old, C57BLKS/J *db*/*db* mice and their age-matched non-diabetic *m*/*m* littermates were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept in a plastic-bottomed cage and exposed to a 12-h light–dark cycle. The room temperature (about 25°C) and humidity (about 60%) were controlled automatically. The mice were allowed free access to laboratory pellet chow (comprising 24.0% protein, 3.5% lipids and 60.5% carbohydrate; CLEA Japan Inc., Tokyo, Japan) and water. After adaptation, glucose and total cholesterol levels of blood taken from the tail vein were measured, and then *db*/*db* mice were divided into three groups. Treatment with Kangen-karyu was initiated after confirming the induction of obesity and hyperglycaemia in the *db/db* mice by the weight (36.0 ± 0.9 g) and serum glucose (300 ± 10 mg/dl), respectively. Mice in the *db*/*db* vehicle group ($n = 8$) were given water orally, while the other two groups ($n = 8$ per group) were orally administered with Kangen-karyu extracts daily for 18 weeks at a dose of 100 mg or 200 mg/kg body weight, respectively. The non-diabetic *m/m* mice ($n = 6$) as normal group were compared with diabetic groups. When the *db/db* mice reached 12–24 weeks of age, there was reduction of body weight and blood glucose, β-cell necrosis and diminished hyperinsulinaemia.[15] Therefore, we decided that the time point of cessation of treatment of *db/db* mice should be 24 weeks of age. Food and water intake were determined every day during the experimental period. After administration for 18 weeks, blood samples were collected by cardiac puncture from anaesthetized mice. Serum was separated immediately by centrifugation. Subsequently, each mouse was perfused with ice-cold physiological saline, and then the liver was harvested, snap-frozen in liquid nitrogen and stored at −80°C until analyses.

Measurement of serum parameters

Glucose, TG, total cholesterol and non-esterified fatty acid (NEFA) were measured using a commercial kit (Glucose CII-

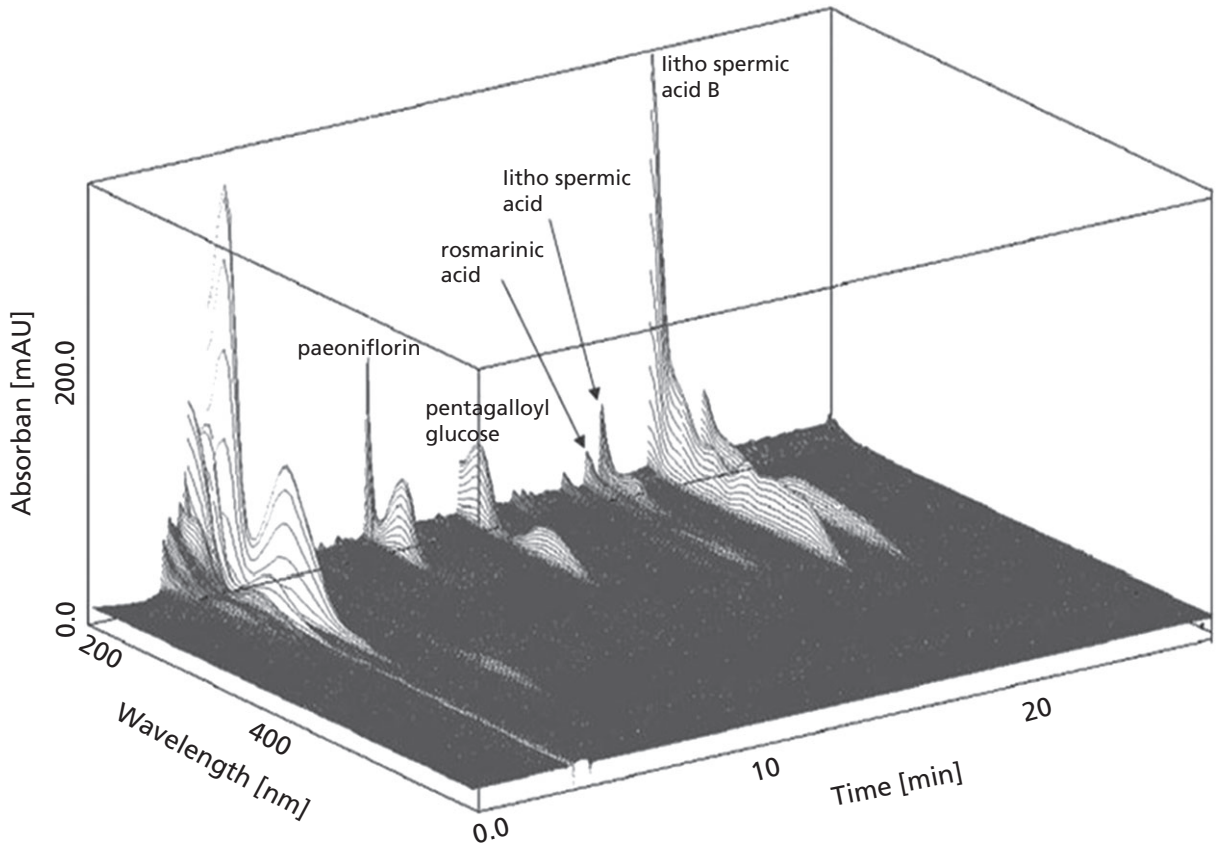

Figure 1 Three-dimensional HPLC of a sample of Kangen-karyu showing its major compounds.

Test, Triglyceride E-Test, Cholesterol E-Test and NEFA C-Test from Wako Pure Chemical Industries, Ltd, Osaka, Japan). Leptin, insulin (Morinaga Institute of Biological Science, Yokohama, Japan) and adiponectin (CycLex Co., Ltd, Nagano, Japan) levels were measured based on enzyme-linked immunosorbent assays. High-density lipoprotein (HDL), very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol distribution were measured using a BioVision kit (BioVision Inc., Mountain View, USA). Reactive oxygen species (ROS) level was determined using the method of Ali *et al*.[16] and TBA-reactive substance (TBARS) concentration was examined by the method of Naito and Yamanaka.[17] Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using commercial reagent: GPT-UV Test Wako (Wako Pure Chemical Industries, Ltd).

Measurement of hepatic TG and total cholesterol contents

The liver of each mouse was homogenized and total lipids of the liver homogenates were extracted with a mixture of chloroform and methanol (2 : 1, v/v) according to the method of Folch *et al*.[18] Then, the amounts of TG and total cholesterol were determined using the commercial kit, as described previously.

Assessment of ROS generation and TBARS levels

ROS generation was measured by the method of Ali *et al*.[16] Liver tissue was homogenized on ice with 1 mM EDTA–50 mM sodium phosphate buffer (pH 7.4). In brief, 25 mM DCFH-DA was added to homogenates, and after 30 min, the changes in fluorescence were determined at an excitation wavelength of 486 nm and emission wavelength of 530 nm. TBARS level was determined according to the method of Mihara and Uchiyama.[19]

Preparation of nuclear and post-nuclear fractions

To prepare nuclear fractions, hepatic tissues were homogenized with ice-cold lysis buffer containing 5 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 15 mM $CaCl_2$ and 1.5 M sucrose, and then 0.1 M dithiothreitol (DTT) and protease inhibitor fluid were added. After centrifugation (10 500*g* for 20 min at 4°C), the pellet was suspended with extraction buffer containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazyl] ethanesulfonic acid (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA and 25% (v/v) glycerol, and then 0.1 M DTT and protease inhibitor cocktail were added. The mixture was placed on ice for 30 min. The nuclear fraction was prepared by centrifugation at 20 500*g* for 5 min at 4°C. The post-nuclear fraction was extracted from the liver of each mouse as described below. In brief, hepatic tissue was homogenized with ice-cold lysis buffer (pH 7.4) containing 137 mM NaCl, 20 mM Tris–HCl, 1% Tween 20, 10% glycerol, 1 mM PMSF and protease inhibitor mixture DMSO solution. The homogenate was then centrifuged at 2000 *g* for 10 min at 4°C. The protein concentration of each fraction was determined using a commercial kit (Bio-Rad Laboratories, Hercules, USA).

Western blot analyses

For the determination of PPARα, SREBP-1, SREBP-2 and NF-κBp65, 30 μg protein of each nuclear fraction was electrophoresed through 8% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk solution for 1 h, and then incubated with primary antibodies to PPARα, SREBP-1, SREBP-2, NF-κBp65 and β-actin, respectively, overnight at 4°C. After the blots were washed, they were incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibody for 1.5 h at room temperature. Also, 30 μg protein of each post-nuclear fraction for COX-2 and iNOS was electrophoresed through 8% SDS-PAGE. Each antigen–antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-4000 (Fujifilm, Tokyo, Japan). Band densities were determined using ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to β-actin. The protein level was expressed relative to those of *m/m* mice (represented as 1).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from hepatic tissue using Trizol reagent (Invitrogen Life Technologies, Carlsbad, USA) and quantified using NanoDrop (Thermo Scientific, Wilmington, USA). The cDNAs were synthesized from 5 μg of RNA employing reverse transcriptase (QIAGEN, Tokyo, Japan). For the real-time PCR, triplicate aliquots of serially diluted cDNA samples were used in a reaction mixture that contained 1 μM of each primer in a reaction volume of 50 μl employing the SYBR Green Real-time PCR kit (QIAGEN, Tokyo, Japan) using a fluorometric thermal cycler (Mx3000P™; Stratagene, La Jolla, USA). Reaction mixtures were incubated for an initial denaturation at 95°C for 15 min, followed by 45 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. Primers used were as follows: acetyl-CoA carboxylase (ACC, sense: CCCAGCAGAATAAAGCTACTTTGG, antisense: TCCTTTTGTGCAACTAGGAACGT), fatty acid synthase (FAS, sense: CCTGGATAGCATTCCGAACCT, antisense: AGCACATCTCGAAGGCTACACA), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, sense: AGCCGAAGCAGCACATGAT, antisense: CTTGTGGAATGCCTTGTGATTG). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The ΔC_T method was employed for relative quantification. The ΔC_T value for each sample was determined by calculating the difference between the C_T value of the target gene and that of the GAPDH reference gene. The normalized target gene expression level in the sample was calculated using the formula $2^{-\Delta\Delta CT}$ as the fold change over the control.

Statistical analysis

Data are expressed as means ± SE. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Dunnett's test. Values of $P < 0.05$ were considered significant.

Results

Haematological analysis

Table 1 shows the levels of serum constituents (glucose, TG, total cholesterol, HDL cholesterol, LDL/VLDL cholesterol and NEFA), lipid metabolism-related hormones (insulin, leptin and adiponectin) and biomarkers associated with oxidative stress (ROS and TBARS). All serum constituents, except serum adiponectin, and biomarkers were elevated in vehicle-treated *db/db* mice compared with *m/m* mice. Kangen-karyu 200 mg/kg-administered *db/db* mice groups showed decreased serum glucose and leptin, but no alterations of insulin and adiponectin. Furthermore, 18-week administration of Kangen-karyu to *db/db* mice significantly improved

Table 1 Haematological analyses

Item	*m/m*	*db/db*		
		Vehicle	K-100	K-200
Glucose (mg/dl)	136.6 ± 4.1***	483.3 ± 10.3	442.8 ± 24.6	436.6 ± 13.4*
Leptin (ng/ml)	1.95 ± 0.31***	19.87 ± 0.91	19.27 ± 1.11	15.43 ± 0.33**
Insulin (ng/ml)	1.61 ± 0.03***	3.12 ± 0.21	3.98 ± 0.39	4.96 ± 0.78
TG (mg/dl)	128.0 ± 16.3**	201.3 ± 14.6	124.6 ± 18.6**	98.4 ± 13.1***
Total cholesterol (mg/dl)	80.1 ± 1.4***	155.7 ± 9.9	160.5 ± 15.4	151.3 ± 18.7
HDL-C (mg/dl)	40.0 ± 2.2**	53.2 ± 2.7	53.2 ± 1.6	52.1 ± 3.5
LDL/VLDL-C (mg/dl)	10.3 ± 0.8***	20.0 ± 1.2	11.7 ± 1.0***	11.4 ± 0.9***
Adiponectin (ng/ml)	18.63 ± 1.03***	6.19 ± 0.27	6.79 ± 0.92	7.50 ± 0.91
NEFA (mEq/l)	0.62 ± 0.02***	1.26 ± 0.09	1.01 ± 0.06	1.03 ± 0.05
TBARS (nmol/ml)	3.02 ± 0.71***	16.97 ± 0.99	6.77 ± 0.65***	6.12 ± 0.67***
ROS (fluorescence/min/ml serum)	636.0 ± 38.8**	882.0 ± 90.5	781.7 ± 62.3	596.0 ± 64.3**
ALT (IU/l)	36.80 ± 2.37*	92.92 ± 15.07	83.81 ± 8.24	80.85 ± 12.95
AST (IU/l)	11.17 ± 0.46***	56.74 ± 3.97	50.96 ± 6.61	42.19 ± 7.34

m/m, Misty; Vehicle, vehicle-treated *db/db* mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice; HDL-C, high-density lipoprotein-cholesterol; LDL/VLDL-C, low-density lipoprotein/very-low-density lipoprotein-cholesterol; NEFA, non-esterified fatty acid; TBARS, thiobarbituric acid-reactive substances; ROS, reactive oxygen species; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Results are shown as the mean ± SE. Significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs *db/db* vehicle-treated values.

Table 2 Hepatic lipids and biomarkers associated with oxidative stress

Item	*m/m*	*db/db*		
		Vehicle	K-100	K-200
TG (mg/g tissue)	3.25 ± 0.14***	17.59 ± 1.15	15.90 ± 0.66	14.23 ± 0.84*
Total cholesterol (mg/g tissue)	2.20 ± 0.22***	4.25 ± 0.21	3.96 ± 0.26	3.37 ± 0.28*
TBARS (MDA nmol/mg protein)	1.95 ± 0.11*	2.74 ± 0.23	2.03 ± 0.19*	1.57 ± 0.08***
ROS (fluorescence/min/mg protein)	2171 ± 63***	2830 ± 68	2503 ± 72**	2307 ± 150**

m/m, Misty; Vehicle, *db/db* vehicle-treated mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; ROS, reactive oxygen species. Results are shown as the mean ± SE. Significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs *db/db* vehicle-treated values.

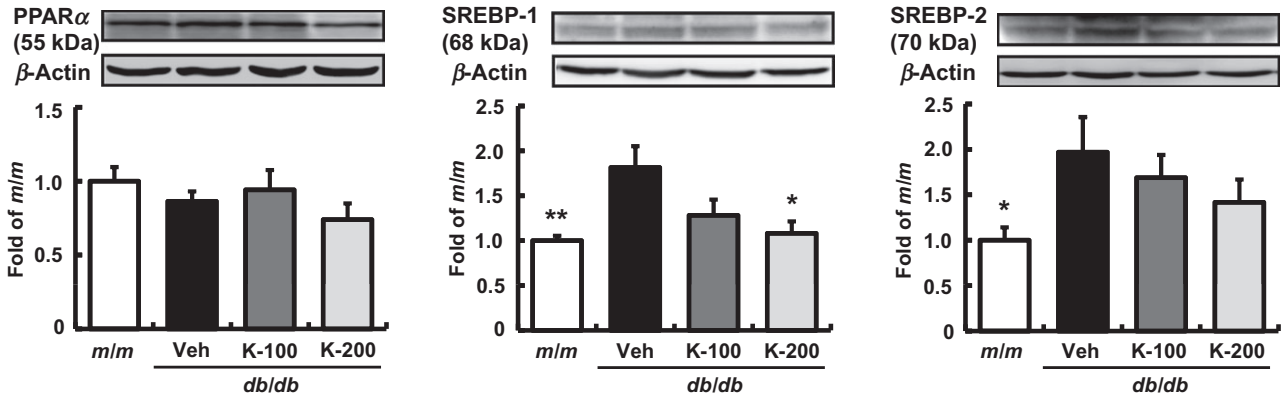

Figure 2 PPARα, SREBP-1 and SREBP-2 expressions in liver. Significance: $*P < 0.05$, $**P < 0.01$ vs *db/db* vehicle-treated values. *m/m*, Misty; Veh, *db/db* vehicle-treated mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice.

TG and LDL/VLDL cholesterol compared with *db/db* control group. In addition, serum levels of TBARS and ROS in *db/db* mice were significantly increased compared with *m/m* mice. However, the elevated serum ROS levels were significantly decreased in *db/db* mice by the administration of Kangen-karyu. In particular, Kangen-karyu significantly reduced TBARS level from a dose of 100 mg.

Hepatic TG and total cholesterol contents

As shown in Table 2, the levels of hepatic TG and total cholesterol in the *db/db* control group showed a marked increase compared with the *m/m* group. However, the hepatic contents of TG and total cholesterol were significantly decreased by Kangen-karyu administration at a dose of 200 mg/kg body weight/day.

Biomarkers associated with oxidative stress in the liver

Table 2 also shows the effect of Kangen-karyu against oxidative stress. TBARS levels were markedly increased in the *db/db* control group compared with the *m/m* group. However, the groups administered Kangen-karyu showed significant decreases in the TBARS levels at doses of 100 and 200 mg/kg. In addition, an elevation in ROS generation was observed in the *db/db* control group compared with the *m/m* group, while it was significantly decreased by Kangen-karyu administration.

Hepatic PPARα, SREBP-1 and SREBP-2 expressions

As shown in Figure 2, protein expressions of hepatic SREBP-1 and SREBP-2 was increased markedly in the *db/db* control group compared with the *m/m* group. However, the administration of Kangen-karyu 200 mg/kg led to a significant down-regulation of SREBP-1 expression. On the other hand, the groups administered Kangen-karyu showed a tendency to reduce SREBP-2 expression. In addition, the *db/db* control group exhibited a decrease in hepatic PPARα expression. However, in the case of hepatic PPARα, no significant difference among the groups was observed.

Hepatic mRNA expressions involved in lipid metabolism

The effects of Kangen-karyu administration on the mRNA levels of genes involved in lipid metabolism in the hepatic tissue were examined by quantitative real-time PCR. As shown in Figure 3, over-expressions of ACC, FAS and HMGR mRNA were seen in the hepatic tissue of *db/db* vehicle group, compared with *m/m* group. However, the administration of Kangen-karyu at a dose of 200 mg/kg body weight/day significantly inhibited the expressions of ACC, FAS and HMGR in *db/db* mice.

Hepatic NF-κBp65, COX-2 and iNOS expressions

NF-κBp65 expression in the *db/db* control group was up-regulated, while it was suppressed significantly by the

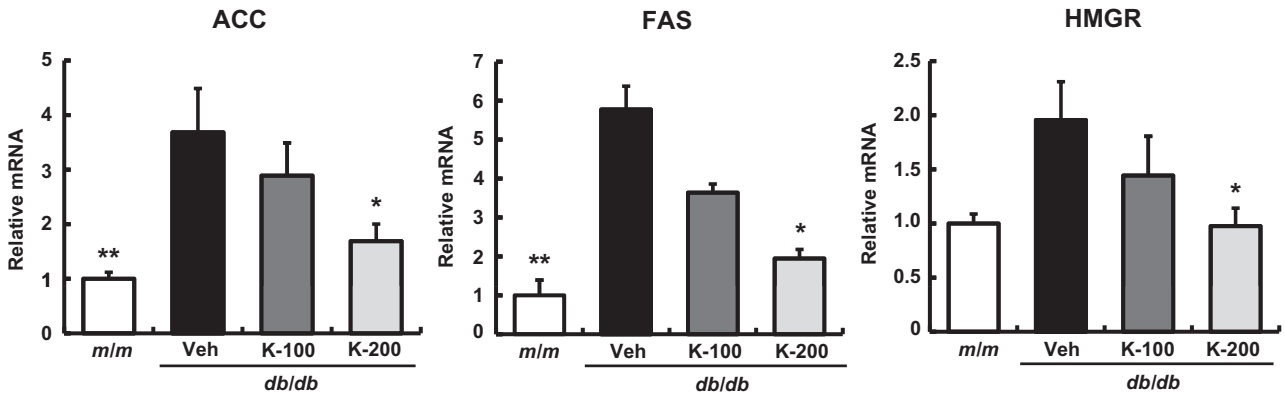

Figure 3 ACC, FAS and HMGR mRNA expressions in liver. Significance: $*P < 0.05$, $**P < 0.01$ vs *db/db* vehicle-treated values. *m/m*, Misty; Veh, *db/db* vehicle-treated mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice.

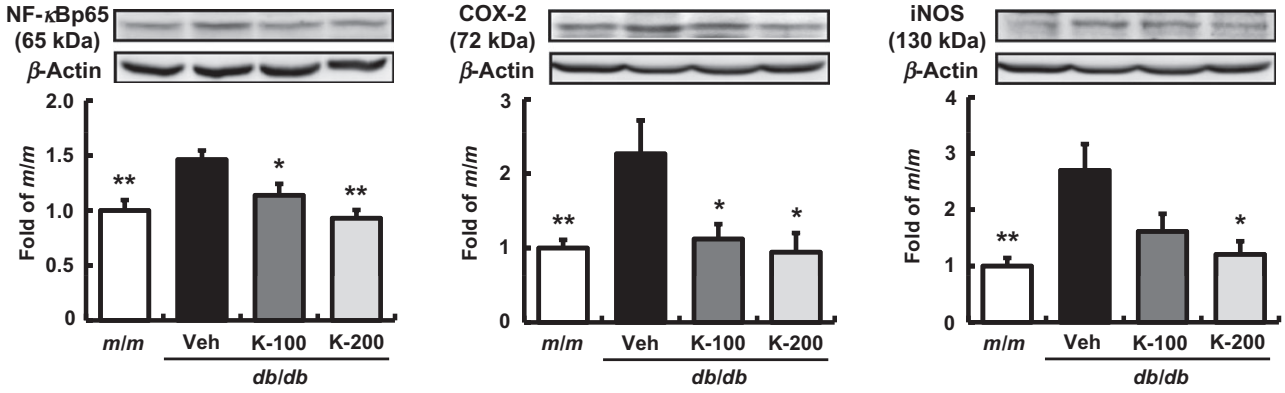

Figure 4 NF-κBp65, COX-2 and iNOS expressions in liver. Significance: $*P < 0.05$, $**P < 0.01$ vs *db/db* vehicle-treated values. *m/m*, Misty; Veh, *db/db* vehicle-treated mice; K-100, Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; K-200, Kangen-karyu 200 mg/kg body weight-treated *db/db* mice.

administration of Kangen-karyu (Figure 4). In addition, significant increases in COX-2 and iNOS expressions in the *db/db* control group were also observed compared with the *m/m* group. However, Kangen-karyu administration significantly suppressed the protein expressions of COX-2 and iNOS nearly to the normal values.

Discussion

Diabetes is associated with oxidative stress and inflammation due to hyperglycaemia and hyperlipidaemia. They induce generation of free radicals, inflammatory responses and oxidative stress reactions account for the complications and mortality of obesity and type 2 diabetes. C57BLKS/J-*db/db* mice were used in this study to identify the effect of Kangen-karyu on dyslipidaemia and oxidative stress in type 2 diabetes. The *db/db* mice develop diabetes mellitus due to a failure to respond to leptin, resulting from a mutation in their receptor gene expressed in the hypothalamus, although gene expression and leptin secretion are markedly augmented in these mice, resulting in leptin resistance.[12] The *db/db* mice were also characterized by obesity, sustained hyperglycaemia, hyperlipidaemia and hyperinsulinaemia as a result of destroyed leptin receptors.[20] Subsequently, the genotypes of *db/db* mice lead to a lack of signalling by leptin, which regulates food intake and systemic fuel metabolism. The administration of Kangen-karyu for 18 weeks to *db/db* mice caused a significant decrease in serum glucose and leptin levels (Table 1). These results imply that Kangen-karyu can ameliorate diabetic pathological conditions induced by hyperglycaemia and metabolic disorders by leptin resistance in type 2 diabetes.

Type 2 diabetes mellitus leads to the abnormal metabolism of glucose, FFA and other reactive metabolites caused by insulin-deficient and -resistant states.[21] Especially in the liver, insulin resistance elevates the hepatic output of TG-rich particles. When the FFA supply exceeds utilization, non-adipose tissues start accumulating TG, which is aggravated by the simultaneous presence of hyperglycaemia. The formation of reactive, long-chain fatty acyl-CoAs and toxic metabolites such as ceramide, the activation of protein kinase C-δ and increase of oxidative stress, may all contribute to apoptosis and the decline of β-cells.[3,22] Thus, the regulation of hyperlipidaemia would play an important role in the etiology of diabetes and the complication of hyperglycaemia. In this

study, the effects of Kangen-karyu on lipid levels, such as TG, total cholesterol, HDL cholesterol, LDL/VLDL cholesterol and NEFA, were examined. We found that *db/db* mice showed hyperlipidaemia. However, the administration of Kangen-karyu reduced hyperlipidaemia through lowering TG and LDL/VLDL cholesterol (Table 1). Also, to investigate the effects of Kangen-karyu on hepatic damage induced by abnormal lipid synthesis, the level of hyperlipidaemia in the liver of *db/db* mice was also examined. The hepatic contents of TG and total cholesterol were significantly decreased by the administration of Kangen-karyu (Table 2). These results mean that Kangen-karyu may improve hyperlipidaemia with the regulation of lipid metabolism, such as TG and total cholesterol synthesis, in type 2 diabetes.

Hyperglycaemia and elevated FFA levels result in the generation of ROS and, consequently, increase oxidative stress. ROS not only directly damage cells by oxidizing DNA, proteins and lipids, but also indirectly damage them by activating a variety of stress-sensitive intracellular signalling pathways, such as NF-κB, p38 mitogen-activated protein kinase (MAPK), NH_2-terminal Jun kinase/stress-activated protein kinase, hexosamines, protein kinase C, advanced glycation end-products (AGE)/receptor for AGE (RAGE) and others. Activation of these pathways results in the increased expression of numerous gene products that cause cellular damage and play a major role in the etiology of the later-stage complications of diabetes.[23] Thus, the up-regulation of endogenous antioxidative systems and suppression of oxidative stress are important factors in the amelioration of diabetes and its complications. In this study, we investigated ROS generation and lipid peroxidation as biomarkers associated with oxidative stress in serum and liver. Lipid peroxidation also leads to oxidant production from many molecules and thus amplifies oxidative damage.[24] Our results showed that the levels of ROS generation and lipid peroxidation in serum and liver were increased in *db/db* mice, which implies *db/db* mice may show increased oxidative damage due to an elevation of ROS generation induced by hyperglycaemia and hyperlipidaemia. However, Kangen-karyu administration exerted its antioxidant activity, decreasing ROS and TBARS levels in serum and hepatic tissue of *db/db* mice (Tables 1 and 2). This suggests that the administration of Kangen-karyu would ameliorate oxidative stress in type 2 diabetes through the inhibition of ROS generation and lipid peroxidation and, thus, would result in the improvement of hepatic disorders caused by oxidative stress.

Lipid homoeostasis is regulated by a family of membrane-bound transcription factors called SREBPs. SREBP-1 is a key transcription factor that nutritionally regulates the hepatic gene expression of lipogenic enzymes and TG deposition in the liver.[25] On the other hand, SREBP-2 regulates genes involved in cholesterol synthesis through the cleavage of its precursor form to an active nuclear form via interaction with SREBP cleavage activating protein and protease in a sterol-dependent manner.[26] Up-regulation of SREBP-1 and SREBP-2 was reported in leptin-resistant mice, such as *ob/ob* mice and $FVB^{db/db}$ mice.[27,28] In this study, the increase in hepatic SREBP-1 in *db/db* mice was down-regulated by the administration of Kangen-karyu. This was probably related to the inhibition of hepatic TG and total cholesterol accumulation. Furthermore, PPARs, with three isoforms (α, δ and γ), are also involved in the long-term regulation of lipid metabolism, and their activity is modulated by endogenous lipid-derived ligands. When PPARα is activated, it promotes fatty acid oxidation, ketone body synthesis and glucose sparing.[29] In our study, hepatic PPARα was decreased in *db/db* mice; it was increased slightly, but not significantly, by Kangen-karyu administration. However, we found that Kangen-karyu exhibited a significant effect on regulation of SREBP-1 (Figure 2). These results suggest that Kangen-karyu has an ameliorating effect on dyslipidaemia in type 2 diabetic mice through the regulation of impaired hepatic SREBPs.

SREBPs have been demonstrated to regulate the transcription of the genes for the LDL receptor, HMGR, HMG-CoA synthase, squalene synthase, FAS and stearoyl-CoA desaturase, as well as other genes with sterol regulatory elements in their regulatory regions.[30] It is well known that SREBP-1 primarily controls genes involved in fatty acid synthesis, whereas SREBP-2 plays a major role in the regulation of cholesterol synthesis.[31–34] Such genes include those for ACC and FAS in the fatty acid synthesis pathway, and for HMGR in the cholesterol synthesis pathway.[30,35,36] Type 2 diabetes is associated with increased de-novo lipogenesis, decreased plasma fatty acid oxidation and increased fatty acid flux from peripheral tissues to the liver.[37] Therefore, we examined the effect of Kangen-karyu on regulation of lipogenic enzyme genes, such as ACC, FAS and HMGR involved in cholesterol and fatty acid synthesis, in the *db/db* mice liver using a real-time quantitative PCR technique. Our results strongly suggest that Kangen-karyu exerts its TG and cholesterol lowering action by reducing the expressions of ACC, FAS and HMGR, thereby inhibiting fatty acid and cholesterol synthesis in type 2 diabetic mice (Figure 3).

In type 2 diabetes, the stress-sensitive intracellular signalling pathway is altered. In particular, one major intracellular target of hyperglycaemia and oxidative stress is the transcription factor NF-κB. NF-κB can be activated by a wide array of exogenous and endogenous stimuli, including hyperglycaemia, elevated FFA, ROS, tumour necrosis factor-α, interleukin-1β, other pro-inflammatory cytokines, AGE-binding RAGE and p38 MAPK. In particular, AGEs trigger the activation of NF-κB via interaction with RAGE, leading to its translocation to the nucleus where it induces transcription, and the promoter region of the RAGE gene contains NF-κB binding sites, potentially producing a self-perpetuating pathway.[38] The aberrant regulation of NF-κB is associated with a number of chronic diseases including diabetes and atherosclerosis.[39] That is, NF-κB regulates the expression of a large number of genes, including growth factors, pro-inflammatory cytokines and others.[39,40] Especially, it is well-known to be involved in the regulation of COX-2 and iNOS expressions, which mediates the inflammatory process.[41] In addition, NF-κB activation induces insulin resistance by lipid/fatty acid infusion and the inhibition of insulin signalling by lipid metabolites, such as diacylglycerol and ceramide.[42] In our Western blotting analysis, experimental type 2 diabetes resulted in the increased expressions of NF-κBp65, COX-2 and iNOS proteins, whereas the expressions of these three proteins were markedly reduced by Kangen-karyu administration. These results showed that the anti-inflammatory effects of

Kangen-karyu may be associated with down-regulation of COX-2 and iNOS followed by inhibition of NF-κB transcription stimulated oxidative stress in the liver of type 2 diabetic mice.

Conclusions

The present results show that Kangen-karyu ameliorated dyslipidaemia and oxidative stress in *db/db* type 2 diabetic mice model. The Kangen-karyu reversed hyperlipidaemia by regulations of hepatic SREBP-1 and lipogenic enzyme gene expressions, such as ACC, FAS and HMGR. These results suggest that the administration of Kangen-karyu can improve abnormal lipid metabolism in type 2 diabetic mice. In addition, oxidative stress in type 2 diabetes was attenuated by Kangen-karyu through the reduction of ROS generation and lipid peroxidation. Kangen-karyu ameliorated the up-regulation of NF-κB and also attenuated COX-2 and iNOS expressions. These results suggest that Kangen-karyu would act as regulators in inflammatory reactions caused by oxidative stress under type 2 diabetes. We concluded that Kangen-karyu with multi-components, such as lithospermic acid and rosmarinic acid (Figure 1), would exhibit its potential via interaction of active compounds with multiple targets. The present study suggests that Kangen-karyu may be associated with ameliorations of oxidative stress and abnormal lipid metabolism in type 2 diabetes.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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