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Protective effect of Chinese prescription Kangen-karyu and its crude drug Tanjin against age-related lipidosis in rats

Eun Ju Cho, Takako Yokozawa and Takuya Okamoto

Abstract

We have investigated the effect of the Chinese prescription Kangen-karyu and its crude drug Tanjin against age-related lipidosis in-vivo in a rat model. The serum and hepatic triglyceride levels were remarkably elevated in 12-month-old compared with two-month-old rats. However, the administration of Kangen-karyu and Tanjin extracts significantly decreased these levels. This suggested a protective role against related pathological conditions as well as hyperlipidaemia. On the other hand, the reduction of the levels of adiponectin in serum with ageing did not show significant changes in rats given diets supplemented with Kangen-karyu and Tanjin extracts. Furthermore, the expression of transcription factors in nuclear hepatic tissue related to lipid metabolism was investigated. The decline in the expression of nuclear peroxisome proliferator-activated receptor α protein in hepatic tissue with age was ameliorated by the administration of Kangen-karyu and Tanjin supplements. On the other hand, the overexpression of sterol regulatory element-binding proteins (SREBP)-1 and SREBP-2 in old rats compared with young rats showed a tendency to decrease with Kangen-karyu and Tanjin administration. The decline of hepatic function with ageing was attenuated by Kangen-karyu and Tanjin, suggesting the beneficial role of Kangen-karyu and Tanjin on lipid metabolism through the improvement of hepatic function. This study has demonstrated that Kangen-karyu and Tanjin inhibited the accumulation of triglyceride with regulation of related protein expressions and they improved hepatic function. Evidence has been provided for the anti-ageing activity of Kangen-karyu and its crude drug Tanjin against age-related lipidosis.

Introduction

Ageing is considered to be a physiological process that can be potentially accelerated by degenerative processes associated with lipotoxic insult. The accumulation of lipids in tissues has the potential to cause deleterious metabolic effects that compromise their normal functionality and increase the susceptibility to degenerative diseases, including inflammatory diseases, dyslipidaemia, atherosclerosis, obesity and diabetes (Kahn & Flier 2000; Unger & Orci 2001; Unger 2002; Slawik & Vidal-Puig 2006). Aged mammals show increased plasma concentrations of lipids and reduced mitochondrial β-oxidation of fatty acids (Toth & Tchernof 2000). In addition, dyslipidaemia can be one of the indicators to define ageing on the basis of cytological, pathological and clinical phenomena, since dyslipidaemia results in age-related pathological conditions (Martin 1978). Therefore, agents that can attenuate age-related lipidosis may show a promising protective potential against the ageing process.

Traditional Chinese prescriptions have attracted much attention due to their beneficial effects observed with clinical experience accumulated over time. Among them, Kangen-karyu (Guan-Yuan-Ke-Li), composed of six crude drugs, has been prescribed with modification of the Chinese prescription developed as an agent against cardiovascular and cerebrovascular disease. It has received much attention due to its numerous biological activities, such as inhibition of platelet aggregation and suppression of hypertension (Takahashi 1991; Gao et al 2001; Makino et al 2002). Furthermore, our previous work demonstrated that Kangen-karyu counteracted oxidative stress by the inhibition of reactive oxygen species generation and regulation of the antioxidative status, and it ameliorated tissue damage associated with ageing in an in-vivo system of senescence-accelerated mice and a cellular senescence model

Department of Food Science and Nutrition, Pusan National University, 30 Jangjeon-dong, Geumjeong-gu, Busan 609-735, South Korea

Eun J. Cho

Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

Takako Yokozawa

Iskra Industry Co., Ltd., 1-14-2 Nihonbashi, Chuo-ku, Tokyo 103-0027, Japan

T. Okamoto

Correspondence: T. Yokozawa, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. E-mail: yokozawa@inm.u-toyama.ac.jp

(Satoh et al 2004a,b, 2005). Moreover, its crude drug, Tanjin, prevented oxidative damage and mitochondrial dysfunction with ageing via reduction in the expression of nuclear factor kappa B and inhibition of the leakage of superoxide in mitochondria. On the basis of this evidence, we expected a beneficial role of Kangen-karyu and Tanjin against alterations in lipid metabolism with ageing. Therefore, this study focused on the effect of Kangen-karyu and Tanjin against age-related lipidosis in an in-vivo rat model.

Materials and Methods

Materials

Nonidet P-40 (NP-40), phenylmethane sulfonyl fluoride (PMSF), 2-amino-2-hydroxymethyl-1,3-propanediol (Tris-Cl), and protease inhibitor mixture dimethyl sulfoxide solution (protease inhibitor fluid) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Polyclonal antibodies to peroxisome proliferator-activated receptor α (PPARα), sterol regulatory element-binding proteins (SREBP-1 and -2), and polyclonal goat anti-rabbit immunoglobulin G (IgG) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The other chemicals and reagents used were of a high quality and were obtained from commercial sources.

Preparation of Kangen-karyu extract

The composition of Kangen-karyu used in this study was 2.25 g Paeoniae Radix (a root of *Paeonia lactiflora* PALLAS), 2.25 g Cnidii Rhizoma (a rhizome of *Cnidium officinale* MAKINO), 2.25 g Carthami Flos (a petal of *Carthamus tinctorius* L.), 1.125 g Cyperi Rhizoma (a rhizome of *Cyperus rotundus* L.), 1.125 g Aucklandiae Radix (a root of *Aucklandia lappa* DCNE.), and 4.5 g Salviae Miltiorrhizae Radix (a root of *Salvia miltiorrhiza* BUNGE). These herbs were extracted with 25 vol 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. For analysis of the components of Kangen-karyu, the aqueous extract was dissolved in aqueous ethanol (50% v/v) with sonication, and filtered through a Cosmonice filter (PVDF, 0.45 μm, Nakarai Tesque, Inc.). Reverse-phase HPLC analysis was performed using a Cosmosil 5C_{18}-AR II column (250×4.6 mm i.d., Nakarai Tesque, Inc.) with elution gradients of 4–30% v/v (39 min) and 30–75% v/v (15 min) CH_3CN in 50 mM H_3PO_4 at a flow rate of 0.8 mL min^{-1}. The ultraviolet (UV) absorbance from 200 to 400 nm was monitored and the three-dimensional data were processed by a JASCO photodiode array detector MD-910. All assigned peaks were identified by carrying out co-injection tests with authentic samples and comparing with UV spectral data. Figure 1 shows the three-dimensional HPLC of Kangen-karyu. The major compounds detected were paeoniflorin, pentagalloyl glucose, rosmarinic acid, lithospermic acid, and lithospermic acid B.

Preparation of Tanjin extract

The roots (50 g) of Tanjin were boiled gently in 1000 mL water for 1 h. After filtration, the solution was evaporated under reduced pressure to give an extract at a yield of 30%, by weight, of the starting materials. Analysis of the Tanjin components was performed according to the method used for the Kangen-karyu extract described above. The major components of Tanjin detected were lithospermic acid B, lithospermic acid, and rosmarinic acid.

Animals and treatments

The animal experiments were ethically approved and followed the "Guidelines for Animal Experimentation" offered by the University of Toyama. Male Wistar rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). The rats were kept under conventional conditions at 23 ± 1°C with an alternating 12-h light/dark cycle, with food and water freely available. From eight-months old the diet was supplemented with one of the extracts at 0.5% or 1.0%, by weight, for four months. Twelve rats were used for each experimental group. During this experimental period, the diet in a pair-feeding schedule was given to all rats (18 g/rat/day), and the intake of Kangen-karyu or Tanjin extract was estimated to be approximately 90 and 180 mg/rat, respectively. Body weight was monitored every week over the four-month period. However, the initial and final body weights, and body weight gains of each group were approximately the same. At the end of the experimental period, blood samples were collected from the aorta abdominalis and the serum was separated immediately by centrifugation. The liver was extirpated, immediately frozen by immersion in liquid nitrogen, and kept at −80°C until analysis. For the experiment, two-month-old male rats were used for the young age group.

Determination of triglyceride levels in serum

Serum triglyceride levels were determined using commercial reagents: Triglyceride E-Test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan) based on the Spayd et al (1978) method.

Determination of adiponectin levels in serum

Adiponectin levels in serum were measured by sandwich enzyme-linked immunosorbent assay as described by Arita et al (1999).

Determination of triglyceride levels in hepatic tissue

Total lipids were extracted by a modified method of Folch et al (1957), using chloroform/methanol (2:1, v/v). After a 0.25 mL extraction of lipids was evaporated, the lipid content was determined using commercial reagents: Triglyceride E-Test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan).

Preparation of nuclear fractions

According to the method of Sakurai et al (1997), hepatic tissue was homogenized with 10 mM 2-[4-(2-hydroxyethyl)-1-piperazyl] ethanesulfonic acid (HEPES) buffer (pH 7.9) containing (in mM)10 KCl, 0.1 EDTA, 1 DTT, 0.5 PMSF, and

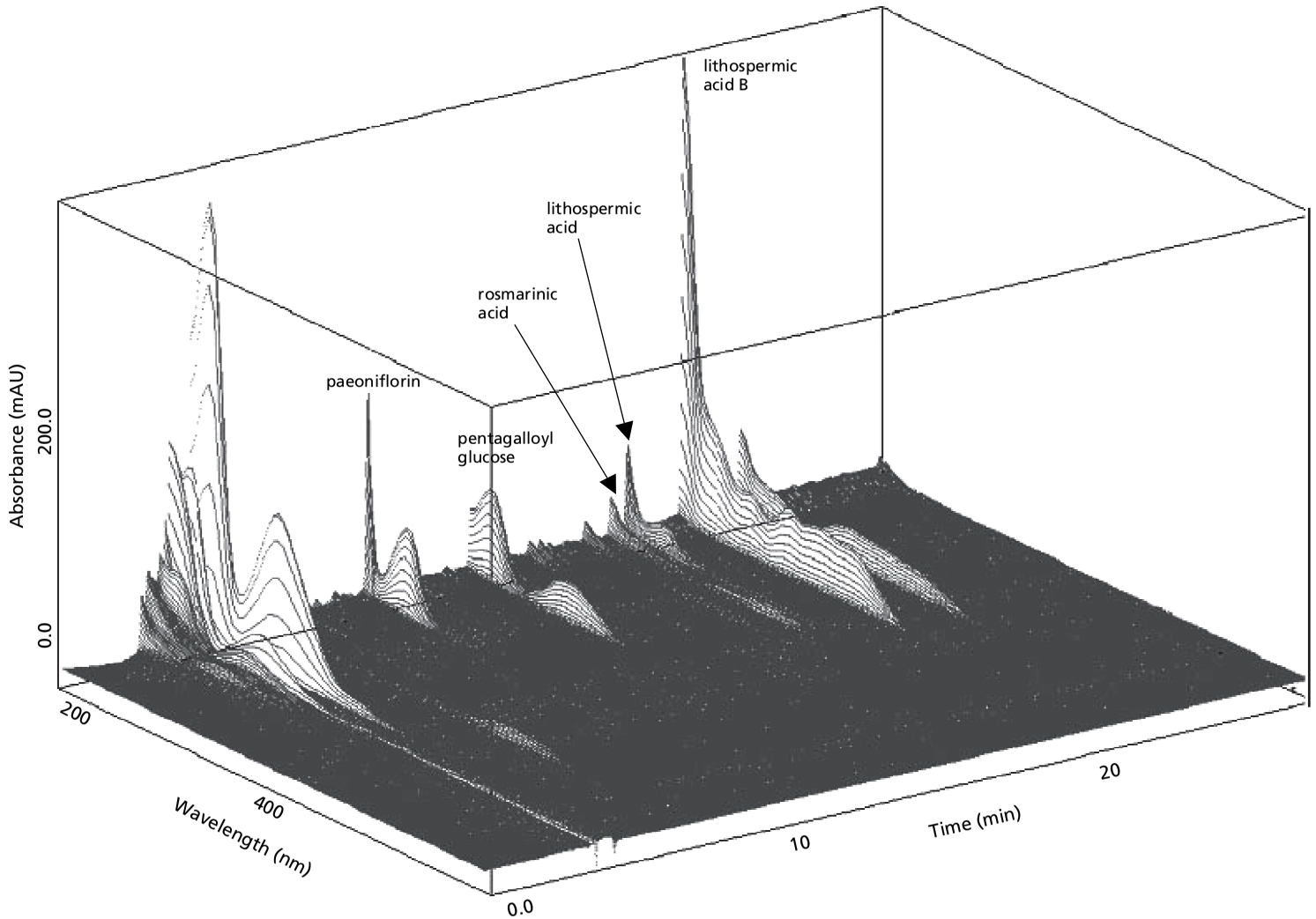

Figure 1 Three-dimensional HPLC of a sample of Kangen-karyu showing its major compounds.

protease inhibitor fluid. The homogenate was chilled on ice for 15 min and then 10% NP-40 was added. The mixture was vortexed vigorously for 10 s and the supernatant was obtained (cytoplasmic fraction) by centrifugation at 15 000 *g* for 5 min at 4°C. The pellet was resuspended in 20 mM HEPES buffer (pH 7.9) containing 0.4 M NaCl, 1 mM EDTA, 1 mM DDT, 1 mM PMSF, and protease inhibitor fluid. The mixture was left on ice for 15 min with frequent agitation. Nuclear extract was prepared by centrifugation at 15000 *g* for 5 min at 4°C. The protein concentration of each fraction was quantified using a commercial kit (Bio-Rad Laboratories, Hercules, CA).

Protein expression

Western blot analysis was performed with 30 μg protein from the nuclear fraction. The protein was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrophoretically transferred to a membrane, blocked with 5% nonfat dry milk solution for 1 h, and then incubated with the corresponding primary anti-PPARα, SREBP-1, SREBP-2, and β-actin antibody overnight at 4°C. After the blots were washed, they were incubated with goat anti-rabbit and/or goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies for 90 min at room temperature. Each antigen–antibody complex was visualized using enhanced chemiluminescence Western blotting detection reagents and detected by chemiluminescence with LAS-1000plus (Fujifilm, Tokyo, Japan). The identification of each protein was estimated by comparison with the protein markers of known molecular weight. Band densities were determined by Scion Image software (Scion Corporation, Frederick, MD) and quantified as a ratio of the density of the β-actin band.

Determination of hepatic functional parameters

Hepatic function was determined by the measurements of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercial reagents: GPT-UV Test Wako (Wako Pure Chemical Industries Ltd, Osaka, Japan).

Statistics

The results were expressed as mean ± s.e. values (n = 12 rats/group). The effect on each parameter was examined using one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test and those at $P < 0.05$ were considered significant.

Results

Triglyceride levels in serum

The effect of Kangen-karyu and Tanjin extracts on the serum triglyceride level with ageing is shown in Table 1. Although the level of triglyceride in serum dramatically increased with age, the administration of Kangen-karyu and Tanjin extracts at an oral dose of 1.0% decreased it from 202.6 mg dL^{-1} to 124.9 and 103.5 mg dL^{-1}, respectively.

Table 1 Effect of Kangen-karyu and Tanjin on triglyceride levels in serum

Age (months)	Group	Triglyceride (mg dL^{-1})
2	–	66.9 ± 3.6
12	–	202.6 ± 15.6$^{\#}$
	Kangen-karyu 0.5%	133.1 ± 6.3$^{\#,*}$
	Kangen-karyu 1.0%	124.9 ± 9.8$^{\#,*}$
	Tanjin 0.5%	109.3 ± 4.8$^{\#,*}$
	Tanjin 1.0%	103.5 ± 6.2$^{\#,*}$

$^{\#}P < 0.001$ compared with control values of two-month-old rats; $^{*}P < 0.001$ compared with control values of 12-month-old rats.

Adiponectin levels in serum

Table 2 shows the effect of Kangen-karyu and Tanjin extracts on adiponectin, which is related to carbohydrate and lipid metabolism. Although the adiponectin level of 12-month-old rats was dramatically lower compared with two-month-old rats, their administration increased the adiponectin level, but not significantly.

Triglyceride in hepatic tissue

As shown in Figure 2, the triglyceride level in hepatic tissue of 12-month-old rats increased remarkably compared with that of two-month-old rats. The levels of triglyceride in the liver of 12-month-old rats (38.9 mg (g tissue)$^{-1}$) decreased to 25.9 and 25.4 mg (g tissue)$^{-1}$ by the administration of 1.0% Kangen-karyu and Tanjin extracts, respectively.

Expression levels of nuclear PPARα, and SREBP-1 and –2 proteins

Figures 3 and 4 show the expression of transcriptional factors related to lipid metabolism on nuclear hepatic tissue. The expression of nuclear PPARα protein in hepatic tissue was reduced with age, while the administration of Kangen-karyu and Tanjin extracts increased the level (Figure 3). On the other hand, SREBP-1 and SREBP-2 proteins, which are related to the metabolism of fatty acids, triglyceride, and cholesterol, showed elevations in expression with age. The rats administered Kangen-karyu and Tanjin showed a significant decrease in the level of SREBP-1 and SREBP-2 (Figure 4).

Table 2 Effect of Kangen-karyu and Tanjin on adiponectin levels in serum

Age (months)	Group	Adiponectin (μg mL^{-1})
2	–	7.28 ± 0.56
12	–	3.10 ± 0.14$^{\#}$
	Kangen-karyu 0.5%	3.45 ± 0.10$^{\#}$
	Kangen-karyu 1.0%	3.31 ± 0.14$^{\#}$
	Tanjin 0.5%	3.52 ± 0.17$^{\#}$
	Tanjin 1.0%	3.42 ± 0.11$^{\#}$

$^{\#}P < 0.001$ compared with control values of two-month-old rats.

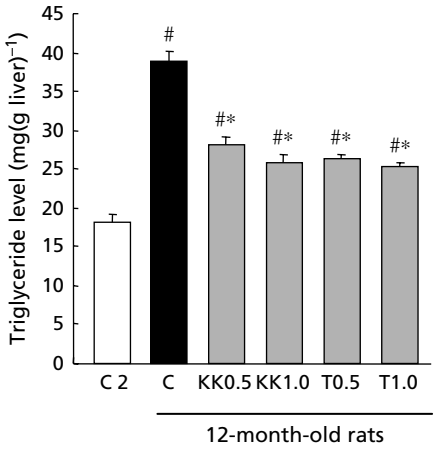

Figure 2 Triglyceride in hepatic tissue. Rats were administered one of the following: Kangen-karyu 0.5%, KK0.5; Kangen-karyu 1.0%, KK1.0; Tanjin 0.5%, T0.5; or Tanjin 1.0%, T1.0. Control rats 2-month-old, C2; control rats 12-month-old, C. $^{\#}P < 0.001$ compared with control values of two-month-old rats; $^{*}P < 0.001$ compared with control values of 12-month-old rats.

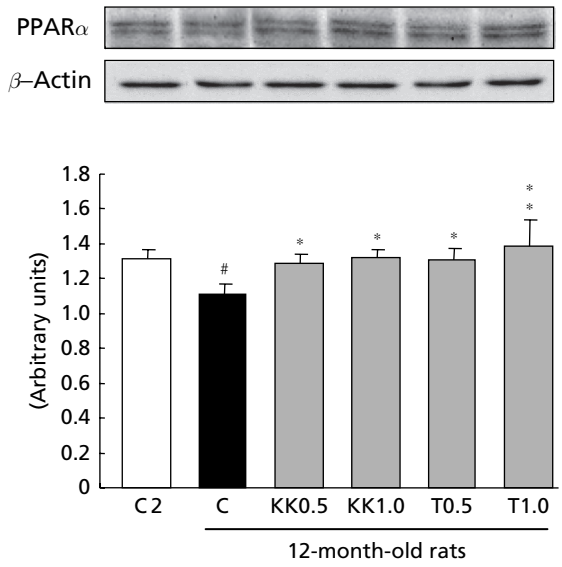

Figure 3 Western blot analysis of PPARα protein expression in the hepatic nucleus. The molecular mass of PPARα was approximately 55 kDa. Rats were administered one of the following: Kangen-karyu 0.5%, KK0.5; Kangen-karyu 1.0%, KK1.0; Tanjin 0.5%, T0.5; or Tanjin 1.0%, T1.0. Control rats 2-month-old, C2; control rats 12-month-old, C. $^{\#}P < 0.001$ compared with control values of two-month-old rats; $^{*}P < 0.01$, $^{**}P < 0.001$ compared with control values of 12-month-old rats.

Hepatic functional parameters

Figure 5 represents the effect of Kangen-karyu or Tanjin on hepatic function with age. While the serum ALT and AST levels, as parameters of hepatic function, of 12-month-old rats increased significantly compared with those of two-month-old rats, they were significantly decreased by the administration of Kangen-karyu or Tanjin extracts (Figure 5), especially the serum AST level, which showed a tendency to decline to the normal value after the administration of Kangen-karyu or Tanjin.

Discussion

Protection from lipidosis with age is considered to ameliorate the pathological conditions related to ageing. This is because the accumulation of lipids such as triglyceride, cholesterol and fatty acids is responsible for degenerative diseases related to ageing, including cardiovascular disease, diabetes, coronary heart disease, hepatitis, renal failure and dementia (James 2001; De Caterina et al 2006). Therefore, to investigate the protective role of Kangen-karyu and its crude drug Tanjin from ageing, this study focused on their effects against age-related lipidosis under an in-vivo rat model.

The elevation of serum lipids is a major risk factor of age-related disease or organ dysfunction. The results demonstrated that the level of triglyceride in serum dramatically increased with ageing, while the administration of Kangen-karyu or Tanjin led to a reduction in the triglyceride level. The accumulation of triglyceride with ageing could be a cause of pathological conditions such as atherosclerosis. Therefore, the decreasing effect on triglyceride level would play a protective role against its related pathological conditions. In addition, Yokozawa et al (2006) reported the protective effects of Kangen-karyu and Tanjin against hypercholesterolaemia through their regulation of cholesterol levels and inhibition of lipid peroxidation. Kangen-karyu led to a decrease in the atherogenic index, along with a decrease in low density lipoprotein cholesterol and increase in high density lipoprotein cholesterol. This suggested that Kangen-karyu would probably play a beneficial role against hyperlipidaemia and its related degenerative diseases.

One of the proteins that decrease with ageing is adiponectin. Adiponectin is the most abundant secretory protein in adipocytes and has been shown to enhance insulin sensitivity, improve the plasma clearance of fatty acids, glucose and triglyceride, and suppress hepatic glucose production (Berg et al 2001; Zhu et al 2004). This suggested that carbohydrate and lipid metabolism with ageing could be affected by adiponectin. The results showed the decline of adiponectin in serum with ageing (Table 2). The administration of Kangen-karyu or Tanjin increased the level, but not significantly. Calorie restriction is one dietary factor that elevates the level. Zhu et al (2004) reported that rats fed a calorie-restricted diet exhibited a significant increase in plasma adiponectin accompanied with a significant decline in the plasma triglyceride level, which may have contributed to a decline in tissue triglyceride accumulation via modulation of the expression of key transcription target genes involved in fatty acid oxidation and energy use. The administration of Kangen-karyu and Tanjin showed an inhibitory effect against the accumulation of triglyceride with ageing, whereas they elevated the adiponectin level, but not significantly.

Among the fatty acid-regulated nuclear receptors, PPAR is the most extensively characterized. PPARα plays an important role in fatty acid metabolic homeostasis through regulation of

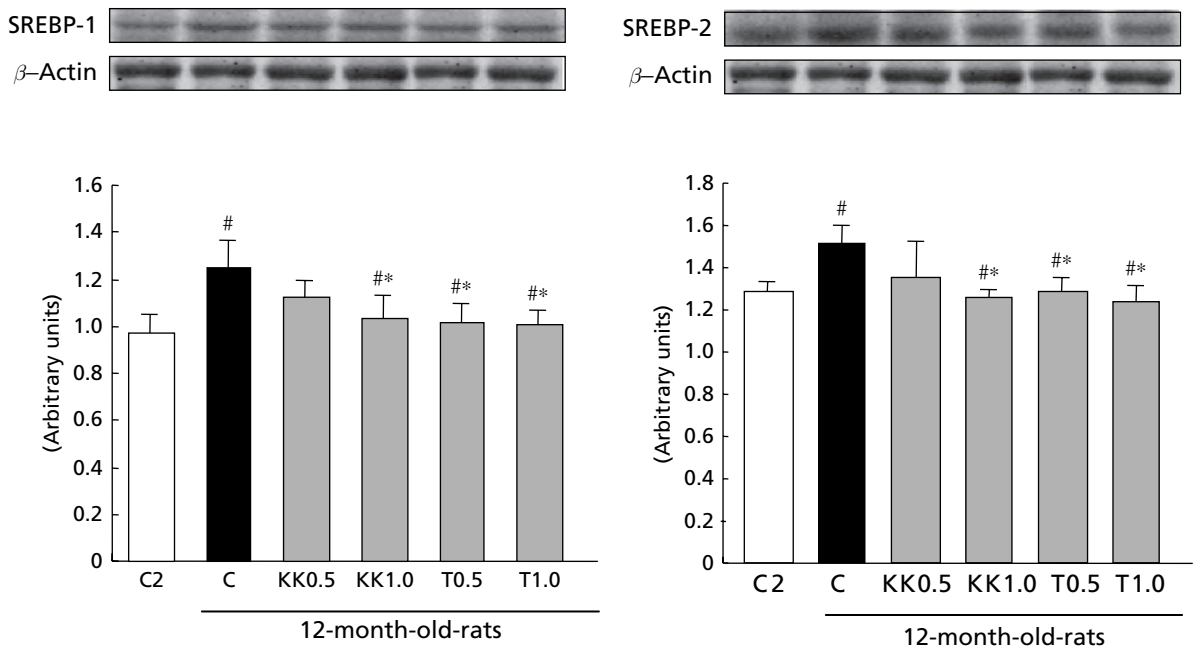

Figure 4 Western blot analysis of SREBP-1 and −2 protein expression levels in the hepatic nucleus. Rats were administered one of the following: Kangen-karyu 0.5%, KK0.5; Kangen-karyu 1.0%, KK1.0; Tanjin 0.5%, T0.5; or Tanjin 1.0%, T1.0. Control rats 2-month-old, C2; control rats 12-month-old, C. $^{\#}P < 0.001$ compared with control values of two-month-old rats; $^{*}P < 0.001$ compared with control values of 12-month-old rats.

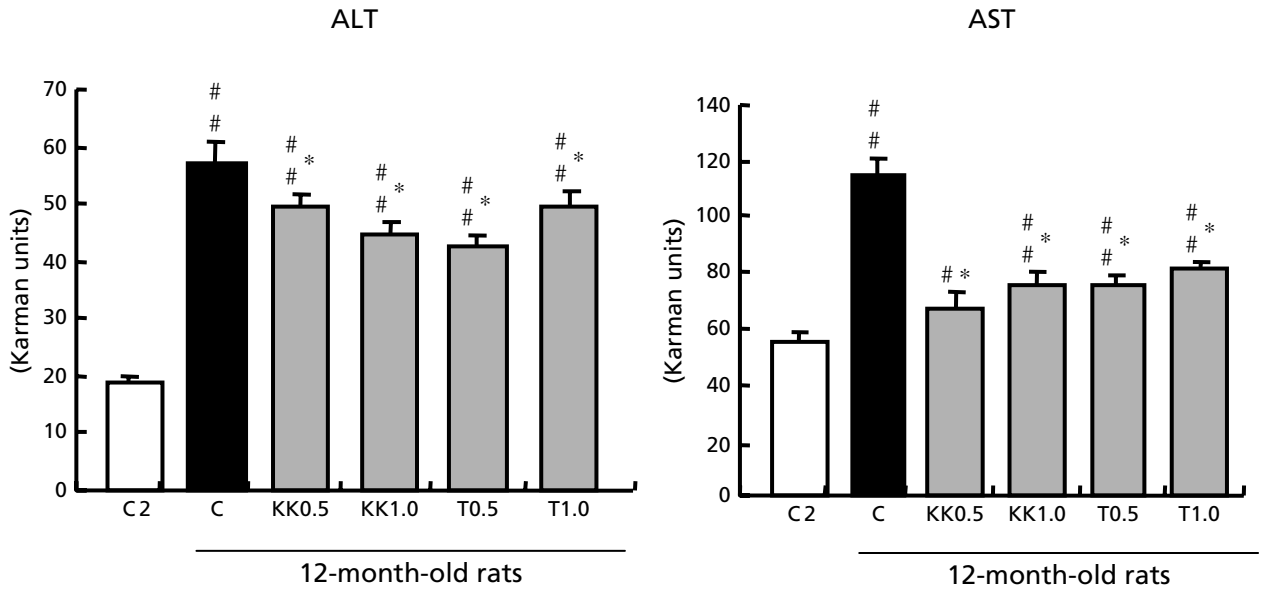

Figure 5 The effect of Kangen-karyu and Tanjin on hepatic function parameters with age. ALT, alanine aminotransferase; AST, aspartate aminotransferase. Rats were administered one of the following: Kangen-karyu 0.5%, KK0.5; Kangen-karyu 1.0%, KK1.0; Tanjin 0.5%, T0.5; or Tanjin 1.0%, T1.0. Control rats 2-month-old, C2; control rats 12-month-old, C. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ compared with control values of two-month-old rats; $^{*}P < 0.001$ compared with control values of 12-month-old rats.

target genes encoding enzymes for fatty acid β-oxidation and fatty acid transporters (Schoonjans et al 1997; Aoyama et al 1998; Palmer et al 1998; Leone et al 1999). Among three isoforms of PPAR that have been cloned, PPARα is mainly expressed in the liver, digestive tract, and kidney (Desvergne & Wahli 1999; Kaplan et al 2001). In addition, the administration of agents capable of activating PPARα can restore the cellular redox balance by lowering of lipid peroxidation in tissue, and an elimination of constitutively active nuclear factor kappa B loss in the spontaneous production of inflammatory

cytokines (Erol 2005). This indicated that the prevention of lipidosis in the ageing process might be an important mechanism for anti-ageing; thus, PPARα activation may be a good candidate as an anti-ageing agent. Therefore, the effect of Kangen-karyu and Tanjin on the expression of PPARα protein has been observed. Consistent with other studies, the expression of PPARα protein was found to decrease in old rats compared with young ones. However, the administration of Kangen-karyu or Tanjin elevated the protein levels, suggesting their role as PPARα activators.

The regulation of gene transcription by fatty acids is attributed to changes in the activity or abundance of at least four transcriptional factor families: PPAR, liver X receptor (LXR), hepatocyte nuclear factor-4α (HNF-4α), and SREBPs. These transcription factors are members of the superfamily of sterol and thyroid hormone nuclear receptors (Pegorier et al 2004). Among three SREBP isoforms, SREBP-1c preferentially enhanced the transcription of genes involved in fatty acid, triglyceride and phospholipid synthesis, whereas SREBP-1a and SREBP-2 activated genes involved in cholesterol synthesis. Consistent with evidence that SREBPs in the liver, kidney, adipose tissue, and brain were increased with ageing (Jiang et al 2005a, b), we found in this study that SREBP-1 and -2 proteins in the liver were increased with age, but Kangen-karyu and Tanjin suppressed the expressions of these proteins (Figure 4). Therefore, the results suggested that administration of Kangen-karyu or Tanjin extracts protected against an accumulation of triglyceride and cholesterol in hepatic tissues with ageing through their regulation of transcription factors, such as PPARα and SREBPs. SREBPs directly repress transcription of insulin receptor substrate-2, and inhibit hepatic insulin signalling that regulates lipogenesis by insulin and glucose (Ide et al 2004). SREBP-1c controls gene expression of lipogenic enzymes and its expression is nutritionally regulated in the liver and adipose tissue (Shimano 2001; Horton et al 2002). Thus, it has been regarded as a mediator for insulin action on gene transcription. The effect of Kangen-karyu and Tanjin on SREBPs suggested a promising role to regulate the insulin sensitivity and lipid metabolism, although the further study on insulin level has to be supported. Furthermore, to elucidate clearly the effects on the lipid profile in hepatic tissue with ageing, studies on other transcriptional factors, such as LXRα and HNF-4α, and diacylglycerol acyltransferase, which is a key enzyme exclusively devoted to the synthesis of triglyceride (Coleman et al 2000; Sanguino et al 2004), must be conducted.

The ageing process leads to several characteristics, such as changes in the biochemical composition, function of tissues, and progressive decline in the physiological capacity, eventually resulting in an increase in the susceptibility and vulnerability to diseases (Troen 2003). In particular, one of the most remarkable age-related changes is the decline in hepatic function and size. The liver plays a key role to facilitate mitochondrial β-oxidation of fatty acids, and increased lipid accumulation in the liver develops with ageing. This change in hepatic function with ageing accelerates the states of reduced β-oxidation and insulin resistance; therefore, the regulation of lipid metabolism with the ageing process may be one of the reasons for the anti-ageing activity. We investigated hepatic function by measuring serum levels of ALT and AST. Old rats showed elevations in the levels of ALT and AST compared with young rats, indicating hepatic functional changes with ageing. On the other hand, the decline of hepatic function with ageing was attenuated by Kangen-karyu and Tanjin supplements (Figure 5). The results support the beneficial role of Kangen-karyu and Tanjin in lipid metabolism through the improvement of hepatic function.

Conclusion

Kangen-karyu and Tanjin showed protective activity from age-related lipidosis under a rat model. The administration of Kangen-karyu and Tanjin extracts inhibited the accumulation of triglyceride with ageing. They also ameliorated the decline in the expression of PPARα protein in hepatic tissue with age. On the other hand, the overexpression of SREBP-1 and SREBP-2 proteins in old rats compared with young rats showed the tendency to decrease, and the decline of hepatic function with ageing was attenuated by Kangen-karyu and Tanjin. This study has provided evidence for the anti-ageing activity of Kangen-karyu and its crude drug Tanjin against age-related lipidosis through their beneficial effects on lipid metabolism and improvement of liver function. Tanjin is attributed to the synergistic and/or additive anti-ageing effect of Kangen-karyu. The active components of Kangen-karyu which lead to its anti-ageing activity have to be elucidated, along with their related mechanisms.

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