Effects of the Chinese prescription Kangen-karyu and its crude drug Tanjin on ageing process in rats

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Abstract

The effects of the Chinese prescription Kangen-karyu and its crude drug Tanjin on the ageing process were investigated in rats. Diets supplemented with Kangen-karyu and Tanjin extracts decreased glycosylated protein levels in serum, a risk marker of ageing and ageing-related diseases. In addition, they inhibited the levels of thiobarbituric acid reactive substance in the serum and liver; Kangen-karyu in particular led to a strong decrease in hepatic mitochondrial thiobarbituric acid reactive substance. The decline in the reduced glutathione/oxidized glutathione ratio in the liver observed with ageing was ameliorated by Kangen-karyu and Tanjin, while these groups attenuated the increase in glutathione peroxidase activity of hepatic tissue against ageing. This suggests that Kangen-karyu and Tanjin regulate the glutathione redox cycle that maintains the cellular redox condition against age-related oxidative stress. Moreover, the overexpression of cytoplasmic cytochrome c observed with ageing was attenuated by Kangen-karyu and Tanjin. This provides new evidence that Kangen-karyu and Tanjin inhibit leakage of superoxide in mitochondria and attenuate cellular oxidative damage. Furthermore, Kangen-karyu and Tanjin would maintain mitochondrial function with ageing through the regulation of related protein expression such as bax and bcl-2 proteins. In addition, Kangen-karyu reduced the expression of nuclear factor kappa B; Kangen-karyu and Tanjin did not affect the expression of inhibitor kappa B. The present study demonstrated that Kangen-karyu prevented oxidative damage and mitochondrial dysfunction with ageing. Furthermore, Kangen-karyu showed a stronger protective effect against ageing by oxidative stress than Tanjin, probably through synergistic and/or additive effects.

Introduction

Since ageing is an inevitable phenomenon in living organisms, much effort has been focused on delaying the ageing process. Among the various theories of ageing, the free radical theory is the most convincing. The free radical theory indicates that the production of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$), is inevitable in aerobic organisms, and the accumulation of injuries caused by ROS is an important factor in ageing. Therefore, the attenuation of oxidative damage by ROS is considered to be the key to retarding the ageing process and its related phenomena. Limiting food or calorie intake has been shown to extend longevity in a wide range of species and in rodents. In an in-vivo model, Masoro (2000) reported that the rate of oxidant generation in mitochondria under calorie restriction was significantly lower than in un-restricted counterparts, and calorie restriction reduced the age-associated accumulation of oxidatively damaged proteins, lipids and DNA. Additionally, administration of antioxidant agents can prevent the development of age-associated disorders such as cancer (Kim et al 1998), cardiovascular disorders (Inoue et al 1990; Kondo et al 1994; Yokozawa et al 1998) and some neurodegenerative disorders (Sano et al 1997). This evidence suggests the possibility of disrupting the ageing process through the regulation of oxidative stress.

Kangen-karyu, a Chinese prescription comprised of six crude drugs, has received much attention because of its numerous biological activities, such as the inhibition of platelet aggregation, suppression of hypertension and its anti-ageing effect (Takahashi 1991; Gao et al 2001; Makino et al 2002). Takahashi et al (1992) demonstrated that Kangen-karyu

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affected the recovery of learning and memory impairment in senescence-accelerated mice (SAM) by preserving the activities of choline acetyltransferase and superoxide dismutase in the cerebellum. Our previous study also showed that Kangen-karyu extract inhibited the oxidative stress-related ageing process in SAM through enhancing antioxidative enzyme activity and scavenging ROS (Satoh et al 2004a). In addition, we reported that Kangen-karyu protected against cellular senescence by reducing oxidative damage through the inhibition of ROS generation and regulation of the antioxidative status (Satoh et al 2004b). These investigations suggest that Kangen-karyu may delay the ageing process by virtue of its antioxidative effect. Nomura et al (1997) also reported that Tanjin, the main extract with the greatest amount of Kangen-karyu, effectively protects against learning deficits caused by ageing in SAM. Therefore, Tanjin extract is also expected to play a crucial role in retarding the ageing process.

To verify the anti-ageing and related effects of Kangen-karyu extract, the present investigation was carried out using rats that proceed along the natural and physiological ageing process with time. Changes in antioxidative status with ageing and the related protein expression were observed, and a comparison of the anti-ageing effects of Kangen-karyu and Tanjin was carried out.

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 dimethylsulfoxide solution (protease inhibitor cocktail) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). The monoclonal antibodies to nuclear factor kappa B (NF-κB) (p65), inhibitor kappa B (IκB), cytochrome c, cyclooxygenase (COX)-1, COX-2, heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), bax, bcl-2 and monoclonal goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The other chemicals and reagents used were of high quality and 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 vols 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. 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 high performance liquid chromatography 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$_3$CN in 50 mM H$_3$PO$_4$ at a flow rate of 0.8 mL min^{-1}. The UV absorbance from 200 to 400 nm was monitored and the three-dimensional data processed by a JASCO photodiode array detector MD-910. All assigned peaks were identified by carrying out a co-injection test with authentic samples and compared with the UV spectral data. By three-dimensional high performance liquid chromatography, lithospermic acid B, paeoniflorin, pentagalloyl glucose, lithospermic acid and rosmarinic acid were detected as the major compounds of Kangen-karyu; albiflorin, carthamin, cyperol and α-cyperone were also observed.

Preparation of Tanjin extract

To prepare Tanjin extract, the roots (50 g) of Tanjin were boiled gently in 1000 mL of 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 of Kangen-karyu extraction described above. The major components of Tanjin detected were lithospermic acid B, lithospermic acid and rosmarinic acid.

Animals and treatment

The Guidelines for Animal Experimentation approved by University of Toyamo were followed during these experiments. Male Wistar rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept under conventional conditions at 23 ± 1°C with an alternating 12-h light/dark cycle and allowed access to food and water ad libitum. Each extract, supplemented in the diet at 0.5% or 1.0%, by weight, was administered for 4 months from 8 months of age. Twelve rats were used for each experimental group. During the experimental period, the diet in a pair-feeding schedule was given to all rats (18 g/rat per day), and the intake of Kangen-karyu or Tanjin extract intake was estimated to be about 90 and 180 mg/rat, respectively. The body weight was monitored every week during the 4-month experimental 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 then extirpated, immediately frozen by immersion in liquid nitrogen and kept at −80°C until analysis. Two-month-old males were used in this experiment as representative younger rats.

Determination of glycosylated protein level

The glycosylated protein level was determined using the modified thiobarbituric acid (TBA) assay of Fluckiger & Winterhalter (1976), in which non-enzymatically bound glucose is released as 5-hydroxymethylfurfural (5-HMF) and quantified colorimetrically. Briefly, serum (100 μL) was diluted to 1 mL with H$_2$O and mixed with 500 μL oxalic acid (1 M), hydrolysed for 4.5 h at

100°C and then, after reaction with TBA, glycosylated haemoglobin was quantified by measuring the absorbance at 443 nm.

Determination of TBA reactive substance level

Serum TBA reactive substance was measured by the method of Naito & Yamanaka (1978). Serum (0.15 mL) was mixed with 0.05 MHCl and 0.67% TBA, and boiled for 30 min. After cooling in ice water, n-BuOH (including 15% MeOH) was added, mixed vigorously and separated by centrifugation at 4000 g for 10 min. The obtained BuOH layer was measured spectrophotometrically at 535 nm. Hepatic TBA reactive substance was assayed according to the method of Mihara & Uchiyama (1978). Briefly, the tissue was homogenized with a 9-fold volume of ice-cold physiological saline. Mitochondria was prepared from liver homogenate by differential centrifugation (800 g and 12 000 g, respectively) in a refrigerated centrifuge (4 °C) according to the methods of Johnson & Lardy (1967) and Jung & Pergande (1985), respectively. The pellet was resuspended in preparation medium. A sample of homogenate or pellet suspension was mixed with 1% H_3PO_4 and 0.67% TBA, and boiled for 45 min. After cooling in ice water, the reaction mixture was extracted with n-BuOH. The absorbance of the n-BuOH phase was measured at 535 nm and 520 nm.

Determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) levels

According to the method of Floreani et al (1997), the liver (approx. 250 mg) was homogenized in 1 mL of 25% metaphosphoric acid plus 3.75 mL of 100 mM sodium phosphate–5 mM EDTA buffer (pH 8.0), and then centrifuged at 105 000 g for 30 min at 4°C. Determination of the GSH and GSSG concentrations in the supernatant was performed by the method of Hissin & Hilf (1976), using o-phthalaldehyde as the fluorescent reagent.

Assay of glutathione peroxidase (GSH-Px) activity

The tissue was homogenized with a 9-fold volume of ice-cold physiological saline and the enzyme activity of the homogenate was determined. GSH-Px activity was obtained colorimetrically with 2-nitro-5-thiobenzoic acid, a compound produced by the reaction between GSH and 5,5′-dithiobis 2-nitrobenzoic acid (Ellman 1959).

Determination of the protein level

The protein level was determined by the method of Itzhaki & Gill (1964) with bovine serum albumin as the standard.

Preparation of total, cytoplasmic and nuclear fractions

According to the method of Choi et al (1997), hepatic tissue was homogenized with 25 mM Tris-Cl (pH 7.5) containing 250 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol (DTT) and protein inhibitor cocktail. After adding NP-40, vortexing and incubating on ice for 30 min, the supernatant

was collected by centrifugation at 20 000 g for 15 min, as a total fraction. To obtain the cytoplasmic and nuclear fractions, hepatic tissue was homogenized with 10 mM 2-[4-(2-hydroxyethyl)-1-piperazyl] ethanesulfonic acid (HEPES) buffer (pH 7.9) containing 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail. 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 cocktail. The mixture was left on ice for 15 min with frequent agitation. Nuclear extract was prepared by centrifugation at 15 000 g for 5 min at 4°C. The protein concentration of each fraction was quantified using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

Protein expression

Western blot analysis was performed on 30 μg of protein from each fraction. The protein was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to Trans-blot transfer medium (Bio-Rad Laboratories) at 100 V for 1 h and immunodetection was performed using the enhanced chemiluminescence kit for Western blotting detection (Amercham Pharmacia Biotech, Freiburg, Germany). The identification of each protein was estimated by comparison with the protein markers of known molecular weight.

Determination of hepatic functional parameters

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

Statistics

The results are expressed as mean ± s.e. 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 statistically significant.

Results

Glycosylated protein levels in the serum

Table 1 shows the effects of Kangen-karyu and Tanjin extracts on glycosylated protein with ageing. The serum glycosylated protein levels of 12-month-old rats led to the observed increase to 22.32 nmol (mg protein)$^{-1}$ from 18.73 nmol (mg protein)$^{-1}$ in 2-month-old rats. However, the rats fed 0.5% Kangen-karyu and Tanjin extracts had significantly reduced glycosylated protein serum levels of 19.70 nmol (mg protein)$^{-1}$ and 20.44 nmol (mg protein)$^{-1}$, respectively.

Table 1 Glycosylated protein level in serum

Age (months)	Group	Glycosylated protein (nmol (mg protein)$^{-1}$)
2	–	18.73 ± 0.29
12	–	22.32 ± 0.70[##]
	Kangen-karyu, 0.5%	19.70 ± 0.56[#,*]
	Kangen-karyu, 1.0%	20.59 ± 0.38[##,*]
	Tanjin, 0.5%	20.44 ± 0.54[##,*]
	Tanjin, 1.0%	22.14 ± 0.41[##]

[#]$P < 0.05$, [##]$P < 0.001$, significantly different compared with control values of 2 months of age; *$P < 0.001$, significantly different compared with control values of 12 months of age.

TBA reactive substance in serum, and hepatic tissue homogenate and mitochondrial fractions

The effects of Kangen-karyu and Tanjin extracts on lipid peroxidation with ageing are shown in Table 2. At 12 months of age, the serum TBA reactive substance level was significantly greater than that at 2 months of age. The administration of both Kangen-karyu and Tanjin extracts significantly reduced the serum TBA reactive substance level. In addition, the level in the hepatic homogenate also showed an age-related increase from 0.63 nmol (mg protein)$^{-1}$ to 0.94 nmol (mg protein)$^{-1}$, and the administration of 0.5% Kangen-karyu and Tanjin extracts significantly reduced this level to 0.68 nmol (mg protein)$^{-1}$ and 0.70 nmol (mg protein)$^{-1}$,

respectively. Furthermore, the TBA reactive substance level in the mitochondrial fraction of hepatic tissue markedly increased with age from 0.69 nmol (mg protein)$^{-1}$ to 1.18 nmol (mg protein)$^{-1}$, greater increases than the value for the hepatic tissue homogenate. Only the rats given Kangen-karyu extract showed a significant decrease in the TBA reactive substance of the hepatic mitochondrial fraction (Table 2).

GSH and GSSG levels, the GSH/GSSG ratio and GSH-Px activity

Although there was no significant change in the GSH level with age, the GSSG level of 12-month-old rats was significantly greater than that of 2-month-old rats, as shown in Table 3. A significant decrease in the GSSG level was observed in the group given 0.5% Kangen-karyu extract; the administration of Tanjin extract did not affect this level. The GSH/GSSG ratio also showed an age-related decrease; the administration of Kangen-karyu and Tanjin extracts led to a significant increase in this ratio in the liver. In particular, the rats fed Kangen-Karyu extract showed a higher GSH/GSSG ratio than those fed Tanjin extract. The hepatic GSH-Px activity showed a significant age-related increase; it was significantly lower in the groups given the extracts of Kangen-karyu and Tanjin compared with the control rats at 12 months of age.

Protein expression of hepatic tissue

As shown in Figure 1, the cytoplasmic cytochrome *c* protein of 12-month-old rats was increased compared with that of

Table 2 Thiobarbituric acid (TBA) reactive substance levels in serum, and hepatic tissue homogenate and mitochondrial fraction

Age (months)	Group	Serum TBA reactive substance (nmol mL^{-1})	Hepatic tissue homogenate TBA reactive substance (nmol (mg protein)$^{-1}$)	Hepatic mitochondrial TBA reactive substance (nmol (mg protein)$^{-1}$)
2	–	3.70 ± 0.15	0.63 ± 0.05	0.69 ± 0.02
12	–	5.35 ± 0.38[#]	0.94 ± 0.20[#]	1.18 ± 0.09[#]
	Kangen-karyu, 0.5%	4.68 ± 0.18[#,***]	0.68 ± 0.11**	0.93 ± 0.06[#,***]
	Kangen-karyu, 1.0%	4.58 ± 0.24[#,***]	0.73 ± 0.06*	1.02 ± 0.08[#,**]
	Tanjin, 0.5%	4.62 ± 0.19[#,***]	0.70 ± 0.06***	1.15 ± 0.06[#]
	Tanjin, 1.0%	4.71 ± 0.17[#,**]	0.65 ± 0.04***	1.13 ± 0.06[#]

[#]$P < 0.001$, significantly different compared with control values of 2 months of age; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different compared with control values of 12 months of age.

Table 3 Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels, GSH/GSSH ratio and glutathione peroxidase (GSH-Px) activity in hepatic tissues

Age (months)	Group	GSH (mmol (g tissue)$^{-1}$)	GSSG (mmol (g tissue)$^{-1}$)	GSH/GSSG ratio	GSH-Px (units (mg protein)$^{-1}$)
2	–	10.83 ± 1.11	0.07 ± 0.01	164.8 ± 8.9	114.2 ± 2.9
12	–	10.92 ± 3.27	0.21 ± 0.03[##]	49.1 ± 6.2[##]	138.1 ± 5.6[##]
	Kangen-karyu, 0.5%	10.28 ± 2.96	0.16 ± 0.02[##,*]	69.2 ± 13.2[##,**]	127.7 ± 6.9[#,*]
	Kangen-karyu, 1.0%	14.98 ± 3.01	0.18 ± 0.03[##]	83.4 ± 7.9[##,***]	129.8 ± 4.6[##]
	Tanjin, 0.5%	12.31 ± 2.37	0.18 ± 0.03[##]	68.3 ± 8.6[##,*]	128.1 ± 7.0[#,*]
	Tanjin, 1.0%	12.63 ± 2.44	0.19 ± 0.03[##]	67.7 ± 7.0[##,*]	132.0 ± 3.3[##]

[#]$P < 0.01$, [##]$P < 0.001$, significantly different compared with control values of 2 months of age; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different compared with control values of 12 months of age.
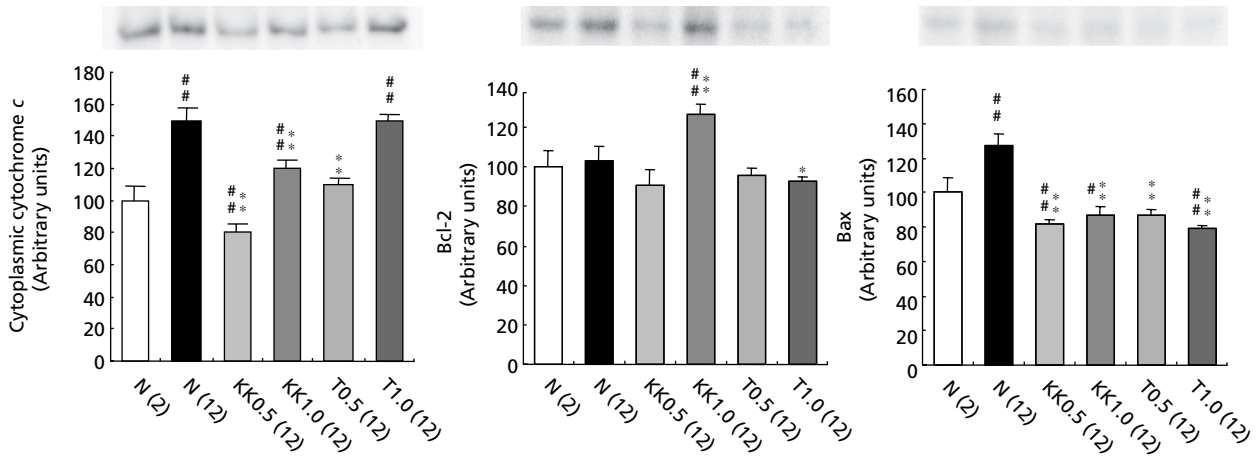
Figure 1 Cytoplasmic cytochrome *c*, bcl-2 and bax proteins. The protein levels were quantified by densitometry. #$P < 0.01$, ##$P < 0.001$, significantly different compared with control values of 2 months of age; *$P < 0.05$, **$P < 0.001$, significantly different compared with control values of 12 months of age.

2-month-old rats; the rats fed 0.5 and 1.0% Kangen-karyu and Tanjin extracts had significantly reduced levels of cytoplasmic cytochrome *c*. The expression of bcl-2 protein showed a tendency to increase with age. The 1.0% Kangen-karyu extract led to an increase in the protein expression of bcl-2, but Kangen-karyu and Tanjin extracts prevented the increase of bax protein with age. Furthermore, nuclear NF-κB protein showed a significant age-related increase, and cytoplasmic IκB protein showed a significant age-related decrease. Whereas the administration of 0.5 and 1.0% Kangen-karyu and 1.0% Tanjin extracts significantly reduced the level of NF-κB protein, they did not affect the protein expression of IκB with age (Figure 2). Figure 3 shows the effects on the protein expression of COX-1, COX-2, iNOS and HO-1. The expression of COX-1 did not change with any treatment and

ageing, whereas the expression of COX-2 showed a tendency to increase with age. Tanjin extract dramatically reduced the COX-2 protein. On the other hand, the expression of iNOS and HO-1 proteins of 12-month-old rats was increased compared with those of 2-month-old rats. Both Kangen-karyu and Tanjin extracts significantly reduced the levels. In this experiment, twelve tissues were used for each protein expression.

Hepatic functional parameters

The serum ALT and AST levels, parameters of hepatic function, of 12-month-old rats were significantly increased compared with those of 2-month-old rats, and they were significantly decreased by the administration of Kangen-karyu and Tanjin extracts (Table 4).
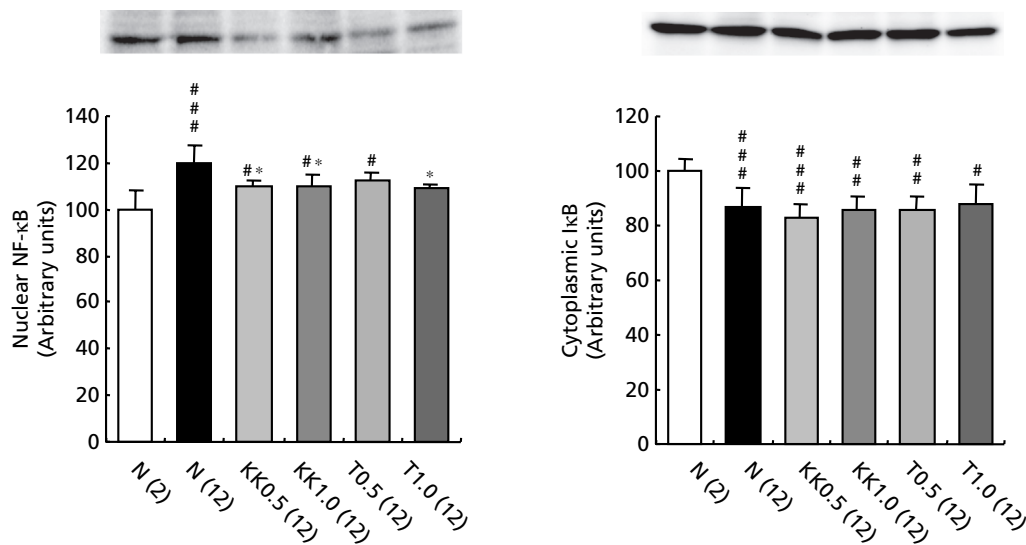

Figure 2 Nuclear factor kappa B (NF-κB) and cytoplasmic inhibitor kappa B (IκB) proteins. The protein levels were quantified by densitometry. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, significantly different compared with control values of 2 months of age; *$P < 0.05$, significantly different compared with control values of 12 months of age.
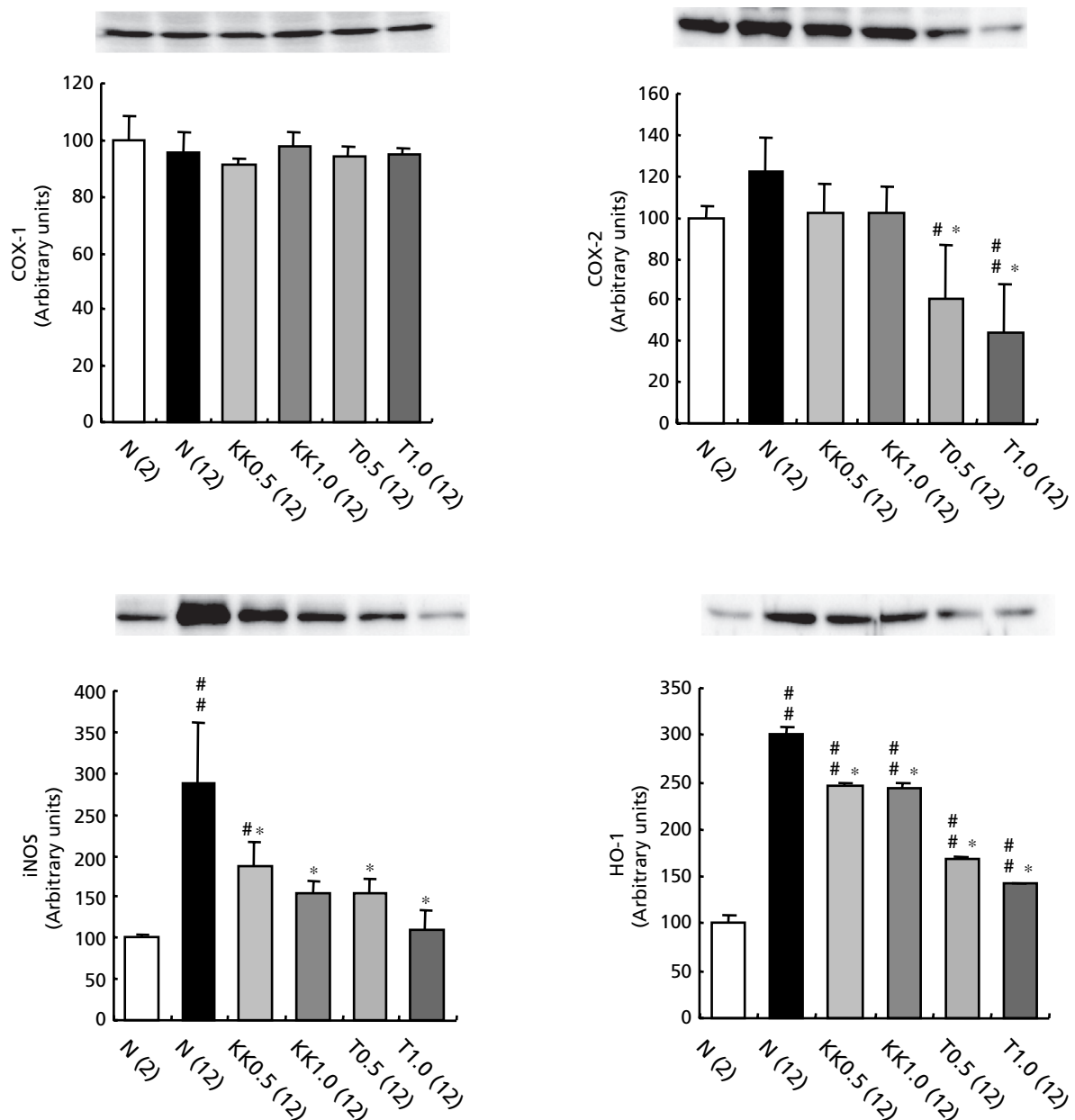
Figure 3   Cyclooxygenase (COX)-1, COX-2, inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) proteins. The protein levels were quantified by densitometry. $^{#}P < 0.01$, $^{##}P < 0.001$, significantly different compared with control values of 2 months of age; $^{*}P < 0.001$, significantly different compared with control values of 12 months of age.

Discussion

The rodent is the most frequently used model of ageing. The lifespan of rats is approximately 36–48 months (natal: 7–10 weeks; young: 3–6 months; adult: 12 months; and old: 36–38 months). According to the free radical theory of ageing, various oxidative reactions occurring in an organism (mainly in the mitochondria) generate free radicals that cause multiple lesions in macromolecules, leading to damage and ageing (Beckman & Ames 1998). Calorie restriction is well established as the most convincing way to retard the ageing pro-

cess and extend lifespan (Yu 1996). Dietary restriction reduces age-related oxidative stress and initiates a wide range of protective actions against oxidative cellular damage. The evidence that calorie restriction leads to an extension of lifespan also suggests that diet and supplemented antioxidative activity would also play promising roles in delaying the ageing process. Recently, we found that Kangen-karyu ameliorated tissue damage and oxidative stress associated with ageing in the SAM model (Satoh et al 2004a). Although it is considered to be a useful in-vivo model for ageing research, the SAM model is not exactly the same as the natural ageing process. Therefore, the present study demonstrated how Kangen-karyu

Table 4 Hepatic function parameters

Age (months)	Group	ALT (Karman units)	AST (Karman units)
2	–	18.94 ± 0.87	55.56 ± 3.07
12	–	$57.23 \pm 3.37^{\#\#}$	$114.72 \pm 6.04^{\#\#}$
	Kangen-karyu, 0.5%	$49.67 \pm 1.93^{\#\#,*}$	$66.55 \pm 6.09^{\#,*}$
	Kangen-karyu, 1.0%	$44.84 \pm 2.22^{\#\#,*}$	$75.69 \pm 3.96^{\#\#,*}$
	Tanjin, 0.5%	$42.54 \pm 1.84^{\#\#,*}$	$75.81 \pm 3.35^{\#\#,*}$
	Tanjin, 1.0%	$49.47 \pm 2.82^{\#\#,*}$	$81.39 \pm 2.76^{\#\#,*}$

ALT, alanine aminotransferase; AST, aspartate aminotransferase. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, significantly different compared with control values of 2 months of age; $^{*}P < 0.001$, significantly different compared with control values of 12 months of age.

affects the ageing process in a model more closely related to human ageing, that is in rats, which proceed along the natural ageing process with time. We focused on the accumulation of damage caused by oxidative stress and protein expression during the ageing process, and evaluated the anti-ageing effects of Kangen-karyu and its crude drug Tanjin.

Biological macromolecules such as proteins, lipids and nucleic acids are the targets of oxidative stress with age (Miquel 2002). In particular, amino groups in proteins react non-enzymatically with reducing sugars to produce to advanced glycation end products (AGEs). AGEs not only modify protein properties but also induce biological damage in-vivo (Kasper & Funk 2001). AGEs are irreversibly formed, accumulate with ageing, arteriosclerosis and diabetes mellitus, and are especially associated with long-lived proteins such as collagens (Frye et al 1998; Ono et al 1998). An increase in serum AGEs with age is a risk marker of ageing and age-related diseases (Sell & Monnier 1989; Nagaraj et al 1996; Odani et al 2001). On the other hand, Hunt et al (1994) reported that in a plasma sample, AGEs and protein glycation appeared to be linearly correlated. 5-HMF is involved in the non-enzymatic browning process and non-enzymatically bound glucose in serum is released as 5-HMF (Bunn et al 1978; McFarland et al 1979). Therefore, we evaluated 5-HMF levels to determine the extent of serum glycosylated protein. The serum glycosylated protein levels of 12-month-old rats increased compared with 2-month-old rats. However, the groups fed Kangen-karyu and Tanjin extracts showed decreased serum glycosylated protein levels, although the reduction in glycosylated protein levels accounts for the comparison with 12-month-old control rats in the present model. These findings suggest that they would be protected from protein modification and biological damage with ageing.

The formation of protein adduct with lipid peroxidation production, TBA reactive substance, is also a useful biological marker of ageing (Kim et al 2002). Our study showed that both serum and hepatic levels of TBA reactive substance at 12 months of age were significantly greater than those at 2 months of age, indicating that the ageing process makes the tissues more susceptible to oxidative stress and this elevation of oxidative stress with ageing induces lipid peroxidation. Interestingly, these increases were greater in mitochondria than in liver homogenate. This supports the role of mitochondria

as primary targets of oxidative damage associated with ageing. Kangen-karyu and Tanjin significantly decreased the levels of TBA reactive substance in serum and hepatic tissue. In particular, Kangen-karyu exerted a greater protective effect on lipid peroxidation with ageing than Tanjin. These results indicate that Kangen-karyu and Tanjin attenuate oxidative stress, and that Kangen-karyu may have an important role in protecting against mitochondrial damage with age.

Since ageing is mainly attributed to oxidative stress, the cellular defence system, including antioxidant and antioxidative enzymes, plays a crucial role in the ageing of aerobic organisms. It has been reported that glutathione and glutathione-related enzymes (glutathione system) play fundamental roles in the cellular defence against ROS with age (Beckman & Ames 1998). GSH is oxidized to GSSG by GSH-Px and GSSG is converted to GSH by glutathione reductase. GSH-Px works together with GSH in the decomposition of H_2O_2 and other organic hydroperoxides. Our study demonstrated that the level of GSSG with age in hepatic tissues increased without changes in the GSH level, and therefore the GSH/GSSG ratio dramatically decreased. In addition, the 12-month-old rats showed greater GSH-Px activity, probably related to increased oxidative damage with age, while Kangen-karyu and Tanjin attenuated the GSH-Px activity of hepatic tissues against ageing. Our results suggest that Kangen-karyu and Tanjin attenuate the glutathione redox cycle to maintain the cellular redox condition against age-related oxidative stress. Miquel (2002) reported that changes in the redox (GSH/GSSG) ratio are much more striking in the mitochondria than in the extra-mitochondrial compartment, and lead to oxidative damage of mitochondrial DNA, which relates to mitochondrial dysfunction (Sastre et al 2002). The effect of Kangen-karyu on the glutathione redox cycle with age would probably also be related to the amelioration of mitochondrial dysfunction.

The expression of several proteins such as HO-1, COXs, iNOS, bax, bcl-2, caspase and cytochrome *c* from the heart, liver, kidney, brain and other tissues lead to changes with ageing (Chung et al 2002). When ROS was produced in mitochondria with ageing, cytochrome *c* was dramatically released from mitochondria into the cytosol (Skulachev 1998). This is an early event in ageing and age-related apoptosis, leading to the generation of O_2^- (Lin et al 2000). The present study demonstrated that the expression of cytosolic cytochrome *c* in 12-month-old rats increased compared with 2-month-old rats, but this increase was prevented in rats fed Kangen-karyu and Tanjin extracts. Kangen-karyu had a greater effect than Tanjin, suggesting that the effect of Kangen-karyu can be attributed partly to the synergistic and/or additive effect of its crude drug. The present investigation also provides new evidence that both Kangen-karyu and Tanjin inhibit the leakage of O_2^- in mitochondria and attenuate the cellular state against oxidative damage.

The increase in age-related NF-κB activity is elicited through the enhanced degradation of IκB induced through phosphorylation by IκB kinase during ageing (Helenius et al 1996; Kim et al 2000). Our study demonstrated that although the expression of cytoplasmic IκB protein in hepatic tissue in 12-month-old rats was lower than that in 2-month-old rats, NF-κB (p65) protein in the nuclear fraction showed an

increase with ageing. Although Kangen-karyu and Tanjin extracts did not affect the expression of cytoplasmic IκB, Kangen-karyu extract significantly reduced the expression of nuclear NF-κB. ROS induces the activation of NF-κB activity, and NF-κB, in turn, up-regulates the synthesis of anti-apoptotic members, the bcl-2 family (Sasaki et al 2001), and increases the transcription of genes that encode protective enzymes such as iNOS and COX-2 (Chung et al 2002). Our study demonstrated that the level of bcl-2 expression in 12-month-old rats had a tendency to increase compared with that of 2-month-old rats, and the level of bax protein increased with ageing. Consistent with the present study, similar observations of the overexpression of bax and down-expression of bcl-2 with ageing have been demonstrated (Ginn-Pease & Whisler 1998; Youssef et al 2003; Lee et al 2004). The present study showed an increase in the proteins of both bcl-2 and bax with ageing. Consistent with the present results, the increase in the bax/bcl-2 ratio with ageing by other studies indicates the possible role of the mitochondrial bax/bcl-2 apoptotic signalling pathway in ageing (Alway et al 2003; Chung & Ng 2005). Further study is needed on the comparison of protein expression of cytoplasmic bax with that of the mitochondrial fraction for a clearer explanation of the role of bax protein in the ageing process. Furthermore, these changes enhance the release of cytochrome c from mitochondria into the cytosol and lead to mitochondrial dysfunction with age (Bernardi et al 1999). Kangen-karyu and Tanjin extracts prevented the changes in bcl-2 and bax protein expression with ageing, indicating that Kangen-karyu and Tanjin could play crucial roles in protecting against mitochondrial dysfunction with ageing through the regulation of bax and bcl-2 protein levels.

Feng et al (1995) reported that ROS induces the expression of COX-2 protein, the key enzyme in pro-inflammatory prostanoid synthesis, and COX-2 is induced readily by cytokines, hormone, growth factor and tumour promoters in selected tissues (Smith et al 1994; Goppelt-Struebe 1995). iNOS is also readily inducible by pro-inflammatory cytokines, and has a close relationship with ROS generation, and the induction of its gene expression increases in aged mice and rats (Kim et al 1998). In addition, HO-1 is ubiquitously expressed and is inducible, which degrades heme to free irons and a pro-oxidant. It also contributes to the antioxidant defence mechanisms of the cells and enhanced oxidative stress during ageing is accompanied by compensatory induction of HO-1 (Lavrovsky et al 2000). Our results showed that COX-2, iNOS and HO-1 protein expression in 12-month-old rats were dramatically increased compared with 2-month-old rats. Although Kangen-karyu extract did not lead to changes in the COX-2 protein expression, iNOS and HO-1 protein expression were significantly reduced by treatment with this prescription. Tanjin extract dramatically reduced the expression of COX-2, iNOS and HO-1 proteins.

This study demonstrated that the ageing process resulted in increases in protein glycation and lipid peroxidation over time, and functional alterations in the liver with changes in related protein expressions. Kangen-karyu and Tanjin attenuated these changes, indicating that they modulate the abnormalities caused by ageing. Furthermore, the present study indicates that Kangen-karyu and Tanjin exert anti-ageing activities in an in-vivo ageing model through different mechanisms.

Kangen-karyu works mainly to maintain mitochondrial function against the leakage of ROS from mitochondria, elevates the level of bcl-2 protein expression and prevents the release of cytochrome c from mitochondria. In addition, Kangen-karyu ameliorates the glutathione redox cycle, prevents accumulating GSSG levels and strongly elevates the GSH/GSSG ratio. Although Tanjin did not have a remarkable role in protecting mitochondria from ageing compared with Kangen-karyu, it did attenuate the protein expression of inducible genes such as COX-2, iNOS and HO-1. Tanjin is attributed to the synergistic and/or additive anti-ageing effect of Kangen-karyu. The active components of Kangen-karyu that lead to its anti-ageing activity have to be elucidated, together with the related mechanisms.

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