J. Trad. Med. 23, 153－165, 2006

Medical and Pharmaceutical Society for WAKAN-YAKU

Review

Contribution of Chinese prescription Kangen-karyu in the oxidative stress-related aging process

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Chinese prescription Kangen-karyu, comprised of six crude drugs, has received much attention due to its numerous biological activities. The present study reports the anti-aging potential of Kangen-karyu and its related mechanisms under *in vivo* conditions with senescence-accelerated mice (SAM) and aging rats, and in a cellular system with stress-induced premature senescence (SIPS). Kangen-karyu attenuated oxidative stress by the inhibition of free radical generation and lipid peroxidation under the SAM model, suggesting its anti-aging potential. In addition, investigation with the *in vivo* natural aging model also showed that Kangen-karyu and its main crude drug, Tanjin, played protective roles against protein modification and lipid peroxidation in serum and tissue with aging. In particular, Kangen-karyu exerted a stronger anti-aging effect than Tanjin, suggesting that Tanjin contributes synergistically to the anti-aging activity of Kangen-karyu. Kangen-karyu also regulated the glutathione redox cycle, working primarily to maintain mitochondrial function against the leakage of reactive oxygen species from mitochondria, and led to the inhibition of nuclear factor-kappa B (NF-κB) to nuclear translocation and regulation of related protein expressions such as cyclooxygenase-2, inducible nitric oxide synthase, heme oxygenase-1, bax, and bcl-2 proteins. Furthermore, under the SIPS cellular model, Kangen-karyu extract showed an anti-aging activity and led to increased longevity through the attenuation of oxidative damage with the inhibition of lipid peroxidation and cell cycle regulation. Its anti-aging activity results from modulations of NF-κB and the expressions of its related proteins. The present study suggests that Kangen-karyu has a promising anti-aging potential against oxidative stress-induced aging processes.

Key words Kangen-karyu, aging, oxidative stress, stress-induced premature senescence, senescence-accelerated mice.

Introduction

Aging is an inevitable biological process that affects most living organisms. There are several theories of aging, error-catastrophe, protein modification, free radicals (oxidative stress), mitochondrial DNA, and some developmental-genetic theories including the longevity gene. The current view on oxidative damage induced by free radicals with aging is that aging-related changes are mainly attributed to molecular and cellular damage caused by free radicals.[1,2] The production of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH), is also inevitable in aerobic organisms and the accumulation of injuries caused by ROS is an important factor involved in the determination of the lifespans of living cells and the whole body. Since the oxidative stress theory on aging has been considered as the most reliable theory, it has been speculated that the antioxidative defense system that protects against the oxidative damage caused by ROS is suppressed with aging, resulting in functional disorders or tissue injury related to the aging process. Therefore, it has been suggested that prevention of oxidative damage through enhancement of the antioxidative defense status may counteract aging and age-associated disorders. Many experimental studies support the suggestion that antioxidant agent administration can prevent the development of age-associated disorders such as cancer,[3] cardiovascular disorders,[4-6] and some neurodegenerative disorders.[7]

Recently, great effort has been made to search for antioxidants without toxicity and side effects, such as traditional crude drugs, Chinese medicinal prescriptions, and functional foods. Among the Chinese traditional prescriptions, Kangen-karyu (Guan-Yuan-Ke-Li), composed of six crude drugs (Carthami Flos, Paeoniae Radix, Cnidii Rhizoma, Cyperi Rhizoma, Aucklandiae Radix, and Salviae Miltiorrhizae Radix), has received much attention due to its numerous biological activities such as the inhibition of platelet aggregation and suppression of hypertension.[8-11] Furthermore, Takahashi *et al.*[12] demonstrated that Kangen-karyu improved learning and memory impairment in senescence-accelerated mice (SAM) by preserving the activities of choline acetyltransferase and superoxide dismutase (SOD) in the cerebellum, suggesting that Kangen-karyu has anti-aging properties. The present study reports the anti-aging potential of Kangen-karyu and its related mechanisms under *in vivo* conditions with SAM and aging rats, and in a cellular system with stress-induced premature senescence (SIPS).

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Antioxidative effects related to the anti-aging potential of the Chinese prescription Kangen-karyu in senescence-accelerated mice

Many investigators have developed several models for studying human aging.[13] Among them, SAM, one of the murine models of accelerated aging, established by Takeda *et al.*,[14] is frequently used to study the aging process. Until the age of 4 months, senescence-prone inbred strain (SAMP) animals do not differ from the senescence-resistant inbred strain either behaviorally or morphologically, but later, they start to rapidly accumulate senile changes, including a moderate to severe decline of physical activity, hair loss, lack of hair glossiness, skin coarseness, periophthalmic lesions and cataracts, and increased lordokyphosis of the spine.[15,16] In particular, recently, SAM have attracted considerable attention, because the antioxidative defense systems of SAM strains, i.e., antioxidant enzyme activities and antioxidants, are deteriorated with the aging process.[17] Furthermore, Nakahara *et al.*[18] demonstrated that the aging process in SAM is mainly responsible for the mitochondrial dysfunction induced by oxidative stress from free radicals. Therefore, SAM are considered to be a useful *in vivo* model to evaluate antioxidative activities against aging associated with oxidative stress.

Studies on the generation of O_2^- with aging have produced conflicting results. Sohal *et al.*[19] reported that O_2^- increased with aging; on the other hand, Nakahara *et al.*[18] did not observe an age-related increase in the generation of ROS in SAMP 8 mice. Our present results showed that the serum generation of O_2^- in younger and older SAMP did not differ significantly. However, the administration of Kangen-karyu resulted in lower serum O_2^- levels than the older control group levels.[20] This suggests that the effect of Kangen-karyu on O_2^- generation contributes to its anti-aging properties in this animal model through the attenuation of oxidative stress caused by O_2^-. In addition, the previous study also demonstrated that the nitrite/nitrate level, reflecting the oxidative end production of nitric oxide (NO), increased significantly with age in SAMP. In the light of the reports that SAM strains have phenotypes of impaired immune response,[16] and that the synthesis of NO was regulated by many immunological factors, including tumor necrosis factor-α, interleukin-1, and interferon-α,[21] the impaired immune response could be related to excessive NO generation with age in SAMP. However, the administration of Kangen-karyu resulted in a decrease in the NO level in older SAMP, suggesting that Kangen-karyu attenuates NO-induced oxidative damage with aging through regulation of the immune response.

To investigate the effects on ·OH generation, we measured the urinary levels of creatinine (Cr) and methylguanidine (MG). Since MG is synthesized from Cr by ·OH, the MG/Cr ratio is an indicator of ·OH generation.[22-27] The administration of Kangen-karyu extract inhibited ·OH generation significantly (Table 1), implying its protective effect against ·OH. The above results suggest that Kangen-karyu scavenges O_2^-, NO, and ·OH, and thus, it is expected to display benefits against the aging process in this model.

To counteract oxidative stress from ROS, the human body has a defense system comprising antioxidative enzymes and antioxidants. Although the activities of antioxidative enzymes are not consistently related to anti-aging to prolong the lifespan, it is clear that antioxidative enzyme activities contribute to the prevention of oxidative damage. The result on the elevation of SOD activity by the administration of Kangen-karyu suggests that it has a protective effect against aging through protecting organisms against oxidative insults.[20]

Changes in the antioxidative defense status and oxidative damage by free radicals with aging are considered to induce the accumulation of oxidized intracellular biomolecules such as lipids, proteins, and DNA. Malondialdehyde (MDA) is often used as a biological marker of lipid peroxidation, though it is not a precise marker. Accordingly, the changes in MDA we observed in the present study may not be entirely due to age-related changes in lipid peroxidation *per se*. However, because of the increased levels of other markers we measured, the MDA

Table 1 Cr, MG, and MG/Cr ratio in urine

Age (weeks)	Group	Cr (mg/ml)	MG (μg/ml)	MG/Cr ($x10^{-3}$)
34	-	0.44 ± 0.04*	7.25 ± 0.38**	16.45 ± 0.30**
	Kangen-karyu	0.50 ± 0.05**	6.59 ± 0.22**,a	13.12 ± 0.26**,b
5	-	0.32 ± 0.03	1.05 ± 0.18	3.30 ± 0.32

*p<0.01, **p<0.001 vs. control values of 5 weeks of age; ap<0.05, bp<0.001 vs. control values of 34 weeks of age.

Table 2 MDA levels in hepatic and renal tissues

Age (weeks)	Group	Hepatic MDA (nmol/mg protein)	Renal MDA (nmol/mg protein
34	-	1.20 ± 0.06**	1.69 ± 0.06**
	Kangen-karyu	1.04 ± 0.03*,a	1.63 ± 0.08**
5	-	0.91 ± 0.07	1.32 ± 0.04

*p<0.01, **p<0.001 vs. control values of 5 weeks of age; ap<0.01 vs. control values of 34 weeks of age.

levels likely reflect the extent of oxidatively damaged lipids in SAM. Our data are consistent with other reports[28] showing increased lipid peroxidation due to the oxidative status in various tissues. Our study showed that the hepatic and renal tissue MDA concentrations at 34 weeks of age were significantly higher than those at 5 weeks of age (Table 2), indicating that the aging process makes these tissues more susceptible to oxidative stress, and that increased oxidative stress induces lipid peroxidation with aging in this animal model. In contrast, Kangen-karyu significantly reduced the hepatic MDA concentration, which was associated with the attenuation of oxidative stress through scavenging free radicals and/or enhancing the defense system. It should be noted that although lipid peroxidation increased both in the liver and kidney in these animals, Kangen-karyu decreased only hepatic MDA, and not renal MDA. The difference between different organs may possibly depend on how organs differentially process the accumulated MDA, as the liver has a higher capacity to metabolize MDA, while renal MDA is not readily metabolized.

A further study was carried out to elucidate how Kangen-karyu affects tissue dysfunctions associated with aging, since aging leads to several characteristics, such as changes in the biochemical composition and function of tissue, increased mortality after maturation, a progressive decline in physiological capacity, reduced ability to respond adaptively to environmental stimuli, and increased susceptibility and vulnerability to diseases.[29] Therefore, we investigated hepatic and renal functions by measuring serum levels of alanine amino transferase (ALT), and both urea nitrogen and Cr, respectively. Older SAMP resulted in functional alterations in the liver and kidney in this animal model, and Kangen-karyu attenuated these changes,[20] implying that it modulated these abnormalities of the liver and kidney of SAMP.

The changes with age in some of the parameters examined in this animal model are different from what we know of human aging. As was discussed above, NO generation increased with age in SAM, while in humans, it is known to decline with age. Serum ALT and urea nitrogen levels, which both increased with age in SAM, do not increase in humans during the normal aging process. Further, the selection of animal age, especially young animals (5 weeks of age) may not be optimal to examine age-associated changes in this animal model. With these limitations in mind, the antioxidative activities as demonstrated for these drugs need be carefully interpreted in terms of the human aging process.

Anti-aging activity of the Chinese prescription Kangen-karyu and its mechanisms in human lung fibroblasts

Among the various cell types, proliferative cells, such as human diploid fibroblasts (HDFs), melanocytes, lymphocytes, and retinal pigment epithelial cells, display typical replicative senescence (RS). In particular, the HDFs first described by Hayflick and Moorhead[30] have become a classical experimental model of cellular aging and have been used to study aging-associated molecular changes in human cells. After serial passage, WI-38 human lung fibroblast cells, which are HDFs, lose the ability to proliferate and become senescent, showing cellular changes related to the aging process.[31-36] In addition, HDFs, including WI-38 cells, exhibit the SIPS phenotype after being subjected to many different sub-lethal stresses, including oxidative stress,[37] and this SIPS phenotype is almost identical to the phenotype associated with RS. In particular, Wolf *et al.*[38] reported that H_2O_2-treated WI-38 cells showed changes indicative of increased oxidative DNA damage, such as elevated 8-hydroxy-2'-deoxyguanosine levels, senescence-associated β-galactosidase (SA-β-Gal) activity, and G_0/G_1 cell cycle arrest, indicating RS of the cells. Consistent with these pieces of evidence, our results also showed that H_2O_2-treated WI-38 cells exhibited cellular senescence due to increased oxidative damage. These findings indicate that SIPS of WI-38 cells caused by H_2O_2 is a useful and reasonable cellular aging model for evaluating the anti-aging effects of agents that counteract oxidative stress. We used this well-established model to evaluate the anti-aging effects of Kangen-karyu extract and focused on the antioxidant potential and mechanisms responsible for the anti-aging activity of Kangen-karyu extract.

One of the remarkable changes of HDFs undergoing cellular aging is a senescence-like morphological change almost identical to that observed with SIPS.[39-41] In addition, several studies demonstrated that SA-β-Gal activity increases dramatically during RS both *in vitro* and *in vivo*.[42] Consistent with these findings, HDFs exposed to H_2O_2 displayed stress-induced morphological changes indicative of premature senescence. Moreover, the SA-β-Gal activity was elevated in HDFs exposed to H_2O_2 (Fig. 1). However, Kangen-karyu extract exerted a protective effect against these morphological changes associated with cellular aging and inhibited the H_2O_2-induced increase in SA-β-Gal activity. These results suggest that Kangen-karyu would prevent H_2O_2-induced cellular senescence of HDFs.

The characteristics of RS of HDFs include G_0/G_1 phase arrest of the cell cycle.[38] Our present results also showed that G_0/G_1 phase arrest of WI-38 cells resulted from H_2O_2-induced oxidative stress, whereas treatment with Kangen-karyu extract, which attenuated the oxidative status, normalized the cell cycle by decreasing the proportion of cells in the G_0/G_1 phase (Table 3), implying that the quiescent state of cells including growth arrest is closely related to cell cycle arrest. Our previous report also demonstrated that SIPS caused by H_2O_2 increased intracellular ROS generation.[43,44] In contrast, pretreatment of Kangen-karyu extract to WI-38 cells under conditions of SIPS decreased ROS generation, thereby reducing oxidative stress. Moreover, Kangen-karyu extract reduced the magnitude of the level of lipid peroxidation elevation and it was associated with the attenuation of oxidative stress. These results clearly indicate that Kangen-karyu can prevent H_2O_2-induced growth arrest of HDFs via preventing G_0/G_1 phase arrest under cell cycle distribution as well as reducing ROS

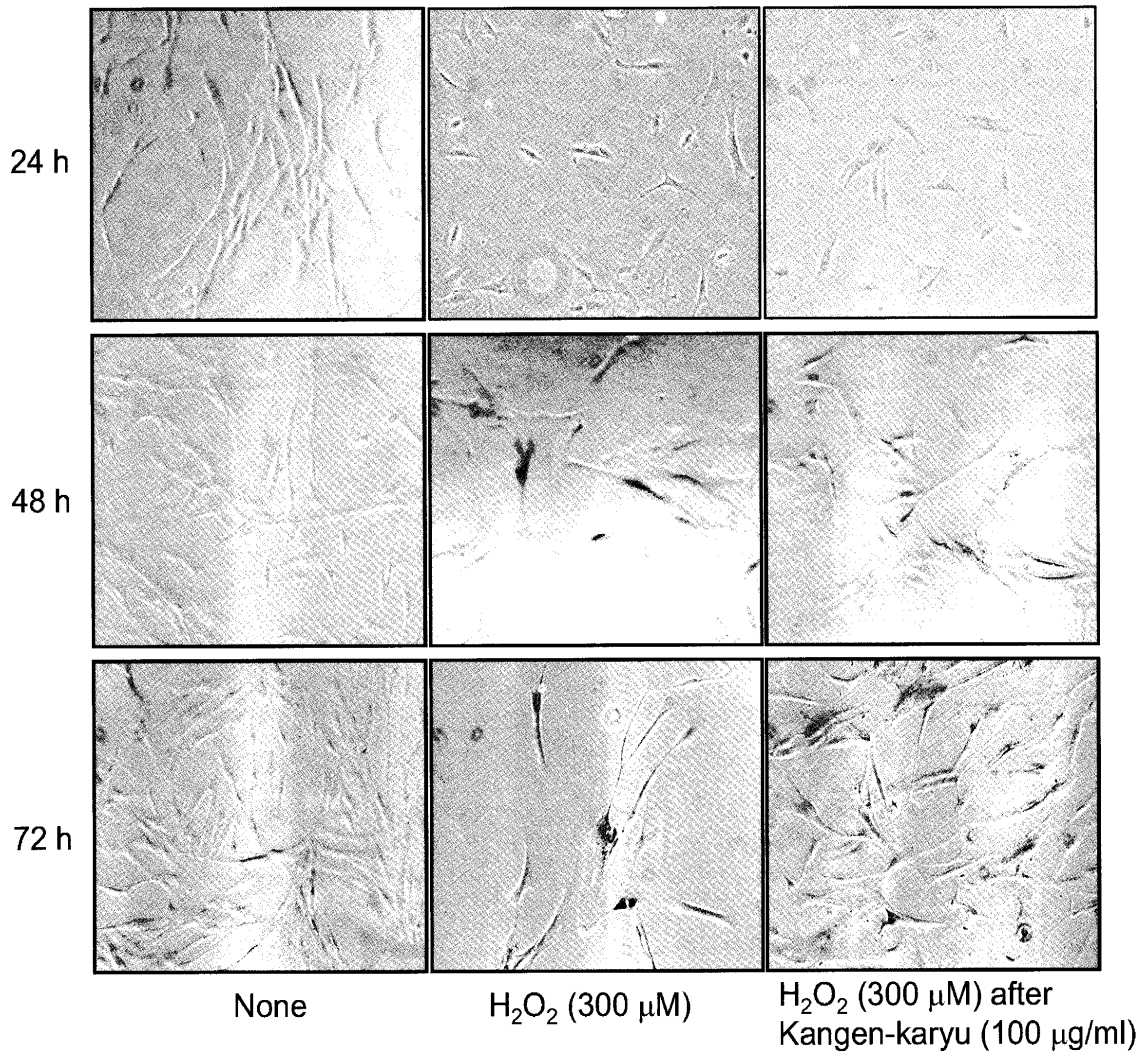

Fig. 1 H_2O_2 induced SA-β-Gal activities. HDFs were pretreated with vehicle (BME media) or Kangen-karyu (100 μg/ml) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were incubated with fresh media for 24, 48, and 72 h, and the cell morphology was determined by SA-β-Gal staining and microscopy.

Table 3 Effect of Kangen-karyu extract on cell cycle

	Percentage of cells in each phase of cell cycle (%)		
	G_0/G_1	S	G_2/M
None	46.1 ± 1.4	30.7 ± 1.4	23.2 ± 0.3
H_2O_2 (300 μM)	54.3 ± 1.4$^{\#\#\#}$	23.0 ± 1.7$^{\#\#}$	22.4 ± 0.4
H_2O_2 (300 μM) plus Kangen-karyu extract (10 μg/ml)	53.3 ± 1.5$^{\#\#\#}$	23.1 ± 1.9$^{\#\#}$	23.6 ± 0.5*
H_2O_2 (300 μM) plus Kangen-karyu extract (100 μg/ml)	49.5 ± 1.7$^{\#,**}$	29.8 ± 2.4**	20.7 ± 0.9$^{\#\#,*}$

$^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. no treatment values; $^{*}p<0.05$, $^{**}p<0.01$ vs. H_2O_2 treatment values.

generation and oxidative damage.

The enhancement of oxidative stress by several factors, including ROS generation, results in a loss of cell viability.[45] The viability of WI-38 cells was reduced by H_2O_2-induced oxidative damage.[44] However, pre-treatment of Kangen-karyu extract improved cell viability by protecting against H_2O_2-induced oxidative damage through the decrease in ROS generation and thiobarbituric acid (TBA)-reactive substance levels. In addition, H_2O_2-induced HDFs lose the ability to proliferate and then reach premature senescence, whereas Kangen-karyu extract treatment prolonged the lifespan of HDF cells (Fig. 2). This indicates that Kangen-karyu has an anti-aging potential to extend longevity. Several studies have demonstrated a positive correlation between an organism's cellular lifespan and its longevity. The proliferative lifespan of fibroblasts decreased with aging and fibroblasts derived from patients with syndromes of premature aging, such as Werner's syndrome and Hutchinson-Gilford syndrome, had a reduced lifespan *in vitro*.[46-50] Therefore, the present finding of the

increased longevity of WI-38 cells by Kangen-karyu suggests that this prescription might prolong not only the lifespan of cells *in vitro* but also the longevity of the whole organism.

To investigate the related mechanisms to cellular anti-aging activity, nuclear factor-kappa B (NF-κB) translocation to the nucleus was observed. NF-κB is a powerful transcriptional factor that plays a pivotal role in the regulation of a number of immune and inflammatory response genes and in the activation of several cellular promoters. In unstimulated cells, the NF-κB dimer is present in the cytosol as an active complex with the inhibitory protein IκB. In response to cell stimulation with agents such as phorbol esters,[51] tumor necrosis factor-α,[52-54] interleukin-1,[55,56] UV light,[57,58] hypoxia,[59] lipopolysaccharide,[60] and H_2O_2, the NF-κB dimer dissociates from IκB and translocates to the nucleus, which is followed by NF-κB activation. Our present study demonstrated that HDFs under the condition of SIPS caused by H_2O_2 showed NF-κB translocation to the nucleus from the cytosol, suggesting that cells undergoing premature RS may display NF-κB translocation following oxidative stress. In addition, oxidative stress was demonstrated to contribute to the age-related increase of NF-κB *in vivo*. Therefore, the prevention of NF-κB translocation resulting from oxidative stress we observed suggests that Kangen-karyu exerts an anti-aging effect through NF-κB modulation (Fig. 3). The translocation into the nucleus and activation of NF-κB induce the expression of several proteins, such as cyclooxygenase (COX), inducible nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1), bax, and bcl-2.[61] Our previous study also demonstrated that Kangen-karyu extract regulates related proteins to NF-κB modulation.[43] It prevented the overexpression of HO-1 and bax proteins induced by H_2O_2, suggesting that the Kangen-karyu extract regulates the expression of these proteins.

Among the theories of aging, the mitochondrial theory proposes that oxidative stress leads to injury of the genome and mitochondrial membranes of somatic differentiated cells,[2] and that such injured cells display a decline in the mitochondrial membrane potential.[62] The fluorescent dye rhodamine 123 is frequently used as a mitochondrial membrane potential probe because it is well characterized, causes

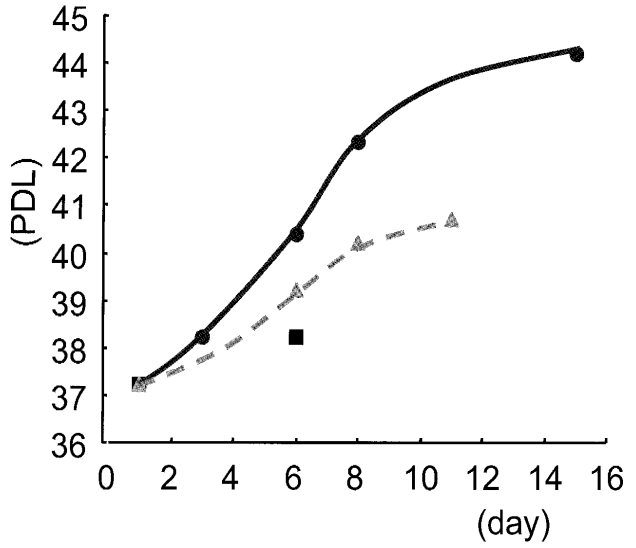

Fig. 2 H_2O_2 induced PDL of HDFs. None, ●; H_2O_2 treatment, ■; H_2O_2 plus Kangen-karyu extract treatment, △.

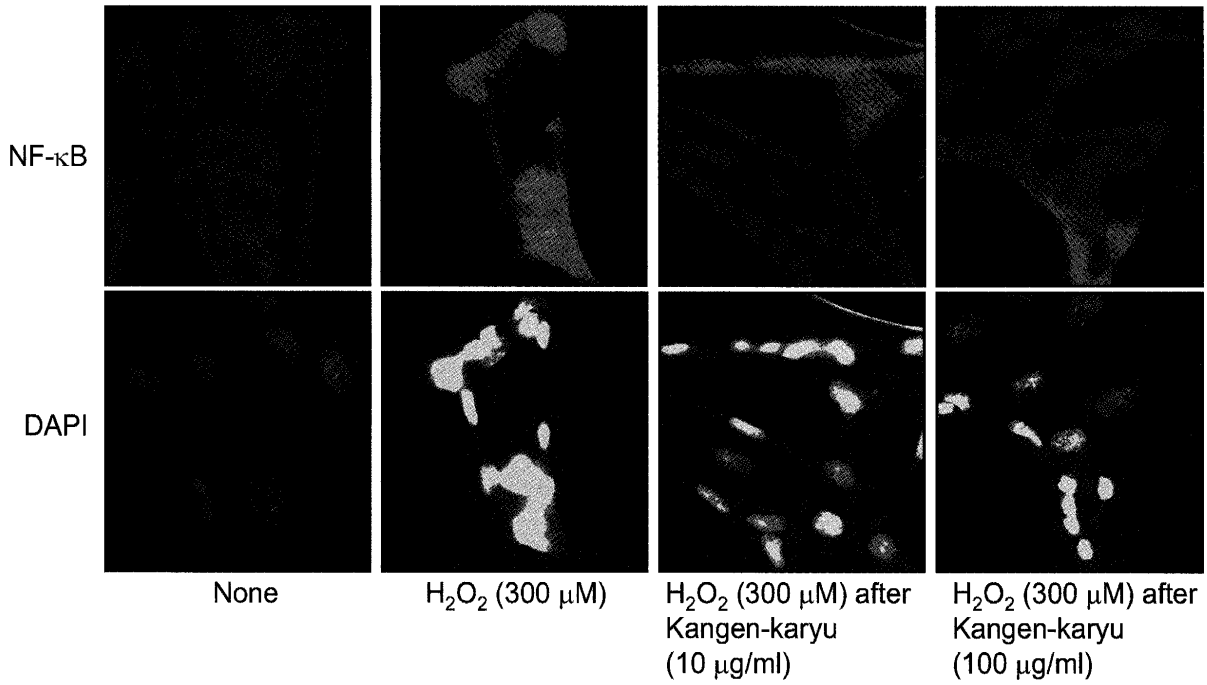

Fig. 3 H_2O_2 induced NF-κB translocations of HDFs. HDFs were pretreated with vehicle (BME media) or Kangen-karyu (10 and 100 μg/ml) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were incubated with fresh media for 0 h, and NF-κB translocations were determined by immunofluorescence and DAPI staining.

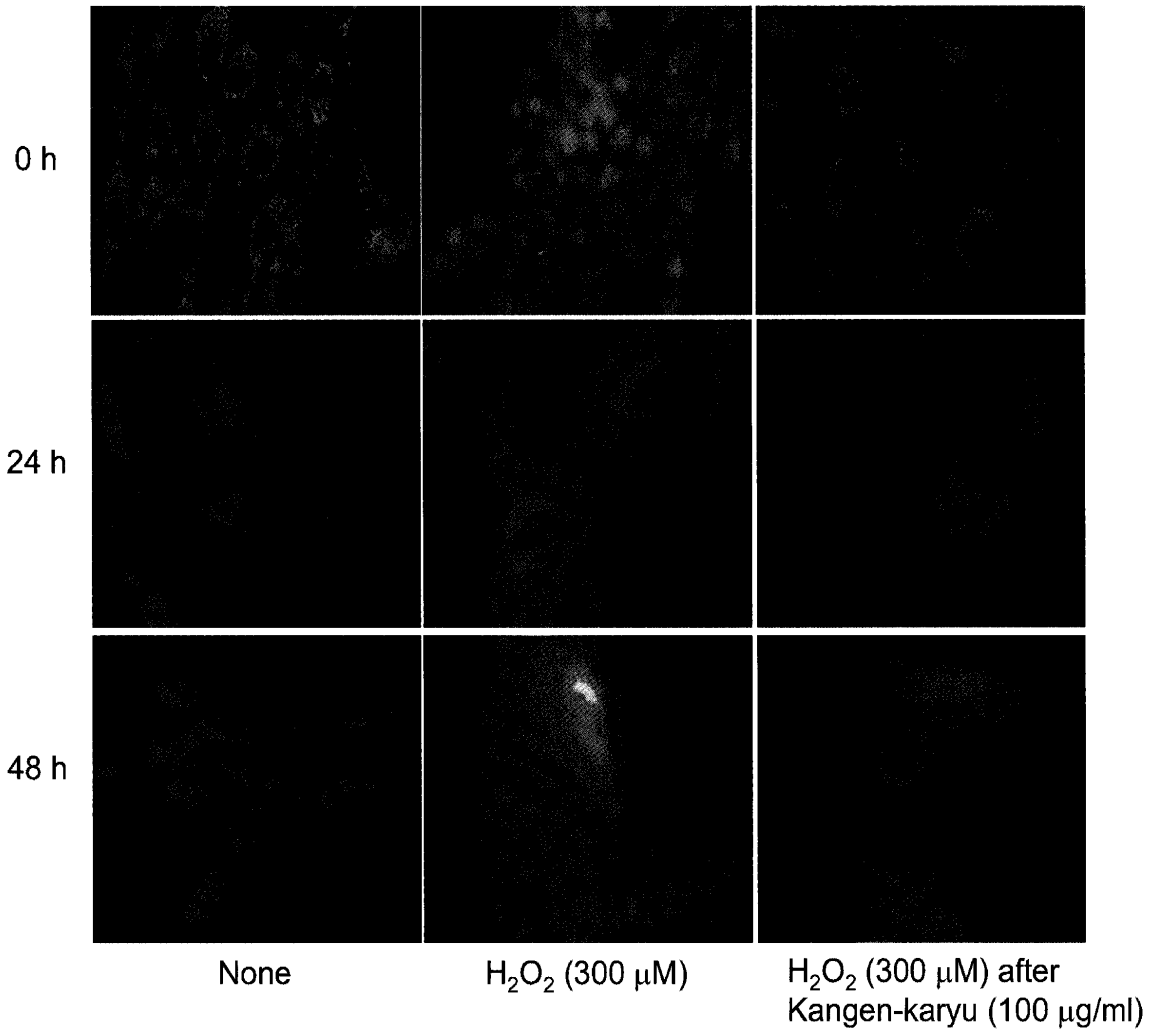

Fig. 4 H_2O_2 induced mitochondrial membrane potentials of HDFs. HDFs were pretreated with vehicle (BME media) or Kangen-karyu (100 μg/ml) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were incubated with fresh media for 0, 24, and 48 h, and mitochondrial membrane potentials were determined by rhodamine123 staining.

no loss of mitochondrial coupling, and is not toxic at low concentrations.[63] Moreover, dye accumulation and the resulting fluorescence intensity of cells are stable, allowing accurate measurement of cellular fluorescence characteristics. In the present study, H_2O_2-treated HDFs certainly showed increased rhodamine 123 fluorescence compared with untreated HDFs (Fig. 4). This is consistent with the results of Lee *et al.*,[45] who showed that premature senescence induced by H_2O_2 led to a decline of the mitochondrial membrane potential. However, Kangen-karyu extract prevented this decline, suggesting that Kangen-karyu extract protects mitochondria from mitochondrial oxidative stress and dysfunction.

Under the cellular senescence model induced by oxidative stress, Kangen-karyu extract showed an anti-aging activity through the attenuation of oxidative damage with the inhibition of lipid peroxidation and cell cycle regulation. Eventually, it could delay the cellular aging process with a protective effect against aging-related morphological changes. Furthermore, the present study also indicated that the anti-aging activity is attributable to the modulation of NF-κB and expression of its related proteins.

Anti-aging properties of the Chinese prescription Kangen-karyu and Tanjin in aging rats

In the previous section, we described the anti-aging activity of Kangen-karyu in an SAM model. We found that Kangen-karyu ameliorated tissue damage and oxidative stress associated with aging in the SAM model.[20] However, the SAM model is not exactly identical to the natural aging process, although it is considered to be a useful *in vivo* model for aging research. Therefore, the present section demonstrated how Kangen-karyu affects the aging process in a model more closely related to human aging using rats allowed to proceed along the natural aging process with time. The rodent model on aging research is the most frequently employed. It is known that the lifespan of rats is approximately 36 to 48 months; 7-10 weeks-old (natal), 3-6 months-old (young), 12 months-old (adult), and 36-38 months-old (old). By the free radical theory of aging, various oxidative reactions occurring in an organism (mainly in mitochondria) generate free radicals which cause multiple lesions in macromolecules, leading to damage and aging.[2] Therefore, we focused on the accumulation of damage

caused by oxidative stress and protein expressions during the aging process, and evaluated the anti-aging effects of Kangen-karyu and its crude drug, Tanjin. Nomura *et al.*[64] also reported that Tanjin, the main extract with the greatest amount of Kangen-karyu, effectively protects against learning deficits caused by aging in SAM. Accordingly, Tanjin extract is also expected to play a crucial role in retarding the aging process.

Biological macromolecules such as proteins, lipids, and nucleic acids are the targets of oxidative stress with age.[65] In particular, amino groups in proteins react non-enzymatically with reducing sugars to produce advanced glycation endproducts (AGEs). AGEs not only modify protein properties but also induce biological damage *in vivo*.[66] AGEs are irreversibly formed, accumulated with aging, arteriosclerosis, and diabetes mellitus, and are especially associated with long-lived proteins such as collagens.[67] An increase in serum AGEs with age is a risk marker of aging and age-related diseases.[68-70] On the other hand, Hunt *et al.*[71] have reported that in plasma samples, AGEs and protein glycation appeared to be linearly correlated. 5-Hydroxymethylfurfural (5-HMF) is involved in the non-enzymatic browning process and non-enzymatically bound glucose in serum is released as 5-HMF.[72,73] Therefore, we evaluated 5-HMF levels to determine the extent of serum glycosylated protein (Table 4). 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 under the present model. This suggests that Kangen-karyu and Tanjin would protect against protein modification and biological damage with aging. Our previous study also showed that both serum and hepatic levels of TBA-reactive substance at 12 months of age were significantly higher than those at 2 months of age, indicating that the aging process makes the tissues more susceptible to oxidative stress, and that this elevation of oxidative stress with aging induces lipid peroxidation (data not shown). In contrast, Kangen-karyu and Tanjin significantly decreased the levels of TBA-reactive substance in serum and hepatic tissue. These results indicate that Kangen-karyu and Tanjin attenuate oxidative stress under the *in vivo* rat model with the natural aging process.

The level of oxidized glutathione (GSSG) with age in hepatic tissues increased without changes in the reduced glutathione (GSH) level, and therefore the GSH/GSSG ratio dramatically decreased (Table 5). In addition, the 12-month-old rats showed higher GSH-Px activities, probably related to increased oxidative damage with age, while Kangen-karyu and Tanjin attenuated the GSH-Px activities of hepatic tissues against aging. The present results suggest that Kangen-karyu and Tanjin regulate the glutathione redox cycle to maintain the cellular redox condition against age-related oxidative stress. Miquel[65] reported that changes in the redox (GSH/GSSG) ratio are much more striking in the mitochondria than in the extramitochondrial compartment and lead to oxidative damage of mitochondrial DNA, which relates to mitochondrial dysfunction.[74] The effect of Kangen-karyu on the glutathione redox cycle with age would also probably be related to the amelioration of mitochondrial dysfunction.

The expressions 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 led to changes with aging.[61] When ROS was produced in mitochondria with aging, cytochrome *c* was dramatically released from mitochondria into the cytosol.[75] This is an early event in aging and age-related apoptosis, leading to the generation of O_2^{-}.[76] The present study demonstrated that the expression of cytosolic cytochrome *c* in 12-month-old rats increased compared with 2-month-old rats, but its increase was prevented in rats fed Kangen-karyu and Tanjin

Table 4 Glycosylated protein level in serum

Age (months)	Group	Glycosylated protein (nmol/mg protein)
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$^{\#\#}$

$^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. control values of 2 months of age;
$^{*}p<0.001$ vs. control values of 12 months of age.

Table 5 GSH and GSSG levels, GSH/GSSH ratio, and GSH-Px activity in hepatic tissues

Age (months)	Group	GSH (mmol/g tissue)	GSSG (mmol/g tissue)	GSH/GSSG ratio	GSH-Px (unit/mg protein)
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$^{\#\#}$

$^{\#}p<0.01$, $^{\#\#}p<0.001$ vs. control values of 2 months of age; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control values of 12 months of age.

extracts (Fig. 5). A stronger effect with Kangen-karyu than Tanjin was observed, suggesting that the effect of the prescription 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 aging.[77,78] 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 aging. 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 (Fig. 6). 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,[79] and increases the transcription of genes that encode protective enzymes such as iNOS and COX-2.[61] The present result showed that the level of bcl-2 expression in 12-month-old rats showed a tendency to increase compared with that of 2-month-old rats, and the level of bax protein increased with aging (Fig. 5). Consistent with the present study, similar observations of the overexpression of bax and reduced expression of bcl-2 with aging have been well demonstrated.[80-82] The increase in the bax/bcl-2 ratio with aging by other studies indicates the possible role of the mitochondrial bax/bcl-2 apoptotic signaling pathway in aging.[83,84] Kangen-karyu and Tanjin extracts prevented the changes in bcl-2 and bax protein expressions with aging, implying that Kangen-karyu and Tanjin would play crucial roles in protecting against mitochondrial dysfunction with aging through the regulation of bax and bcl-2 protein levels. Furthermore, these changes enhance the release of cytochrome *c* from mitochondria into the cytosol and lead to mitochondrial dysfunction with age, as reported by Bernardi

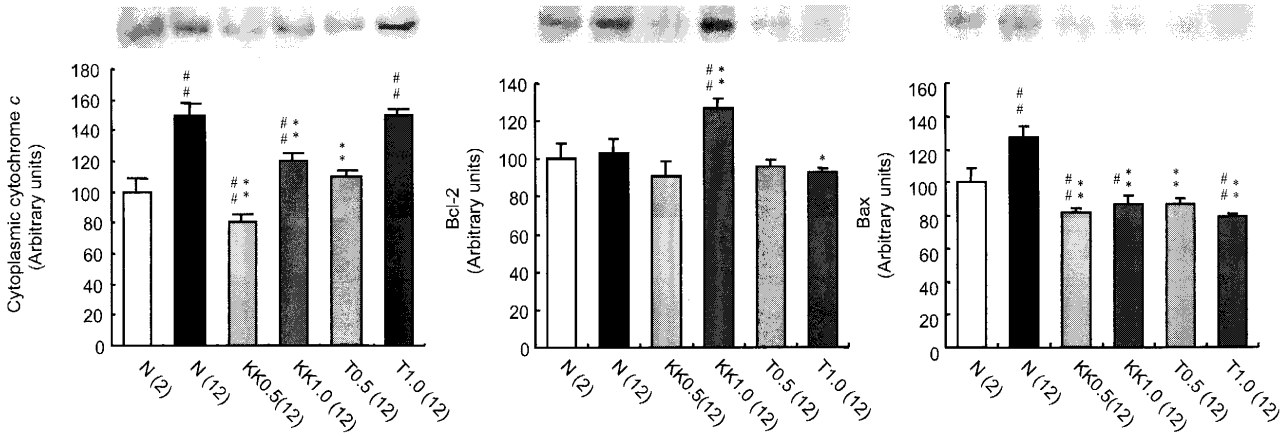

Fig. 5 Effect of Kangen-karyu on cytoplasmic cytochrome *c*, bcl-2, and bax proteins of aged rats. The protein levels were quantified by densitometry. #p<0.01, ##p<0.001 vs. control values of 2 months of age; *p<0.05, **p<0.001 vs. control values of 12 months of age.

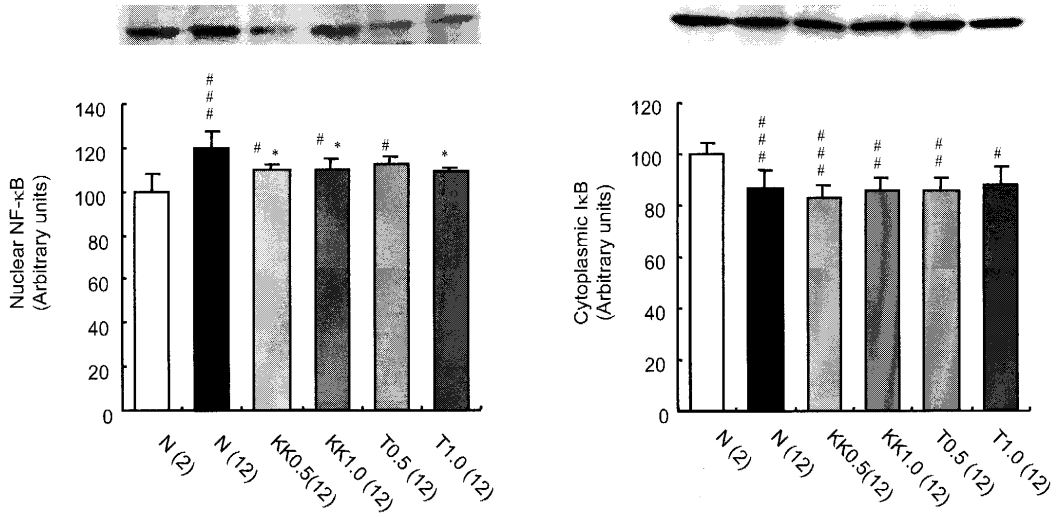

Fig. 6 Effect of Kangen-karyu on nuclear NF-κB and cytoplasmic IκB proteins of aged rats. The protein levels were quantified by densitometry. #p<0.05, ##p<0.01, ###p<0.001 vs. control values of 2 months of age; *p<0.05 vs. control values of 12 months of age.

et al.[85] Further study is also needed on the comparison of protein expression of cytoplasmic bax with that of the mitochondrial fraction under this model for the elucidation of the role of bax protein in the aging process.

Feng *et al.*[86] reported that ROS induces the expression of COX-2 protein, the key enzyme in proinflammatory prostanoid synthesis, and COX-2 is induced readily by cytokines, hormones, growth factors, and tumor promoters in selected tissues.[87,88] iNOS is also readily inducible by proinflammatory cytokines, and has a close relationship with ROS generation, and the induction of its gene expression increases in aged mice and rats.[3] In addition, HO-1 is ubiquitously expressed and is inducible, which degrades heme to free iron and a prooxidant. It also contributes to the antioxidant defense mechanisms of the cells, and enhanced oxidative stress during aging is accompanied by a compensatory induction of HO-1.[89] The present results demonstrated that COX-2, iNOS, and HO-1 protein expressions in 12-month-old rats were dramatically increased compared with 2-month-old rats. Although Kangen-karyu extract administration did not lead to changes in COX-2 protein expression, iNOS and HO-1 protein expressions were reduced significantly by treatment with this prescription (Fig. 7). On the other hand, Tanjin extract dramatically reduced the expressions of COX-2, iNOS, and HO-1 proteins.

This study demonstrated that the aging process over time resulted in increases in protein glycation and lipid peroxidation, and functional alterations in the liver with changes in related protein expressions, whereas Kangen-karyu and Tanjin attenuated these changes, implying that they modulate these abnormalities caused by aging. Furthermore, the present study indicated that Kangen-karyu and Tanjin exert anti-aging activities under the *in vivo* aging model with 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.

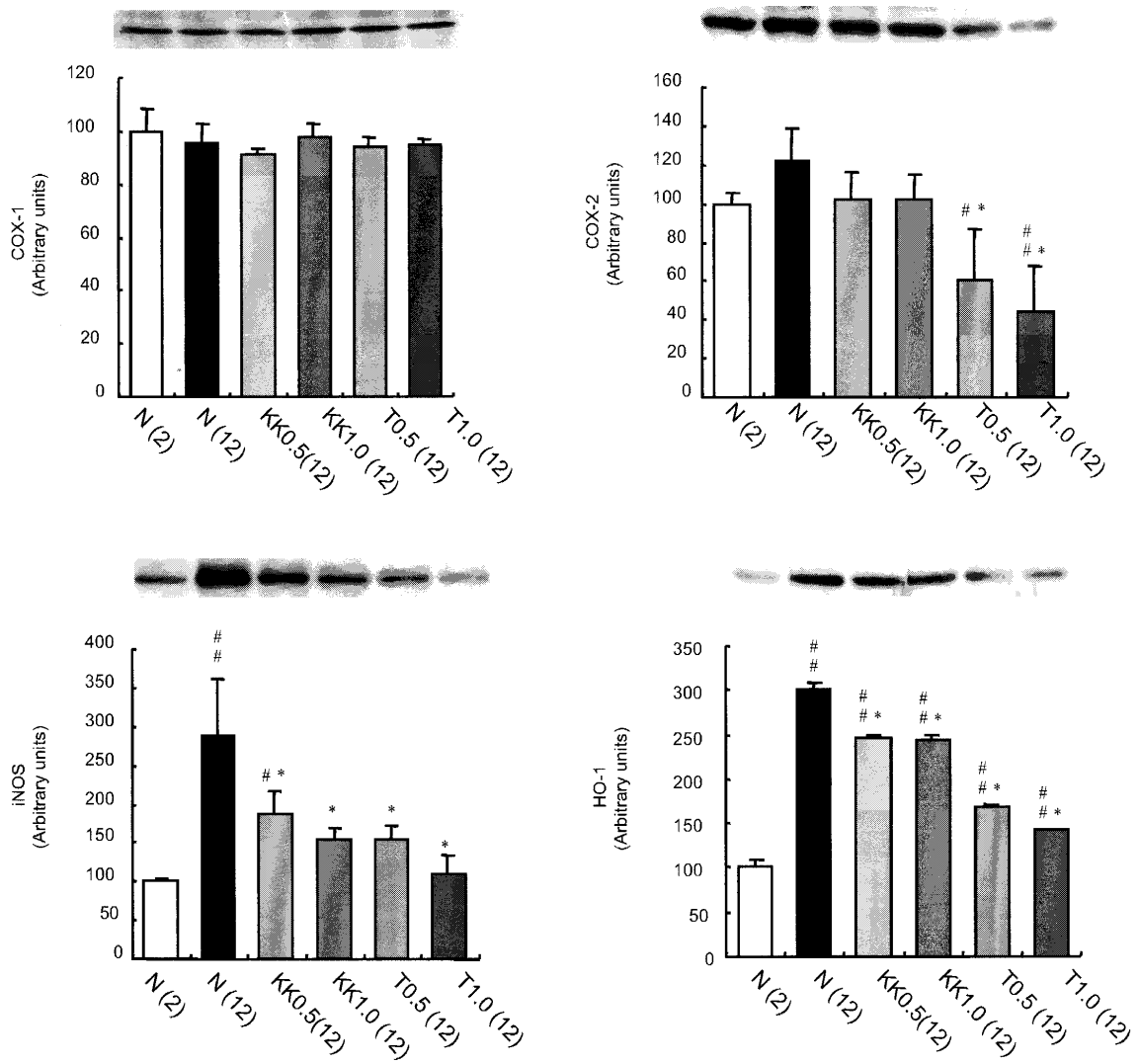

Fig. 7 Effect of Kangen-karyu on COX-1, COX-2, iNOS, and HO-1 proteins of aged rats. The protein levels were quantified by densitometry. #p<0.01, ##p<0.001 vs. control values of 2 months of age; *p<0.001 vs. control values of 12 months of age.

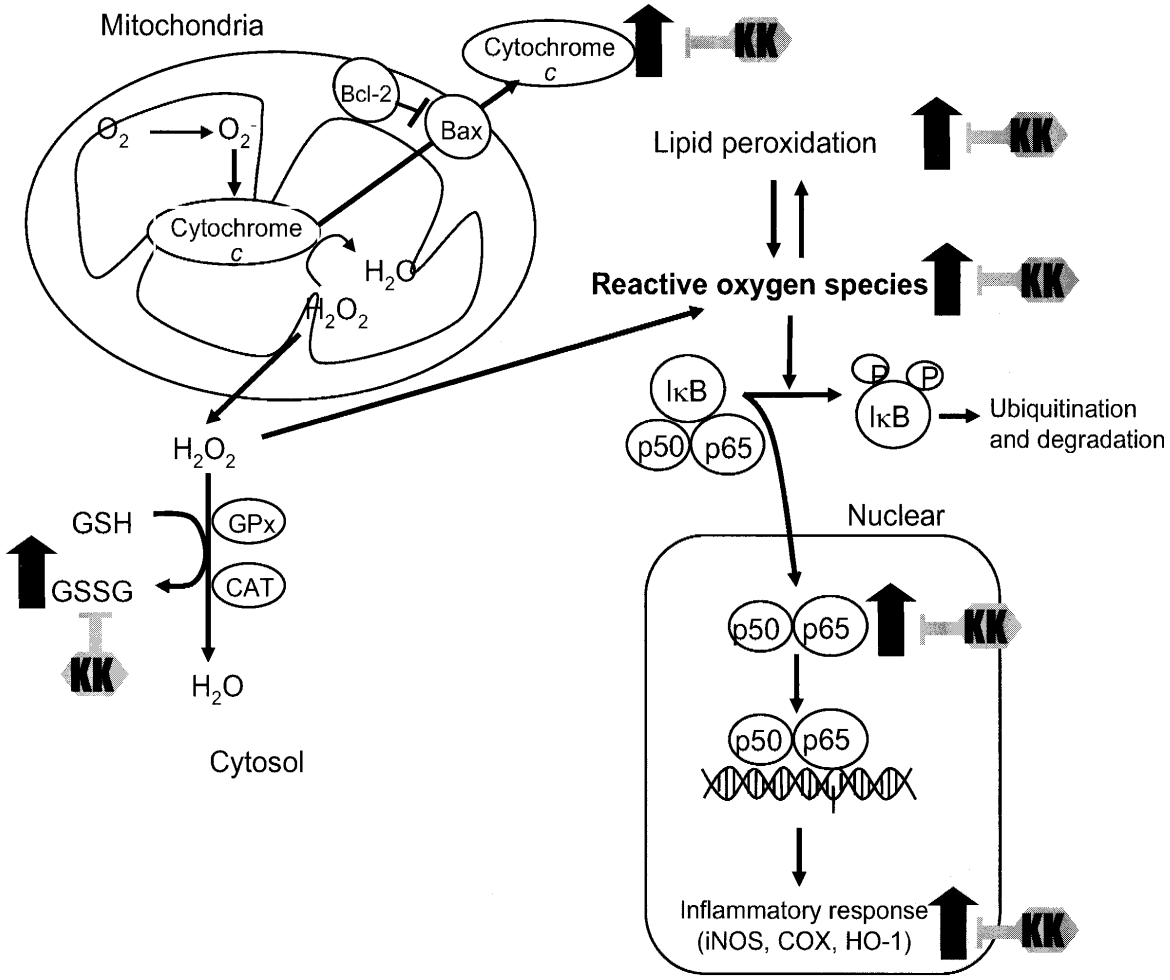

Fig. 8 Schematic overview of the action mechanism of Kangen-karyu against oxidative stress-induced aging.

On the other hand, Tanjin did not have a remarkable role in protecting mitochondria from aging compared to Kangen-karyu, whereas it attenuated the protein expressions of inducible genes such as COX-2, iNOS, and HO-1. Tanjin is attributed to the synergistic and/or additive anti-aging effect of Kangen-karyu.

Conclusion

The serial studies on the anti-aging potential of Kangen-karyu under *in vivo* aging models and the SIPS cellular system indicated that Kangen-karyu exerted anti-aging properties through its attenuation of oxidative stress with aging. Furthermore, we could propose a schematic overview of the possible action mechanisms of Kangen-karyu against oxidative stress-induced aging (Fig. 8). The aging process increases oxidative stress such as ROS generation and accumulation of oxidative damage in tissue/cells. Oxidative stress induces NF-κB translocation from the cytosol to the nucleus (activation of NF-κB), leading to an increase in the translocation of genes related to the proinflammatory reaction such as COX-2 and iNOS. These proteins have a close relationship with ROS generation. Furthermore, these changes enhance the release of cytochrome *c* from mitochondria into the cytosol through the regulation of bax and bcl-2 proteins, eventually leading to mitochondrial dysfunction with aging. However, Kangen-karyu works mainly to maintain mitochondrial function against the leakage of ROS from mitochondria and led to the inhibition of NF-κB nuclear translocation and regulation of related protein expressions such as COX-2, iNOS, HO-1, bax, and bcl-2 proteins. In addition, Kangen-karyu regulates the glutathione redox cycle that maintains the cellular redox condition against age-related oxidative stress. This supports Kangen-karyu as a promising anti-aging agent.

Acknowledgment

We thank Professor Byung Pal Yu of The University of Texas Health Science Center at San Antonio for the helpful comments.

References

1) Harman, D.: The aging process. *Proc. Natl. Acad. Sci. USA* **78**, 7124-7128, 1981.
2) Beckman, K.B., and Ames, B.N.: The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581, 1998.
3) Kim, J.M., Araki, S., Kim, D.J., Park, C.B., Takasuka, N., Baba-Toriyama, H., Ota, T., Nir, Z., Khachik, F., Shimidzu, N., Tanaka, Y.,

Osawa, T., Uraji, T., Murakoshi, M., Nishino, H., and Tsuda, H.: Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis* **19**, 81-85, 1998.

4) Inoue, M., Watanabe, N., and Matsuo, K.: Inhibition of oxygen toxicity by targeting superoxide dismutase to the endothelial cell surface. *FEBS Lett.* **269**, 89-92, 1990.
5) Kondo, K., Matsumoto, A., Kurata, H., Tanahashi, H., Koda, H., Amachi, T., and Itakura, H.: Inhibition of oxidation of low-density lipoprotein with red wine. *Lancet* **344**, 1152, 1994.
6) Yokozawa, T., Liu, Z.W., and Dong, E.: A study of ginsenoside-Rd in a renal ischemia-reperfusion model. *Nephron* **78**, 201-206, 1998.
7) Sano, M., Ernesto, C., Thomas, R.G., Klauber, M.R., Schafer, K., Grundman, M., Woodbury, P., Growdon, J., Cotman, C.W., Pfeiffer, E., Schneider, L.S., and Thal, L.J.: A controlled trial of selegiline, α-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N. Engl. J. Med.* **336**, 1216-1222, 1997.
8) Takahashi, H.: Clinical trial of prescription of Kaketsukao. *Clin. J. Chinese Med.* **12**, 145-151, 1991.
9) Gao, M., Ikeda, K., Noguchi, T., Mori, K., and Yamori, Y.: Studies on the preventive effect of 'Kangen-karyu', Chinese herbal medicine, on stroke in SHR-SP. *J. Trad. Med.* **18**, 245-250, 2001.
10) Makino, T., Wakushima, H., Okamoto, T., Okukubo, Y., Saito, K., and Kano, Y.: Effects of Kangen-karyu on coagulation system and platelet aggregation in mice. *Biol. Pharm. Bull.* **25**, 523-525, 2002.
11) Makino, T., Wakushima, H., Okamoto, T., Okukubo, Y., Deguchi, Y., and Kano, Y.: Pharmacokinetic interactions between warfarin and Kangen-karyu, a Chinese traditional herbal medicine, and their synergistic action. *J. Ethnopharmacol.* **82**, 35-40, 2002.
12) Takahashi, M., Sugaya, K., and Kubota, K.: Kangen-karyu prevents the decrease of cholinergic markers following the nucleus basalis magnocellularis lesion. *Jpn. J. Pharmacol.* **60**, 307-310, 1992.
13) Kuro-o, M.: Disease model: human aging. *Trends. Mol. Med.* **7**, 7179-7181, 2001.
14) Takeda, T., Hosokawa, M., and Higuchi, K.: Senescence-accelerated mouse (SAM): a novel murine model of senescence. *Exp. Gerontol.* **32**, 105-109, 1997.
15) Hosokawa, M., Abe, T., Higuchi, K., Shimakawa, K., Omori, Y., Matsushita, T., Kogushi, K., Deguchi, E., Kishimoto, Y., Yasuoka, K., and Takeda, T.: Management and design of the maintenance of SAM mouse strains: an animal model for accelerated senescence and age-associated disorders. *Exp. Gerontol.* **32**, 111-116, 1997.
16) Takeda, T.: Senescence-accelerated mouse (SAM): a biogerontological resource in aging research. *Neurobiol. Aging* **20**, 105-110, 1999.
17) Boldyrev, A.A., Yuneva, M.O., Sorokina, E.V., Kramarenko, G.G., Fedorova, T.N., Konovalova, G.G., and Lankin, V.Z.: Antioxidant systems in tissues of senescence accelerated mice. *Biochemistry* **66**, 1157-1163, 2001.
18) Nakahara, H., Kanno, T., Inai, Y., Utsumi, K., Hiramatsu, M., Mori, A., and Packer, L.: Mitochondrial dysfunction in the senescence accelerated mouse (SAM). *Free Radic. Biol. Med.* **24**, 85-92, 1998.
19) Sohal, R.S., Ku, H.H., Agarwal, S., Forster, M.J., and Lai, H.: Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mech. Ageing Dev.* **74**, 121-133, 1994.
20) Satoh, A., Yokozawa, T., Cho, E.J., Okamoto, T., and Sei, Y.: Antioxidative effects related to the potential anti-aging properties of the Chinese prescription Kangen-karyu and Carthami Flos in senescence-accelerated mice. *Arch. Gerontol. Geriatr.* **39**, 69-82, 2004.
21) Yao, H.W., Li, J., Jin, Y., Zhang, Y.F., Li, C.Y., and Xu, S.Y.: Effect of leflunomide on immunological liver injury in mice. *World J. Gastroenterol.* **9**, 320-323, 2003.
22) Yokozawa, T., Fujitsuka, N., and Oura, H.: Production of methylguanidine from creatinine in normal rats and rats with renal failure. *Nephron* **56**, 249-254, 1990.
23) Ienaga, K., Nakamura, K., Yamakawa, M., Toyomaki, Y., Matsuura, H., Yokozawa, T., Oura, H., and Nakano, T.: The use of ^{13}C-labelling to prove that creatinine is oxidized by mammals into creatol and 5-hydroxy-1-methylhydantoin. *J. Chem. Soc. Chem. Commun.* 509-510, 1991.
24) Nakamura, K., Ienaga, K., Yokozawa, T., Fujitsuka, N., and Oura, H.: Production of methylguanidine from creatinine via creatol by active oxygen species. Analyses of the catabolism *in vitro*. *Nephron* **58**, 42-46, 1991.
25) Yokozawa, T., Fujitsuka, N., and Oura, H.: Studies on the precursor of methylguanidine in rats with renal failure. *Nephron* **58**, 90-94, 1991.
26) Yokozawa, T., Fujitsuka, N., Oura, H., Mori, A., and Kashiwagi, H.: Determination of radical species in the kidney of rats with chronic renal failure by the spin trapping method. *Nephron* **70**, 382-384, 1995.
27) Yokozawa, T., Fujitsuka, N., Oura, H., Ienaga, K., and Nakamura, K.: *In vivo* effect of hydroxyl radical scavenger on methylguanidine production from creatinine. *Nephron* **75**, 103-105, 1997.
28) Matsugo, S., Kitagawa, T., Minami, S., Esashi, Y., Oomura, Y., Tokumaru, S., Kojo, S., Matsushima, K., and Sasaki, K.: Age-dependent changes in lipid peroxide levels in peripheral organs, but not in brain, in senescence-accelerated mice. *Neurosci. Lett.* **278**, 105-108, 2000.
29) Troen, B.R.: The biology of aging. *Mt. Sinai J. Med.* **70**, 3-22, 2003.
30) Hayflick, L., and Moorhead, P.S.: The several cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585-621, 1961.
31) Hayflick, L.: The cell biology of human aging. *N. Engl. J. Med.* **295**, 1302-1308, 1976.
32) Harley, C.B.: Telomere loss: mitotic clock or genetic time bomb? *Mutat. Res.* **256**, 271-282, 1991.
33) Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacock, M., and Campisi, J.: A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl. Acad. Sci. USA* **92**, 9363-9367, 1995.
34) Linskens, M.H., Harley, C.B., West, M.D., Campisi, J., and Hayflick, L.: Replicative senescence and cell death. *Science* **267**, 17, 1995.
35) Campisi, J., Dimri, G.P., Nehlin, J.O., Testori, A., and Yoshimoto, K.: Coming of age in culture. *Exp. Gerontol.* **31**, 7-12, 1996.
36) Campisi, J.: Cancer, aging and cellular senescence. *In Vivo* **14**, 183-188, 2000.
37) Toussaint, O., Medrano, E.E., and von Zglinicki, T.: Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp. Gerontol.* **35**, 927-945, 2000.
38) Wolf, F.I., Torsello, A., Covacci, V., Fasanella, S., Montanari, M., Boninsegna, A., and Cittadini, A.: Oxidative DNA damage as a marker of aging in WI-38 human fibroblasts. *Exp. Gerontol.* **37**, 647-656, 2002.
39) Chen, Q., and Ames, B.N.: Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc. Natl. Acad. Sci. USA* **91**, 4130-4134, 1994.
40) Frippiat, C., Chen, Q.M., Zdanov, S., Magalhaes, J.P., Remacle, J., and Toussaint, O.: Subcytotoxic H_2O_2 stress triggers a release of transforming growth factor-beta 1, which induces biomarkers of cellular senescence of human diploid fibroblasts. *J. Biol. Chem.* **276**, 2531-2537, 2001.
41) Frippiat, C., Dewelle, J., Remacle, J., and Toussaint, O.: Signal transduction in H_2O_2-induced senescence-like phenotype in human diploid fibroblasts. *Free Radic. Biol. Med.* **33**, 1334-1346, 2002.
42) Wang, Y., Meng, A., and Zhou, D.: Inhibition of phosphatidylinostol 3-kinase uncouples H_2O_2-induced senescent phenotype and cell cycle arrest in normal human diploid fibroblasts. *Exp. Cell Res.* **298**, 188-196, 2004.

43) Satoh, A., Yokozawa, T., Kim, Y.A., Cho, E.J., Okamoto, T., and Sei, Y.: The mechanisms underlying the anti-aging activity of the Chinese prescription Kangen-karyu in hydrogen peroxide-induced human fibroblasts. *J. Pharm. Pharmacol.* **57**, 1335-1343, 2004.
44) Satoh, A., Yokozawa, T., Tanaka, T., Okamoto, T., and Sei, Y.: The antioxidative activity of Kangen-karyu extract delays senescence of human lung fibroblasts. *J. Trad. Med.* **21**, 87-93, 2004.
45) Lee, H.C., Yin, P.H., Lu, C.Y., Chi, C.W., and Wei, Y.H.: Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. *Biochem. J.* **348**, 425-432, 2000.
46) Hayflick, L.: Current theories of biological aging. *Fed. Proc.* **34**, 9-13, 1975.
47) Allsopp, R.C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E.V., Futcher, A.B., Greider, C.W., and Harley, C.B.: Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**, 10114-10118, 1992.
48) Oshima, J., Campisi, J., Tannock, T.C., and Martin, G.M.: Regulation of c-fos expression in senescing Werner syndrome fibroblasts differs from that observed in senescing fibroblasts from normal donors. *J. Cell Physiol.* **162**, 277-283, 1995.
49) Adelfalk, C., Lorenz, M., Serra, V., von Zglinicki, T., Hirsch-Kauffmann, M., and Schweiger, M.: Accelerated telomere shortening in Fanconi anemia fibroblasts-a longitudinal study. *FEBS Lett.* **506**, 22-26, 2001.
50) Yegorov, Y.E., and Zelenin, A.V.: Duration of senescent cell survival *in vitro* as a characteristic of organism longevity, an additional to the proliferative potential of fibroblasts. *FEBS Lett.* **541**, 6-10, 2003.
51) Sen, R., and Baltimore, D.: Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. *Cell* **47**, 921-928, 1986.
52) Baeuerle, P.A., and Henkel, T.: Function and activation of NF-kappa B in the immune system. *Ann. Rev. Immunol.* **12**, 141-179, 1994.
53) Siebenlist, U., Franzoso, G., and Brown, K.: Structure, regulation and function of NF-kappa B. *Ann. Rev. Cell Biol.* **10**, 405-455, 1994.
54) Baldwin, A.S. Jr.: The NF-kappa B and I kappa B proteins: new discoveries and insights. *Ann. Rev. Immunol.* **14**, 649-683, 1996.
55) Beg, A.A., Finco, T.S., Nantermet, P.V., and Baldwin, A.S. Jr.: Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol. Cell. Biol.* **13**, 3301-3310, 1993.
56) DiDonato, J.A., Mercurio, F., and Karin, M.: Phosphorylation of I kappa B alpha precedes but is not sufficient for its dissociation from NF-kappa B. *Mol. Cell. Biol.* **15**, 1302-1311, 1995.
57) Stein, B., Kramer, M., Rahmsdorf, H.J., Ponta, H., and Herrlich, P.: UV-induced transcription from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat and UV-induced secretion of an extracellular factor that induces HIV-1 transcription in nonirradiated cells. *J. Virol.* **63**, 4540-4544, 1989.
58) Stein, B., Rahmsdorf, H.J., Steffen, A., Litfin, M., and Herrlich, P.: UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-fos, and metallothionein. *Mol. Cell. Biol.* **9**, 5169-5181, 1989.
59) Schmedtje, J.F. Jr., Ji, Y.S., Liu, W.L., DuBois, R.N., and Runge, M.S.: Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J. Biol. Chem.* **272**, 601-608, 1997.
60) Cordle, S.R., Donald, R., Read, M.A., and Hawiger, J.: Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-Rel and related NF-kappa B proteins in human monocytic THP-1 cells. *J. Biol. Chem.* **268**, 11803-11810, 1993.
61) Chung, H.Y., Kim, H.J., Kim, K.W., Choi, J.S., and Yu, B.P.: Molecular inflammation hypothesis of aging based on the anti-aging mechanism of calorie restriction. *Microsc. Res. Tech.* **59**, 264-272, 2002.
62) Ames, B.N.: Delaying the mitochondrial decay of aging. *Ann. N.Y. Acad. Sci.* **1019**, 406-411, 2004.
63) Johnson, L.V., Walsh, M.L., and Chen, L.B.: Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. USA* **77**, 990-994, 1980.
64) Nomura, Y., Arima, T., Namba, T., Hattori, M., and Kadota, S.: Ameliorating effects of Dan-Shen and its major ingredient calcium/magnesium lithospermate B on cognitive deficiencies in senescence-accelerated mouse. *Folia Pharmacol. Jpn.* **110** Suppl I, 142-147, 1997.
65) Miquel, J.: Can antioxidant diet supplementation protect against age-related mitochondrial damage? *Ann. N.Y. Acad. Sci.* **959**, 508-516, 2002.
66) Kasper, M., and Funk, R.H.: Age-related changes in cells and tissues due to advanced glycation end products (AGEs). *Arch. Gerontol. Geriatr.* **32**, 233-243, 2001.
67) Ono, Y., Aoki, S., Ohnishi, K., Yasuda, T., Kawano, K., and Tsukada, Y.: Increased serum levels of advanced glycation end-products and diabetic complications. *Diabetes Res. Clin. Pract.* **41**, 131-137, 1998.
68) Sell, D.R., and Monnier, V.M.: Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J. Biol. Chem.* **264**, 21597-21602, 1989.
69) Nagaraj, R.H., Shipanova, I.N., and Faust, F.M.: Protein cross-linking by the Maillard reaction. Isolation, characterization, and *in vivo* detection of a lysine-lysine cross-link derived from methylglyoxal. *J. Biol. Chem.* **271**, 19338-19345, 1996.
70) Odani, H., Iijima, K., Nakata, M., Miyata, S., Kusunoki, H., Yasuda, Y., Hiki, Y., Irie, S., Maeda, K., and Fujimoto, D.: Identification of N(ω)-carboxymethylarginine, a new advanced glycation endproduct in serum proteins of diabetic patients: possibility of a new marker of aging and diabetes. *Biochem. Biophys. Res. Commun.* **285**, 1232-1236, 2001.
71) Hunt, J.V., Skamarauskas, J.T., and Mitchinson, M.J.: Protein glycation and fluorescent material in human atheroma. *Atherosclerosis* **111**, 255-265, 1994.
72) Bunn, H.F., Gabbay, K.H., and Gallop, P.M.: The glycosylation of hemoglobin: relevance to diabetes mellitus. *Science* **200**, 21-27, 1978.
73) McFarland, K.F., Catalano, E.W., Day, J.F., Thorpe, S.R., and Baynes, J.W.: Nonenzymatic glucosylation of serum proteins in diabetes mellitus. *Diabetes* **28**, 1011-1014, 1979.
74) Sastre, J., Borras, C., Garcia-Sala, D., Lloret, A., Pallardo, F.V., and Vina, J.: Mitochondrial damage in aging and apoptosis. *Ann. N.Y. Acad. Sci.* **959**, 448-451, 2002.
75) Skulachev, V.P.: Cytochrome *c* in the apoptotic and antioxidant cascades. *FEBS Lett.* **423**, 275-280, 1998.
76) Lin, H.Z., Yang, S.Q., Chuckaree, C., Kuhajda, F., Ronnet, G., and Diehl, A.M.: Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat. Med.* **6**, 998-1003, 2000.
77) Helenius, M., Hanninen, M., Lehtinen, S.K., and Salminen, A.: Changes associated with aging and replicative senescence in the regulation of transcription factor NF-κB. *Biochem. J.* **318**, 603-608, 1996.
78) Kim, H.J., Kim, K.W., Yu, B.P., and Chung, H.Y.: The effect of age on cyclooxygenase-2 gene expression: NF-κB activation and IκBα degradation. *Free Radic. Biol. Med.* **28**, 683-692, 2000.
79) Sasaki, M., Kumazaki, T., Takano, H., Nishiyama, M., and Mitsui, Y.: Senescent cells are resistant to death despite low Bcl-2 level. *Mech. Ageing Dev.* **122**, 1695-1706, 2001.
80) Ginn-Pease, M.E., and Whisler, R.L.: Redox signals and NF-κB activation in T cells. *Free Radic. Biol. Med.* **25**, 346-361, 1998.
81) Youssef, J.A., Bouziane, M., and Badr, M.Z.: Age-dependent effects of nongenotoxic hepatocarcinogens on liver apoptosis *in vivo*. *Mech. Ageing Dev.* **124**, 333-340, 2003.
82) Lee, J.H., Jung, K.J., Kim, J.W., Kim, H.J., Yu, B.P., and Chung, H.Y.: Suppression of apoptosis by calorie restriction in aged kidney. *Exp. Gerontol.* **39**, 1361-1368, 2004.
83) Always, S.E., Degens, H., Krishnamurthy, G., and Chaudhrai, A.:

Denervation stimulates apoptosis but not Id2 expression in hindlimb muscles of aged rats. *J. Gerontol. Ser. A: Biol. Sci. Med. Sci.* **5**, 687-697, 2003.

84) Chung, L., and Ng, C.: Age-related alterations in expression of apoptosis regulatory proteins and heat shock proteins in rat skeletal muscle. *Biochim. Biophys. Acta* **1762**, 103-109, 2005.

85) Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di Lisa, F.: Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur. J. Biochem.* **264**, 687-701, 1999.

86) Feng, L., Xia, Y., Garcia, G.E., Hwang, D., and Wilson, C.B.: Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide. *J. Clin. Invest.* **95**, 1669-1675, 1995.

87) Smith, W.L., Meade, E.A., and DeWitt, D.L.: Pharmacology of prostaglandin endoperoxide synthase isozymes-1 and -2. *Ann. N.Y. Acad. Sci.* **714**, 136-142, 1994.

88) Goppelt-Struebe, M.: Regulation of prostaglandin endoperoxide synthase (cyclooxygenase) isozyme expression. *Prostaglandins Leukot. Essent. Fatty Acids* **52**, 213-222, 1995.

89) Lavrovsky, Y., Song, C.S., Chatterjee, B., and Roy, A.K.: Age-dependent increase of heme oxygenase-1 gene expression in the liver mediated by NF-κB. *Mech. Ageing Dev.* **114**, 49-60, 2000.

Japanese abstract

冠元顆粒は6種類の生薬からなる漢方方剤で，種々の生物活性を有することから注目されている。本研究では，冠元顆粒の抗老化の可能性とその機序について，老化促進モデルマウス（SAM）と加齢ラット，さらにstress-induced premature senescence（SIPS）を持った細胞系を用い検討した結果を報告する。SAMにおいて，冠元顆粒はフリーラジカル産生と脂質過酸化を抑制して酸化ストレス状態を緩和し，抗老化作用を有することが示唆された。生理的老化モデルを用いた実験においても，冠元顆粒とその主薬の丹参が，加齢によって生じる蛋白修飾や脂質過酸化に対し保護作用を示した。このような作用は，冠元顆粒の方が丹参より強い作用を示し，冠元顆粒の相乗作用が示唆された。冠元顆粒はまた，グルタチオンレドックスサイクルの調節やミトコンドリアからの活性酸素種の漏出を防御してミトコンドリア機能を維持し，NF-κBの核への移行の抑制とシクロオキシゲナーゼ-2，誘導型一酸化窒素合成酵素，ヘムオキシゲナーゼ-1，bax，bcl-2等の蛋白発現を制御していた。さらに，SIPS細胞モデルを用いた実験で，冠元顆粒が脂質過酸化の抑制による酸化傷害の改善や細胞周期を制御し，抗老化作用と細胞寿命の延長を示していたが，このような作用はNF-κBと蛋白発現の調節に起因していた。以上，本研究において冠元顆粒が酸化ストレスから誘導される老化過程に対し，抗老化作用を有する可能性が示唆された。

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