JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 1335–1343

Received March 7, 2005
Accepted June 7, 2005
DOI 10.1211/jpp.57.10.0013
ISSN 0022-3573

The mechanisms underlying the anti-aging activity of the Chinese prescription Kangen-karyu in hydrogen peroxide-induced human fibroblasts

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Abstract

Our previous study showed that Kangen-karyu extract protected against cellular senescence by reducing oxidative damage through the inhibition of reactive oxygen species generation and regulation of the antioxidative status. Although these findings suggest that Kangen-karyu could delay the aging process, the mechanisms responsible for protection against aging have rarely been elucidated. Therefore, this study was focussed on the mechanisms responsible for the anti-aging activity of Kangen-karyu extract using hydrogen peroxide (H_2O_2)-induced human diploid fibroblasts, a well-established experimental model of cellular aging. Kangen-karyu extract exerted a protective effect against the morphological changes induced by H_2O_2 treatment and inhibited senescence-associated β-galactosidase activity. In addition, the beneficial effects of Kangen-karyu extract on cell viability and lifespan indicated that Kangen-karyu extract could delay the cellular aging process. The observation that Kangen-karyu extract prevented nuclear factor kappa B (NF-κB) translocation in response to oxidative stress suggested that Kangen-karyu exerted its anti-aging effect through NF-κB modulation and prevention of H_2O_2-induced overexpression of haem oxygenase-1 protein. Moreover, pretreatment with Kangen-karyu extract reduced overexpression of bax protein and prevented the mitochondrial membrane potential decline, suggesting that Kangen-karyu extract may protect mitochondria from mitochondrial oxidative stress and dysfunction. These findings indicate that Kangen-karyu is a promising potential anti-aging agent that may delay, or normalize, the aging process by virtue of its protective activity against oxidative stress-related conditions.

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

Introduction

Human diploid fibroblasts (HDFs) 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, HDFs lose the ability to proliferate and become senescent, showing cellular changes related to the aging process, so-called replicative senescence (Hayflick 1976; Harley 1991; Dimri et al 1995; Linskens et al 1995; Campisi et al 1996; Allen et al 1999; Campisi 2000). In addition, HDFs exhibit the stress-induced premature senescence (SIPS) phenotype after being subjected to many different sub-lethal stresses, including oxidative stress (Toussaint et al 1995; Chen et al 1998; Dumont et al 2000), and this SIPS phenotype is almost identical to the phenotype associated with replicative senescence (Dumont et al 2000). In particular, HDFs subjected to hydrogen peroxide (H_2O_2)-induced oxidative stress showed senescence, activation of signal transduction molecules (such as nuclear factor-kappa B (NF-κB) (de Magalhaes et al 2004)), alteration of the expression of genes such as cyclooxygenase (COXs), haem oxygenase-1 (HO-1), bax and bcl-2 (Chen et al 2000), oxidative damage of several components (Chen et al 2000; Lee et al 2000, 2002) and reduction of the mitochondrial membrane potential. Therefore, the aging model of H_2O_2-treated HDFs was employed to investigate the anti-aging effect, and mechanisms responsible for this effect, of Kangen-karyu under conditions of cellular SIPS.

Kangen-karyu, a Chinese prescription comprising six crude drugs, has received much attention due to its wide-ranging biological activity, such as inhibition of platelet aggregation, suppression of hypertension and anti-aging (Takahashi 1991; Gao et al 2001; Makino et al 2002a, b). Takahashi et al (1992) demonstrated that Kangen-karyu affected the recovery of learning and memory impairment in senescence-accelerated mice (SAM) by preserving the activity of choline acetyltransferase and superoxide dismutase in the cerebellum. Our previous study also showed that Kangen-karyu extract inhibited the oxidative stress-related aging process in SAM through enhancing antioxidative enzyme activity and scavenging reactive oxygen species (ROS) (Satoh et al 2004a). In addition, Kangen-karyu extract protected against cellular senescence by reducing oxidative damage through the inhibition of ROS generation and regulation of the antioxidative status (Satoh et al 2004b). Although these investigations suggest that Kangen-karyu extract may delay the aging process by virtue of its antioxidative effects, the mechanisms responsible for protection against aging have rarely been elucidated. Therefore, this study was focused on the mechanisms responsible for the anti-aging activity of Kangen-karyu extract.

Materials and Methods

Reagents and cells

Basal medium of Eagle (BME), paraformaldehyde, Triton X-100 and rhodamine-123 were purchased from Sigma Chemical Co. (St Louis, MO). Calcium- and magnesium-free phosphate-buffered saline (PBS), H_2O_2, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), dimethyl formaldehyde, dimethyl sulfoxide (DMSO), potassium ferrocyanide, potassium ferricyanide, Nonidet P-40 (NP-40), phenylmethane sulfonyl fluoride (PMSF) and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris-Cl) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Foetal bovine serum (FBS), trypsin solution and 5-bromo-4-chloro-3-indoyl-β-D-galactoside were purchased from Life Technologies Inc. (Grand Island, NY, USA), Nakarai (Kyoto, Japan) and Molecular Probes (Eugene, OR, USA), respectively. Normal human lung diploid fibroblasts (WI-38, TIG-1) were purchased from Health Science Research Resources Bank (Osaka, Japan). The monoclonal anti-COX-1 and -2, HO-1, bax and bcl-2 antibodies, goat monoclonal anti-mouse immunoglobulin (IgG) and goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of Kangen-karyu extract

The composition of Kangen-karyu used in this study was: 2.25 g Paeoniae Radix (*Paeonia lactiflora* Pallas root), 2.25 g Cnidii Rhizoma (*Cnidium officinale* Makino rhizome), 2.25 g Carthami Flos (*Carthamus tinctrius* L. petal), 1.125 g Cyperi Rhizoma (*Cyperus rotundus* L. rhizome), 1.125 g Aucklandiae Radix (*Aucklandia lappa* Dcne. root) and 4.5 g Salviae Miltiorrhizae Radix (*Salvia miltiorrhiza* Bunge root). These herbs were extracted with 25 volumes of water at 100°C for 1 h. After filtration through a filter paper, the solution was evaporated under reduced pressure to give an extract at a yield of 44% (w/w) of the starting materials. For analysis of the components of Kangen-karyu, the aqueous extract was dissolved in aqueous ethanol (50% v/v) with sonication, and filtered through a Cosmonice filter (PVDF, 0.45 μm, Nakarai Tesque, Inc.). Reverse-phase HPLC analysis was performed using a Cosmosil $5C_{18}$-AR II column (250 × 4.6 mm i.d., Nakarai Tesque, Inc.) with elution gradients of 4–30% v/v (39 min) and 30–75% v/v (15 min) CH_3CN in 50 mM H_3PO_4 at a flow rate of 0.8 mL min^{-1}. The ultraviolet (UV) absorbance from 200 to 400 nm was monitored and the three-dimensional data was 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.

Cell culture and induction of SIPS

TIG-1 and WI-38 HDFs were cultivated in 10-mm culture dishes containing BME supplemented with 10% v/v FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were detached from the dish by 0.05% v/v trypsin-EDTA in PBS and subcultured in culture plates. WI-38 or TIG-1 HDFs at population doubling levels (PDLs) of 27.0 and 37.2, respectively, were seeded at a density of 10^5 cells/mL in 6- or 96-well culture plates and incubated for 2 h. After treatment with or without each concentration (5–100 μg mL^{-1}) of Kangen-karyu extract, the cells were treated with a final concentration of 300 μM H_2O_2 for 60 min. After changing the medium, the cells were incubated for 0–72 h and senescence-associated β-galactosidase (SA-β-Gal) activity, cell viability, NF-κB translocation, Western blotting and mitochondrial membrane potential changes were analysed. In addition, to compare cell lifespans, Kangen-karyu extract-treated and untreated cells were cultivated continuously until they reached crisis.

SA-β-Gal assay

To measure SA-β-Gal activity, we used the method of Dimri et al (1995). After treatment with/without Kangen-karyu extract followed by H_2O_2, cells were washed with PBS, incubated with fresh medium for 24, 48 or 72 h, fixed with 3.7% v/v paraformaldehyde for 2 min, washed with PBS and then incubated at 37°C with SA-β-Gal staining solution (1 mg mL^{-1} 5-bromo-4-chloro-3-indoyl β-D-galactoside (stock solution: 20 mg mL^{-1} dimethylformamide, 40 mM citric acid, sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$)) with evaluation of staining under microscope.

Cell viability

After confirming by preliminary experiments that Kangen-karyu extract did not interfere with the MTT assay, cell viability was assessed using the MTT colorimetric assay (Mosmann 1983). The cells were pretreated with/without Kangen-karyu extract followed by H_2O_2 and then incubated in fresh medium for 0, 24 or 48 h. MTT solution (1 mg mL^{-1}; 100 μL) was added to each well of a 96-well culture plate, which was incubated for 4 h at 37°C and the medium containing MTT was removed. The formazan crystals incorporated into the viable cells were solubilized with 100 μL DMSO and the absorbance at 540 nm of each well was read using a Microplate Reader (Model 3550-UV; Bio-Rad, Tokyo, Japan). The percentage of viable cells was determined according to the formula: viable cells (%) = (λ_{540} of treated cells/λ_{540} of non-treated cells) × 100, where λ_{540} is the absorbance of formazan crystals at 540 nm.

Cell lifespan

The PDL of each culture was determined as follows: current PDL = last PDL + $\log_2$ (collected cell number/seeded cell number) and the cell lifespan was evaluated using the method of Cristofalo & Charpentier (1980).

NF-κB translocation

After pretreatment with Kangen-karyu extract and treatment with H_2O_2 for 60 min, the cells were fixed with 3.7% v/v paraformaldehyde for 10 min at room temperature, washed with PBS and permeabilized with 0.2% v/v Triton X-100 for 10 min at room temperature. Then, the cells were washed with PBS and blocked with 2% w/v bovine serum albumin for 1 h at 4°C. After washing with PBS, the cells were treated with a 1:100 dilution of monoclonal anti-NF-κB antibody (p65) for 3 h at room temperature. The anti-NF-κB-stained cells were washed with PBS, incubated with a 1:250 dilution of fluorescein isothiocyanate-conjugated anti-rabbit IgG for 1 h at room temperature, washed with PBS, stained with a 1:250 dilution of 4,6-diamidino-2-phenylindole (DAPI) for 2 min at room temperature, washed twice with PBS, analysed using a fluorescence microscope, and then stained with anti-NF-κB antibody (p65) for NF-κB (green) and DAPI for nucleus (blue).

Western blot analysis

The cells were treated with Kangen-karyu extract or H_2O_2 (or both) and then incubated with fresh medium for 24 h. The cells attached to the bottom of each well were collected by scraping them off into a lysis buffer (250 mM NaCl, 25 mM Tris-Cl (pH 7.5), 5 mM EDTA and 1% v/v NP-40) containing 1 mM PMSF, 1 mM dithiothreitol and protein inhibitor fluid (containing AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin) and incubated on ice for 30 min. Then, the cells in the supernatants were collected by centrifugation at 7500 g for 15 min and the protein concentrations of these cell extracts were quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Western blot analysis was performed on volumes containing 30 μg protein from each sample. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Trans-blot transfer medium (Bio-Rad Laboratories) at 100 V for 1 h and immunodetection was performed using an enhanced chemiluminescence kit for Western blotting detection (Amersham Pharmacia Biotech., Freiburg, Germany). COX-1 was used as the internal control.

Mitochondrial membrane potential

To measure mitochondrial membrane potential, rhodamine-123 was used as a fluorescent probe. Rhodamine-123 is specifically incorporated into mitochondrial membranes. Fibroblasts were seeded in 96-well plates, treated with Kangen-karyu extract or H_2O_2 (or both) for 60 min, washed with PBS and then incubated with 10 μM rhodamine-123 in media for 30 min at 37°C in the dark. After incubation, the medium was carefully discarded, the cells were rinsed twice with PBS and then incubated for 1 h in normal culture medium under the standard culture conditions described above to eliminate any non-retained intracellular probe. The mitochondrial membrane potential was determined by eluting the dye from stained cells with a solution of 1% v/v acetic acid–50% v/v ethanol (100 μL/well). After mixing, the fluorescence of each plate was rapidly read using specific filters (excitation: 490 nm, emission: 530 nm) on a fluorescence plate reader (Lautraite et al 2003). In addition, cells in 6-well plates were treated with or without Kangen-karyu or H_2O_2, washed with PBS and incubated with 10 μM rhodamine-123 in media. After washing twice with PBS, the red fluorescent cells were observed under a fluorescence microscope. The level of membrane potential was measured by microplate reader.

Statistics

The results for each group were expressed as mean ± s.e. values. The effect of Kangen-karyu extract on each parameter was examined using the one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test and those at $P < 0.05$ were considered to be statistically significant.

Results

Analysis of Kangen-karyu extract by HPLC

By three-dimensional HPLC, 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.

Morphological changes and SA-β-Gal activity

Treatment with 300 μM H_2O_2 for 60 min resulted in senescence-like morphological changes in the WI-38 cells, which became flat and thick (Figure 1). Kangen-karyu

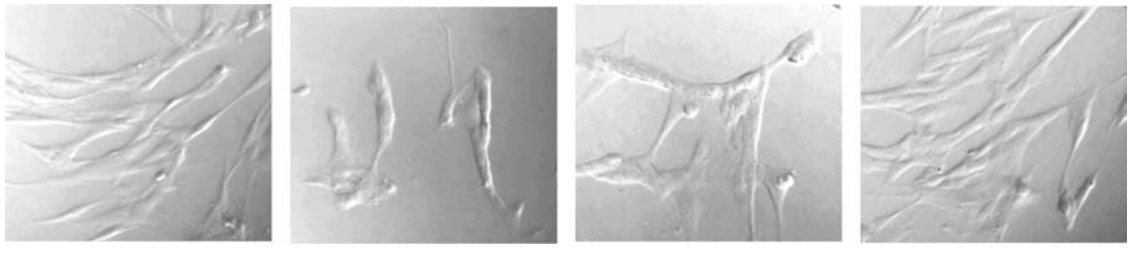

Figure 1 Morphological changes. HDFs were pretreated with Kangen-karyu (10 and 100 μg mL^{-1}) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were incubated with fresh media for 24 h and cell morphology was determined by optical microscopy (magnification ×100).

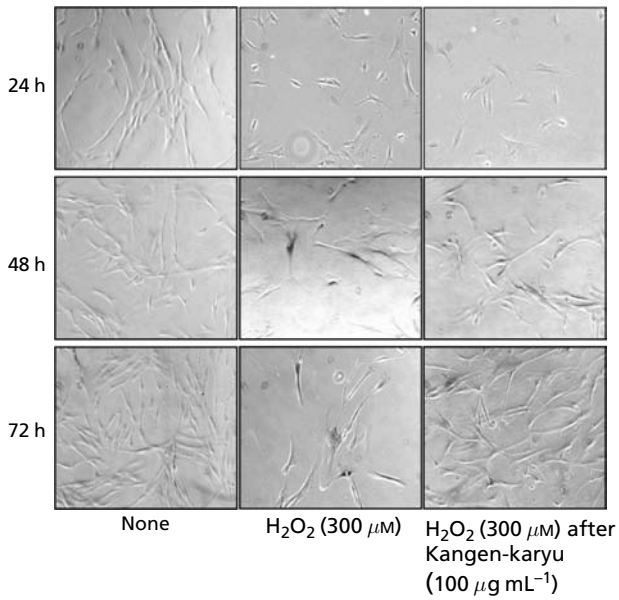

Figure 2 SA-β-Gal activity. HDFs were pretreated with Kangen-karyu (100 μg mL^{-1}) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were incubated with fresh media for 24, 48 or 72 h, incubated with SA-β-Gal staining solution and evaluated by optical microscopy (magnification ×40).

treatment reversed these changes in a concentration-dependent manner. SA-β-Gal activity induced senescence of HDFs in response to H_2O_2 (Figure 2). After H_2O_2 treatment for 48 and 72 h, the cells exhibited increased SA-β-Gal activity, reflected by increased staining intensity. However, Kangen-karyu treatment reduced the SA-β-Gal activity of H_2O_2-treated cells, as shown by a decrease in the staining intensity.

Cell viability

Exposure of HDFs to H_2O_2 led to a time-dependent decrease in cell viability (Table 1). However, pretreatment with Kangen-karyu extract resulted in a significant and concentration-dependent increase in cell viability. The viability of HDFs treated with H_2O_2 for 48 h was 48.2%, while pretreatment with 10, 50 and 100 μg mL^{-1} of Kangen-karyu extract increased viability significantly to 53.1, 58.6 and 60.3%, respectively.

Table 1 The effect of Kangen-karyu on cell viability of HDFs with SIPS induced by H_2O_2 exposure

Group	Incubation time (h)		
	0	24	48
None	61.9 ± 2.1	59.0 ± 1.2	48.2 ± 2.1
Kangen-karyu (5 μg mL^{-1})	65.4 ± 3.4*	61.0 ± 2.4	51.0 ± 1.4*
Kangen-karyu (10 μg mL^{-1})	67.0 ± 3.5**	62.4 ± 2.2*	53.1 ± 1.5**
Kangen-karyu (50 μg mL^{-1})	75.0 ± 3.6**	68.0 ± 1.2**	58.6 ± 1.4**
Kangen-karyu (100 μg mL^{-1})	77.2 ± 3.1**	70.0 ± 1.2**	60.3 ± 1.6**

Data are means ± s.e.m., n = 8. *$P < 0.05$, **$P < 0.001$ vs H_2O_2 treatment values.

Cell lifespan

To evaluate the effect of Kangen-karyu on the lifespan of HDFs with SIPS due to H_2O_2 treatment, the PDLs of the cultures were compared. The PDL of H_2O_2-exposed WI-38 cells was reduced to 38.2 compared with 45.2 for untreated control cells. In contrast, the lifespan of WI-38 cells pretreated with 100 μg mL^{-1} Kangen-karyu showed extension of the PDL from 38.2 to 40.2.

NF-κB translocation

Figure 3 shows the translocation of NF-κB and nuclear DAPI staining of HDFs with the SIPS phenotype. H_2O_2 treatment of human lung fibroblast cells resulted in the translocation of NF-κB from the cytosol to the nuclei, whereas this did not happen in untreated cells. However, pretreatment with 10 and 100 μg mL^{-1} Kangen-karyu inhibited nuclear translocation of NF-κB, demonstrated by reduced nuclear DAPI staining intensity.

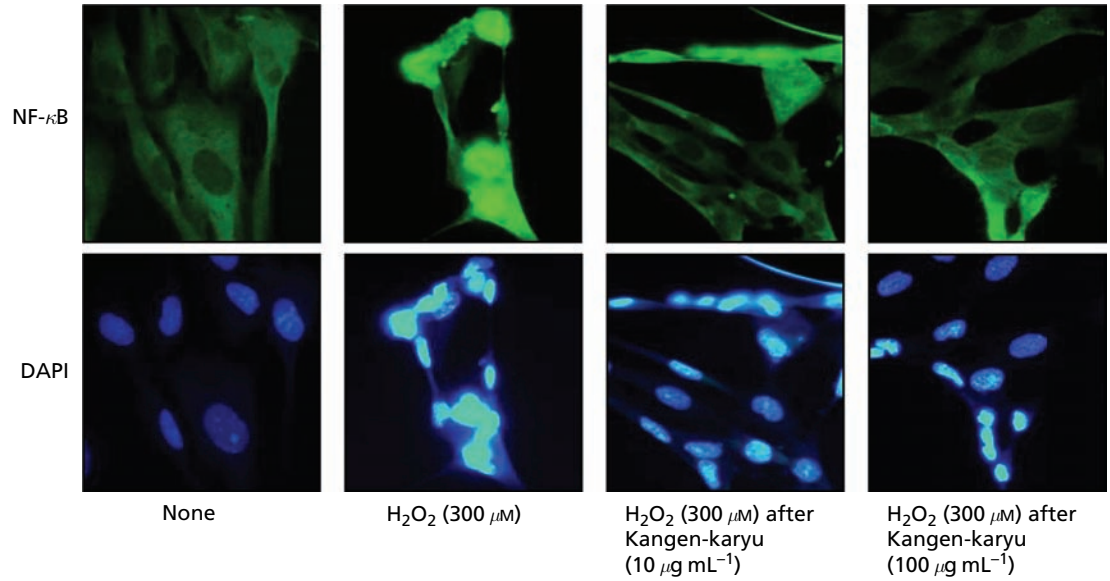

Figure 3 NF-κB translocations. HDFs were pretreated with Kangen-karyu (10 and 100 μg mL^{-1}) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were treated with the anti-NF-κB antibody and then stained with DAPI. NF-κB translocation was analysed using a fluorescence microscope (magnification $\times$100).

Protein expression

The expression levels of COX-1, COX-2 and bcl-2 proteins did not change after treatment with 300 μM H_2O_2 for 24, 48 and 72 h, while those of HO-1 and bax increased after 24 and 72 h. However, pretreatment with 5, 10 and 50 μg mL^{-1} Kangen-karyu extract for 24 h reduced the enhanced expression of HO-1 protein to 78.3, 76.9 and 80.2%, respectively, compared with 100% for untreated control cells. Bax protein was also reduced by Kangen-karyu extract, whereas at concentrations higher than 5 μg mL^{-1} Kangen-karyu treatment did not result in further inhibition (Figure 4).

Mitochondrial membrane potential

The intensity of the red fluorescence observed when rhodamine-123 is incorporated into mitochondria depends on the mitochondrial membrane potential. As shown in Figure 5, the intensity of the red fluorescence increased in a time-dependent manner after exposure of HDFs to H_2O_2 in comparison with untreated HDFs. However, Kangen-karyu-pretreated cells clearly showed reduced fluorescence intensity. As shown in Table 2, the intensity was increased from 100.0 to 181.6% by H_2O_2, and treatment with Kangen-karyu extract at concentrations of 5, 10, 50 and 100 μg mL^{-1} reduced this intensity significantly and concentration-dependently to 155.3, 150.6, 128.3 and 120.5%, respectively (each, $P < 0.001$ vs H_2O_2 treatment).

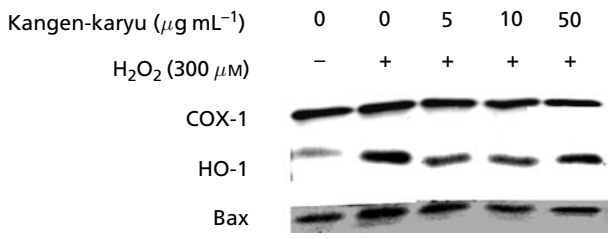

Figure 4 Protein expression. HDFs were pretreated with Kangen-karyu (5, 10, 50 and 100 μg mL^{-1}) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were incubated with fresh media for 24 h. The COX-1, HO-1 and bax proteins were determined by Western blot analysis.

Discussion

H_2O_2-treated HDFs are a well-established experimental model of cellular aging since HDFs exposed to various types of subcytotoxic oxidative stress display a senescence-like phenotype (Toussaint et al 1995; Chen et al 1998; Dumont et al 2000). Some of the many biomarkers of replicative senescence that appear in this state are senescence-like cell morphology, lack of response to mitogenic stimuli and irreversible growth arrest, especially G_0/G_1 phase arrest, a sharp decrease in DNA synthesis and an increase in the population of cells that exhibit SA-β-Gal activity (Dumont et al 2000; Wolf et al 2002; Wang et al 2004). Interestingly, these phenomena were not observed immediately after exposure of HDFs to H_2O_2 (Frippiat et al 2002); therefore, HDFs treated with H_2O_2 for 3 days were used to investigate the effect of Kangen-karyu extract on replicative senescence on the basis of the preliminary study on the time and concentrations of H_2O_2 and Kangen-karyu extract.

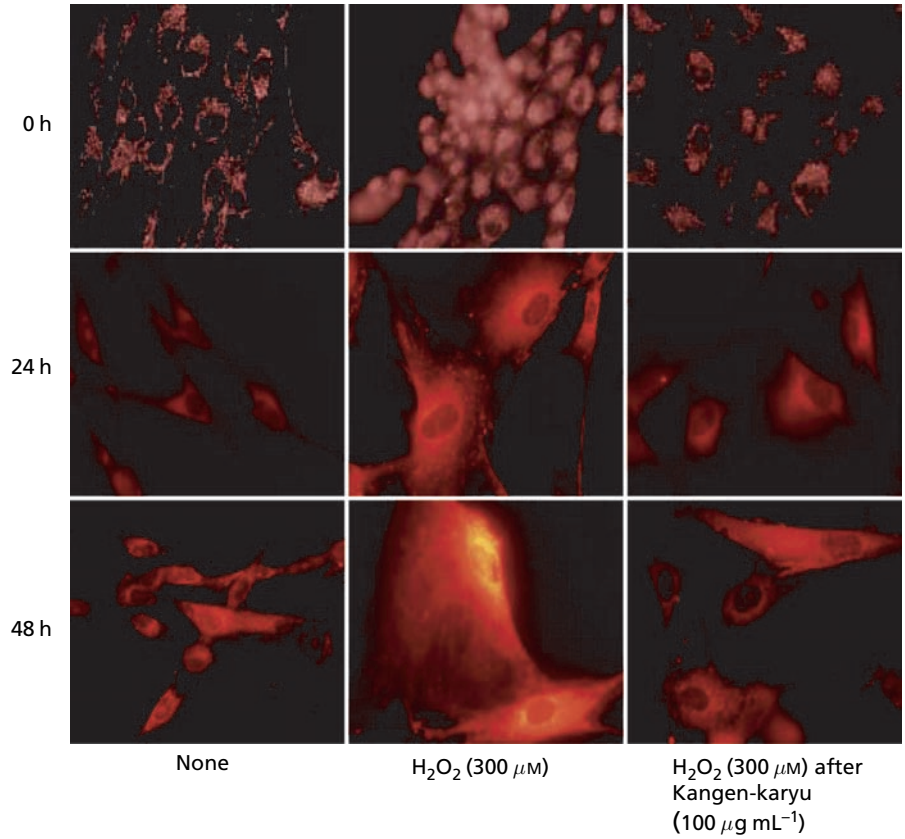

Figure 5 Mitochondrial membrane potential. HDFs were pretreated with Kangen-karyu (100 μg mL^{-1}) for 24 h before 60 min incubation with H_2O_2. After removing H_2O_2, the cells were incubated with fresh media for 0, 24 and 48 h, and mitochondrial membrane potential was estimated under rhodamine-123 staining. The red fluorescent cells were observed under a fluorescence microscope (magnification ×100).

Table 2 The effect of Kangen-karyu on the mitochondrial membrane potential of HDFs with SIPS induced by H_2O_2 exposure

Group	Mitochondrial membrane potential (% of non-treated cells)
None	100.0 ± 5.9
H_2O_2 (300 μM)	$181.6 \pm 14.7^{\#\#}$
H_2O_2 (300 μM) after Kangen-karyu (5 μg mL^{-1})	$155.3 \pm 12.2^{\#\#}$*
H_2O_2 (300 μM) after Kangen-karyu (10 μg mL^{-1})	$150.6 \pm 5.7^{\#\#}$*
H_2O_2 (300 μM) after Kangen-karyu (50 μg mL^{-1})	$128.3 \pm 5.3^{\#\#}$*
H_2O_2 (300 μM) after Kangen-karyu (100 μg mL^{-1})	$120.5 \pm 5.1^{\#}$*

Data are means ± s.e.m., n = 8. $^{\#}P < 0.01$, $^{\#\#}P < 0.001$ vs no treatment values; $^{*}P < 0.001$ vs H_2O_2 treatment values.

One of the remarkable changes in HDFs undergoing cellular aging is a senescence-like morphological change almost identical to that observed with SIPS (Chen & Ames 1994; Frippiat et al 2001, 2002). In addition, several studies demonstrated that SA-β-Gal activity increases dramatically during replicative senescence both in-vitro and in-vivo (Wang et al 2004). Consistent with these findings, HDFs exposed to H_2O_2 displayed the stress-induced morphological changes indicative of premature senescence (Figure 1). Moreover, the SA-β-Gal activity was elevated in HDFs exposed to H_2O_2 (Figure 2). 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 growth rate of HDFs was reported to be delayed under conditions of cellular senescence (von Zglinicki et al 1995). Our results also showed that SIPS due to H_2O_2 treatment led to the loss of cell viability and shortened the cell lifespan (Table 1 and data not shown), suggesting that treatment with H_2O_2 reduced the growth rate of HDFs and these premature replicative senescence phenomena were probably caused by the cell cycle arrest triggered by H_2O_2 and the effect of oxidative stresses on cellular aging. However, the beneficial effects of Kangen-karyu extract on both cell viability and lifespan indicate that Kangen-karyu extract could delay the cellular aging

process. Furthermore, our previous study demonstrated that the antioxidative activity of Kangen-karyu accounted for the delay in the development of the SIPS phenotype of H_2O_2-treated cells, although the mechanisms responsible remain to be elucidated.

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 (Sen & Baltimore 1986), tumour necrosis factor-α (Baeuerle & Henkel 1994; Siebenlist et al 1994; Baldwin 1996), interleukin-1 (Beg et al 1993; DiDonato et al 1995), UV light (Stein et al 1989a, b), hypoxia (Schmedtje et al 1997), lipopolysaccharide (Cordle et al 1993) and H_2O_2, the NF-κB dimer dissociates from IκB and translocates to the nucleus, which is followed by NF-κB activation. This study demonstrated that HDFs under the condition of SIPS caused by H_2O_2 showed NF-κB translocation to the nucleus from the cytosol (Figure 3), suggesting that cells undergoing premature replicative senescence 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 (Radak et al 2004). Therefore, the prevention of NF-κB translocation resulting from oxidative stress suggests that Kangen-karyu exerts an anti-aging effect through NF-κB modulation.

The translocation into the nucleus and activation of NF-κB induce the expression of several proteins, such as COXs, inducible nitric oxide synthase, HO-1, bax and bcl-2 (Chung et al 2002). Therefore, protein expression from 0 to 72 h after H_2O_2 exposure was observed. The induction of high levels of HO-1 and bax proteins was probably related to translocation into the nucleus and subsequent activation of NF-κB. Expression of HO-1, which is one of the stress-response proteins and a rate-limiting enzyme in haem catabolism (Panchenko et al 2000), is also induced by H_2O_2, UV irradiation, sulfhydryl reagents, divalent metal ions and other oxidative agents known to stimulate free radical formation or glutathione (GSH) depletion in cells (Maines & Kappas 1977; Alam et al 1989). These findings suggest that free radicals generated by oxidative stress may have a signalling role in overexpression of HO-1. In addition, HO-1 expression induced by H_2O_2 and UV irradiation is attenuated by the addition of cellular antioxidants such as GSH and vitamin E (Applegate et al 1991). Our previous study demonstrated that Kangen-karyu extract protected against cellular senescence by reducing oxidative damage through the inhibition of ROS generation and regulation of the antioxidative status (Satoh et al 2004b). Furthermore, the current study demonstrated that Kangen-karyu extract prevented the overexpression of HO-1 protein induced by H_2O_2 (Figure 4), suggesting that the antioxidative properties of Kangen-karyu extract regulate the expression of this protein.

Another protein overexpressed under the condition of SIPS caused by H_2O_2 is bax, which normally localizes at nuclear and mitochondrial membranes (Chen et al 2000). The bax protein can form ion channels on mitochondrial membranes and induce the release of cytochrome c from mitochondria (Stridh et al 1998). In addition, bax is considered to be the death factor, whereas bcl-2 is regarded as the survival factor, in a number of systems. Bcl-2 is thought to form a dimer with bax and prevent the release of cytochrome c from mitochondria (Sasaki et al 2001). In-vitro experiments showed that bax could activate caspase in the cytosol in the presence of cytochrome c (Stridh et al 1998), and H_2O_2 induces mitochondrial membrane transition and releases cytochrome c from mitochondria (Skulachev 1998). Our study showed that treating HDFs with H_2O_2 elevated bax protein levels without change in bcl-2 expression. In contrast, pretreatment with Kangen-karyu extract under conditions of SIPS reduced the overexpression of bax protein (Figure 4), suggesting that Kangen-karyu extract inhibited the induction of bax caused by SIPS by regulating mitochondrial function.

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 (Beckman & Ames 1998) and such injured cells display a decline of the mitochondrial membrane potential (Ames 2004). The fluorescent dye rhodamine-123 is frequently used as a mitochondrial membrane potential probe because it is well characterized, causes no loss of mitochondrial coupling, and is not toxic at low concentrations (Johnson et al 1980). Moreover, dye accumulation and the resulting fluorescence intensity of cells are stable, allowing accurate measurement of cellular fluorescence characteristics. In this study, H_2O_2-treated HDFs certainly showed increased rhodamine-123 fluorescence compared with untreated HDFs (Figure 5). This is consistent with the results of Lee et al (2000), 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.

By treating HDFs with Kangen-karyu extract before H_2O_2 exposure, we could observe the efficacy of Kangen-karyu extract at preventing SIPS in response to H_2O_2. Moreover, our investigation focused on the mechanisms whereby Kangen-karyu protects against the cellular aging process. Our results indicate that Kangen-karyu delays aging by inhibiting NF-κB nuclear translocation from the cytosol. It also modulated the function of mitochondria, which are an important oxygen radical source in cells, by regulating related protein expression.

Further study will also support the effect of Kangen-karyu on the process of aging with the time-schedule treatment of Kangen-karyu before or after H_2O_2 treatment. In addition, the therapeutic efficacy of Kangen-karyu extract by treating cells with Kangen-karyu extract under SIPS by H_2O_2 treatment was evaluated (data not shown). According to the free radical theory of aging, aging phenomena and causes are complicated by several factors: accumulation of oxidative damage, such as DNA damage, and oxidatively modified proteins and lipids

in the cytosol that may lead to aging. Moreover, these disorders lead to chronic damage that is more severe than acute damage and damage associated with the normal aging process. Our additional investigation also demonstrated that Kangen-karyu normalized cells under the aging process induced by oxidative damage through inhibiting oxidative damage and growth arrest (Satoh et al 2004b). These findings indicate that Kangen-karyu has promising potential as an anti-aging agent that will delay or normalize the aging process by virtue of its activity against oxidative stress-related conditions.

In conclusion, our study indicates that Kangen-karyu extract protects against cellular aging. The mechanisms responsible for this effect involve protection against the mitochondrial dysfunction that occurs with senescence by reducing bax overexpression and the consequent oxidative damage, inhibition of NF-κB nuclear translocation from the cytosol and regulation of the expression of stress-response proteins such as HO-1.

Although further study on the main compounds of Kangen-karyu that have anti-aging effects has to be carried out, some evidence supports their biological activity. The main compound of Kangen-karyu, lithospermic acid, was reported as a potent antioxidant with free radical scavenging activity (Chen et al 1999; Zhang et al 2004). In addition, several reports also demonstrated that it ameliorated renal functional impairment and prevented hepatitis and fibrosis after oral administration to rats (Yokozawa et al 1997; Kang et al 2004). Another major compound of Kangen-karyu, paeoniflorin, showed protective effect against senile dementia and aging-induced cognitive dysfunction (Ohta et al 1994). Since the Chinese prescription Kangen-karyu is composed of several compounds with biological effects, including antioxidant and anti-aging, it is regarded to have great potential against the aging process.

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