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Therapeutic efficacy of Kangen-karyu against H_2O_2-induced premature senescence

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Abstract

The anti-aging potential of Kangen-karyu extract was investigated using the mechanisms of the cellular aging model of stress-induced premature senescence (SIPS) in TIG-1 human fibroblasts. SIPS was induced by a sublethal dose of H_2O_2 and chronic oxidative stress with repeat treatment of low-dose H_2O_2. Reactive oxygen species generation, lipid peroxidation, and senescence-associated β-galactosidase activity were elevated in TIG-1 cells under SIPS induced by H_2O_2. However, Kangen-karyu extract led to significant declines in these parameters, suggesting its role in ameliorating oxidative stress-related aging. It was also observed that SIPS due to H_2O_2 treatment led to the loss of cell viability, whereas Kangen-karyu extract improved cell viability by attenuating H_2O_2-induced oxidative damage. TIG-1 cells under the condition of SIPS caused by sublethal and chronic low doses of H_2O_2 showed nuclear factor-κB (NF-κB) translocation to the nucleus from the cytosol, while Kangen-karyu extract inhibited NF-κB nuclear translocation, implying that Kangen-karyu extract could exert an anti-aging effect through NF-κB modulation. In addition, treatment with Kangen-karyu extract under H_2O_2-induced chronic oxidative stress normalized the cell cycle by reducing the number of cells in the G_0/G_1 phase and elevating the proportion of those in the S phase, indicating the role of Kangen-karyu extract in cell cycle regulation. On the other hand, Kangen-karyu extract did not exert such an effect on cell cycle regulation under acute oxidative stress induced by sublethal H_2O_2. Furthermore, treatment with Kangen-karyu extract prolonged the lifespan of TIG-1 cells under SIPS. The present study suggests that Kangen-karyu might play a therapeutic role against the aging process caused by oxidative stress.

Introduction

Kangen-karyu, a Chinese prescription comprising six crude drugs, has been reported to exert numerous biological activities, such as inhibition of platelet aggregation, suppression of hypertension, and anti-aging (Takahashi 1991; Gao et al 2001; Makino et al 2002a, b). Its anti-aging activity in particular has attracted much attention. Takahashi et al (1992) demonstrated that Kangen-karyu affected the recovery of learning and memory impairment in senescence-accelerated mice 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 aging process in senescence-accelerated mice through enhancing antioxidative enzyme activity and scavenging reactive oxygen species (ROS) (Satoh et al 2004). These investigations suggest that Kangen-karyu extract may delay the aging process by virtue of its antioxidative effect.

To study aging-associated molecular changes, the cellular model using human diploid fibroblasts (HDFs) has become a classic experimental model of cellular aging. After serial passage, HDFs lose their ability to proliferate and become senescent, showing cellular changes related to the aging process, so-called replicative senescence (RS) (Hayflick 1976; Harley 1991; Dimri et al 1995; Linskens et al 1995; Campisi et al 1996; Campisi 2000). In addition, HDFs exhibit the stress-induced premature senescence (SIPS) phenotype after being subjected to many different sublethal stresses, including oxidative stress, and this SIPS phenotype is almost identical to the phenotype associated with RS (Toussaint et al 1995; Chen et al 1998; Dumont et al 2000). Wolf et al (2002) reported that H_2O_2-treated HDFs showed senescence-like morphological changes, increases in oxidative DNA damage, senescence-associated β-galactosidase (SA-β-Gal) activity, and G_0/G_1 cell cycle arrest, indicating RS of the cells. This evidence supports that HDFs under the condition of SIPS caused by H_2O_2 is a useful and reasonable cellular aging model for evaluating anti-aging effects that counteract oxidative

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stress. In the present study, after the induction of SIPS by treatment with H_2O_2, the anti-aging potential of Kangen-Karyu in terms of its therapeutic efficacy was investigated with its related mechanisms against cellular aging.

Materials and Methods

Reagents

Basal Medium Eagle, paraformaldehyde, and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO, USA). 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, dimethylsulfoxide, potassium ferrocyanide, potassium ferricyanide, Nonidet P-40 (NP-40), phenylmethane sulfonyl fluoride, and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris-Cl) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Fetal 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, Oregon, USA), respectively. Normal human lung diploid fibroblasts (TIG-1) were purchased from Health Science Research Resources Bank (Osaka, Japan).

Preparation of Kangen-karyu extract

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

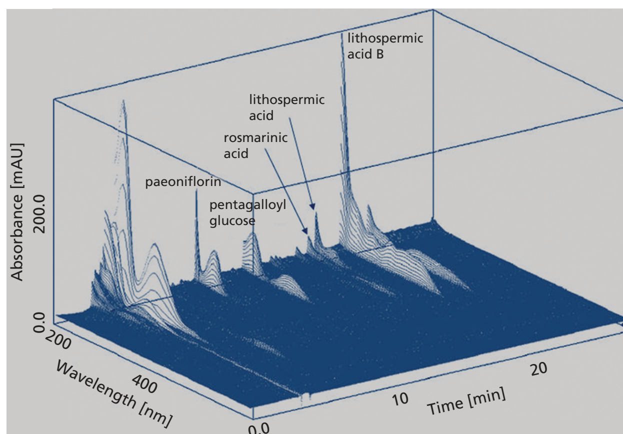

Figure 1 Three-dimensional high-performance liquid chromatogram of Kangen-karyu showing its major compounds.

Cell culture

TIG-1 HDFs were cultivated in 10-mm culture dishes containing Basal Medium Eagle supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured with 0.05% trypsin-EDTA in PBS.

Induction of SIPS by treatment with H_2O_2

TIG-1 HDFs at a population doubling level (PDL) of 27.0 were seeded at a density of 10^5 cells mL^{-1} in 6- or 96-well culture plates and incubated for 2 h. After treatment with 50 μM of H_2O_2 for 60 min to induce acute oxidative stress by a sublethal dose of H_2O_2, the cells were treated with or without each concentration (5–100 μg mL^{-1}) of Kangen-karyu extract. Chronic oxidative stress was induced by adding 5 μM of H_2O_2 for 60 min a day for 3 days.

ROS generation

To measure ROS generation in cells, we used the methods of Wang & Joseph (1999). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) is adapted for the detection of ROS and electron transfer processes. This assay measures the oxidative conversion of stable, non-fluorescent DCFH-DA to the highly fluorescent 2′,7′-dichlorofluorescin in the presence of ROS. After Kangen-karyu extract administration to seeded TIG-1 fibroblast cells, 100 μM (final concentration) of DCFH-DA was added, the cells were incubated at 37°C for 15 min, and then exposed to oxidative stress with H_2O_2 for 60 min. The fluorescent reaction products were assayed on a microplate reader (Tecan, Zurich, Switzerland) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Thiobarbituric acid (TBA)-reactive substance levels

TBA-reactive substance levels in media were determined as described by Mihara & Uchiyama (1978). After treatment with Kangen-karyu extract for 24 h, the media were collected, treated with 0.67% TBA and 20% trichloroacetic acid, and then boiled at 100°C for 45 min. The mixtures were cooled with ice and extracted with *n*-BuOH. After centrifuging at 4000 *g* for 10 min, the fluorescence of the *n*-BuOH layer was measured at an excitation wavelength of 515 nm and an emission wavelength of 553 nm using a fluorescence spectrophotometer (model RF-5300PC; Shimadzu, Kyoto, Japan).

Flow cytometric cell cycle analysis

After collecting cells by centrifugation, they were fixed for at least 30 min at 4°C in 3 mL 70% ice-cold EtOH, washed with PBS twice, incubated with RNase solution for 30 min at 37°C, and then treated with propidium iodide at 4°C for 30 min. The cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) with laser excitation at 488 nm using a 639-nm band pass filter to visualize the red propidium iodide fluorescence. The percentages of cells at various phases of the cell cycle, namely G_0/G_1, S, and G_2/M, were assessed using ModFit LT software (Verity Software House, Topsham, ME, USA) in the analysis data (Block et al 1987; Ho et al 2000).

SA-β-Gal activity

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, TIG-1 cells were washed with PBS, incubated with fresh medium for 72 h, fixed with 3.7% paraformaldehyde for 2 min, 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 $MgCl_2$). Staining was evaluated by microscopy.

Cell viability

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 60 min. A 100-μL aliquot of MTT solution (1 mg mL^{-1}) was added to each well of a 96-well culture plate, incubated for 4 h at 37°C, and then the medium containing MTT was removed. The formazan crystals incorporated into the viable cells were solubilized with 100 μL dimethylsulfoxide and the absorbance at 540 nm of each well was read using a microplate reader (model 3550-UV; Bio-Rad, Tokyo, Japan).

Cell lifespan

To compare cell lifespans, Kangen-karyu extract treated and untreated cells were cultivated continuously until they reached crisis. 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).

Nuclear factor-κB (NF-κB) translocation

After treatment with Kangen-karyu extract, TIG-1 cells under SIPS by H_2O_2 for 60 min were fixed with 3.7% paraformaldehyde for 10 min, washed with PBS, and permeabilized with 0.2% Triton X-100 for 10 min. Then, the cells were washed with PBS and blocked with 2% bovine serum albumin for 1 h at 4°C. After washing with PBS, the cells were treated with the monoclonal anti-NF-κB antibody (p65) for 3 h. The anti-NF-κB-stained cells were washed with PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG for 1 h, washed with PBS, stained with 4,6-diamidino-2-phenylindole (DAPI) for 2 min, washed with PBS twice, and analysed using a fluorescence microscope.

Statistical analysis

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

Effects of Kangen-karyu extract on acute oxidative stress induced by sublethal H_2O_2

Table 1 shows the effect of Kangen-karyu extract on ROS and TBA-reactive substance levels in TIG-1 cells under sublethal doses of H_2O_2. Although ROS generation of H_2O_2-treated TIG-1 cells increased significantly compared with non-treated cells, Kangen-karyu extract led to significant decreases in these levels in a dose-dependent manner. The TBA-reactive substance level, a parameter of lipid peroxidation, of H_2O_2-treated cells increased significantly from 100.0% to 147.0%. However, when TIG-1 cells were cultured with Kangen-karyu extract, the levels were

Table 1 Effect of Kangen-karyu extract on reactive oxygen species (ROS) generation and thiobarbituric acid (TBA)-reactive substance levels in TIG-1 cells treated with sublethal doses of H_2O_2

	ROS generation (% of non-treated cells)	TBA-reactive substance levels (% of non-treated cells)
None	100.0 ± 4.4	100.0 ± 0.9
H_2O_2 (50 μM)	153.1 ± 7.4$^{\#}$	147.0 ± 1.1$^{\#\#}$
H_2O_2 (50 μM) + Kangen-karyu (5 μg mL^{-1})	147.2 ± 7.5$^{\#}$	138.9 ± 2.1$^{\#\#,**}$
H_2O_2 (50 μM) + Kangen-karyu (10 μg mL^{-1})	137.5 ± 5.1$^{\#,*}$	132.4 ± 1.3$^{\#\#,**}$
H_2O_2 (50 μM) + Kangen-karyu (50 μg mL^{-1})	132.3 ± 4.2$^{\#,**}$	128.9 ± 2.1$^{\#\#,**}$
H_2O_2 (50 μM) + Kangen-karyu (100 μg mL^{-1})	120.9 ± 4.9$^{\#,**}$	120.8 ± 2.5$^{\#\#,**}$

$^{\#}P < 0.01$, $^{\#\#}P < 0.001$, significantly different compared with non-treated control cells; $^{*}P < 0.01$, $^{**}P < 0.001$, significantly different compared with H_2O_2-treated cells.

Table 2 Effect of Kangen-karyu extract on cell cycle disturbance in TIG-1 cells treated with sublethal doses of H_2O_2

	G_0/G_1	S	G_2/M
None	61.9 ± 0.4	24.0 ± 1.0	14.1 ± 1.4
H_2O_2 (50 μM)	63.8 ± 0.6$^{\#\#}$	19.2 ± 1.2$^{\#\#}$	17.0 ± 0.9$^{\#}$
H_2O_2 (50 μM) + Kangen-karyu (10 μg mL^{-1})	64.5 ± 0.2$^{\#\#\#}$	22.4 ± 1.4*	13.1 ± 1.6**
H_2O_2 (50 μM) + Kangen-karyu (100 μg mL^{-1})	63.7 ± 0.4$^{\#\#}$	22.3 ± 0.1*	14.0 ± 0.5*

$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significantly different compared with non-treated control cells; $*P < 0.05$, $**P < 0.01$, significantly different compared with H_2O_2-treated cells.

decreased dose dependently. At the concentration of 100 μg mL^{-1}, ROS generation decreased from 153.1% to 120.9% and TBA-reactive substance levels were reduced from 147.0% to 120.8%. The effect of Kangen-karyu extract on the cell cycle disturbance induced by H_2O_2 is represented in Table 2. After treatment with a sublethal dose of H_2O_2, the proportion of TIG-1 cells in the G_0/G_1 phase increased significantly from 61.9% to 63.8%, while cells treated with Kangen-karyu extract showed no changes in this proportion. On the other hand, the percentage of S-phase cells was decreased by H_2O_2 treatment, but Kangen-karyu extract-treated cells showed a higher percentage of S-phase cells compared with H_2O_2-treated control cells. In addition, against the significant increase in the number of H_2O_2-treated control cells in the G_2/M phase, Kangen-karyu extract led to a decline in the percentage of cells in the G_2/M phase. Furthermore, the condition of acute oxidative stress induced by sublethal H_2O_2 resulted in the decline of cell viability to 67.0% compared with 100.0% of cells under the normal condition, as shown in Table 3. However, the treatment with Kangen-karyu extract increased cell viability significantly. At a concentration of 100 μg mL^{-1} Kangen-karyu extract, cell viability was elevated to 84.1%.

As shown in Figure 2, sublethal H_2O_2 treatment of TIG-1 cells resulted in the translocation of NF-κB from the cytosol to the nucleus, whereas this was not observed in untreated cells. However, treatment with 100 μg mL^{-1} Kangen-karyu inhibited the nuclear translocation of NF-κB. In addition, DAPI staining results also showed decreases in nuclear staining intensity in cells treated with Kangen-karyu extract.

Table 3 Effect of Kangen-karyu extract on cell viability of TIG-1 cells treated with sublethal doses of H_2O_2

	Cell viability (% of non-treated cells)
None	100.0 ± 1.1
H_2O_2 (50 μM)	67.0 ± 2.2$^{\#}$
H_2O_2 (50 μM) + Kangen-karyu (5 μg mL^{-1})	76.5 ± 2.9$^{\#,*}$
H_2O_2 (50 μM) + Kangen-karyu (10 μg mL^{-1})	78.2 ± 1.5$^{\#,*}$
H_2O_2 (50 μM) + Kangen-karyu (50 μg mL^{-1})	83.5 ± 2.4$^{\#,**}$
H_2O_2 (50 μM) + Kangen-karyu (100 μg mL^{-1})	84.1 ± 2.4$^{\#,**}$

$^{\#}P < 0.001$, significantly different compared with non-treated control cells; $*P < 0.01$, $**P < 0.001$, significantly different compared with H_2O_2-treated cells.

Figure 3 reveals that SA-β-Gal activity in TIG-1 cells was elevated in response to H_2O_2-induced oxidative stress, while treatment with Kangen-karyu extract reduced the SA-β-Gal activity, as observed with the decrease in the staining intensity.

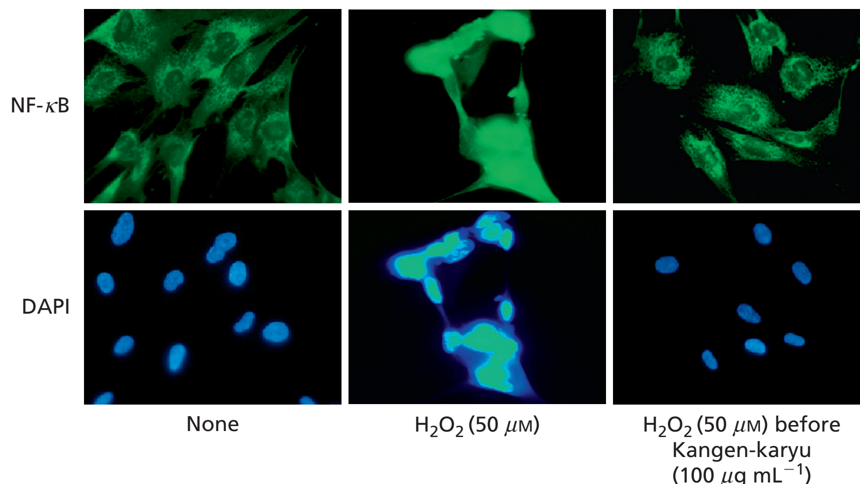

Figure 2 Translocation of nuclear factor-κB (NF-κB) on sublethal H_2O_2-induced premature senescence. After treatment with 50 μM of H_2O_2 for 60 min to TIG-1 human diploid fibroblasts, the cells were treated with vehicle or Kangen-karyu extract. Upper panel, nuclear NF-κB translocation; lower panel, DAPI staining.

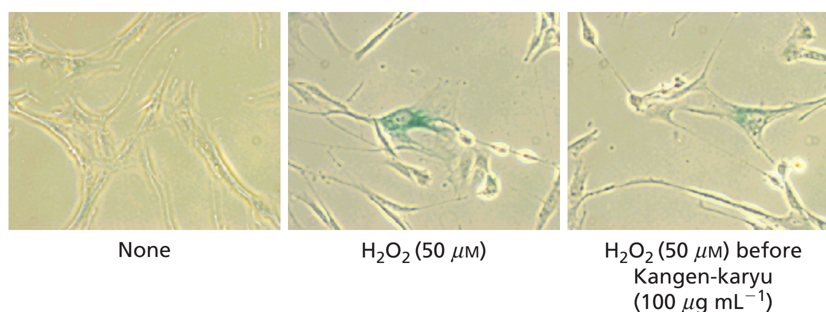

Figure 3 Senescence-associated β-galactosidase (SA-β-Gal) activity on sublethal H_2O_2-induced premature senescence. After treatment with vehicle or Kangen-karyu extract followed by 50 μM of H_2O_2 for 60 min, SA-β-Gal activity was determined by microscopy.

Effect of Kangen-karyu on chronic oxidative stress by repeat treatment of low-dose H_2O_2

Table 4 shows the effect of Kangen-karyu extract on ROS and TBA-reactive substance levels in TIG-1 cells treated repeatedly with low-dose H_2O_2. ROS generation and TBA-reactive substance levels of H_2O_2-treated control TIG-1 cells increased markedly compared with non-treated cells, while they were decreased by Kangen-karyu extract. The treatment with 100 μg mL^{-1} Kangen-karyu extract led to a decrease in ROS generation from 495.9% to 136.2% and TBA-reactive substance levels from 34.35 to 1.34 nmol/10^5 cells. Table 5 also shows the effect of Kangen-karyu extract on cell cycle disturbance under chronic oxidative stress caused by H_2O_2. The arrest of the G_0/G_1 phase was observed in TIG-1 cells treated with H_2O_2, while the disturbance of the cell cycle by oxidative stress was significantly normalized by treatment with Kangen-karyu extract through the decrease in the G_0/G_1 phase and increase in S and G_2/M phases.

As shown in Figure 4, the low-dose H_2O_2 treatment of HDFs led to the translocation of NF-κB from the cytosol to the nucleus; however, treatment with Kangen-karyu extract inhibited the nuclear translocation of NF-κB. This was also confirmed by the reduction in nuclear DAPI staining intensity.

As shown in Figure 5, SA-β-Gal activity in TIG-1 cells under chronic oxidative stress induced by H_2O_2 was elevated. On the other hand, Kangen-karyu treatment reduced the SA-β-Gal activity of H_2O_2-treated cells, as demonstrated by a decrease in the staining intensity.

To evaluate the effect of Kangen-karyu extract on the lifespan of TIG-1 cells under H_2O_2-induced cellular senescence, PDLs were determined. H_2O_2-exposed cells had a reduced lifespan compared with non-treated cells (from PDL 62.0 to PDL 32.0). In contrast, treatment with 100 μg mL^{-1} of Kangen-karyu extract markedly extended the lifespan of TIG-1 cells from PDL 32.0 to PDL 56.0.

Discussion

SIPS of HDFs has become a classic model for aging research, since senescence is caused by the exhaustion of the cellular proliferative potential with a change in the electron transport

Table 4 Effect of Kangen-karyu extract on reactive oxygen species (ROS) generation and thiobarbituric acid (TBA)-reactive substance levels in TIG-1 cells treated repeatedly with low-dose H_2O_2

	ROS generation (% of non-treated cells)	TBA-reactive substance levels (nmol/10^5 cells)
None	100.0 ± 1.8	1.53 ± 0.03
H_2O_2 (5 μM)	495.9 ± 1.9$^{\#\#}$	34.35 ± 0.39$^{\#\#}$
H_2O_2 (5 μM) + Kangen-karyu (10 μg mL^{-1})	138.8 ± 1.5$^{\#\#,*}$	1.39 ± 0.02$^{\#,*}$
H_2O_2 (5 μM) + Kangen-karyu (100 μg mL^{-1})	136.2 ± 1.7$^{\#\#,*}$	1.34 ± 0.03$^{\#,*}$

$^{\#}P < 0.05$, $^{\#\#}P < 0.001$, significantly different compared with non-treated control cells; $^{*}P < 0.001$, significantly different compared with H_2O_2-treated cells.

Table 5 Effect of Kangen-karyu extract on cell cycle disturbance in TIG-1 cells treated repeatedly with low-dose H_2O_2

	G_0/G_1	S	G_2/M
None	57.1 ± 0.2	15.0 ± 0.5	27.9 ± 0.4
H_2O_2 (5 μM)	88.1 ± 0.3$^{\#\#}$	6.6 ± 0.1$^{\#\#}$	5.3 ± 0.1$^{\#\#}$
H_2O_2 (5 μM) + Kangen-karyu (10 μg mL^{-1})	57.2 ± 0.2*	15.7 ± 0.1*	27.1 ± 0.2*
H_2O_2 (5 μM) + Kangen-karyu (100 μg mL^{-1})	59.4 ± 0.5$^{\#,*}$	15.0 ± 0.2*	25.6 ± 0.7$^{\#,*}$

$^{\#}P < 0.05$, $^{\#\#}P < 0.001$, significantly different compared with non-treated control cells; $^{*}P < 0.001$, significantly different compared with H_2O_2-treated cells.

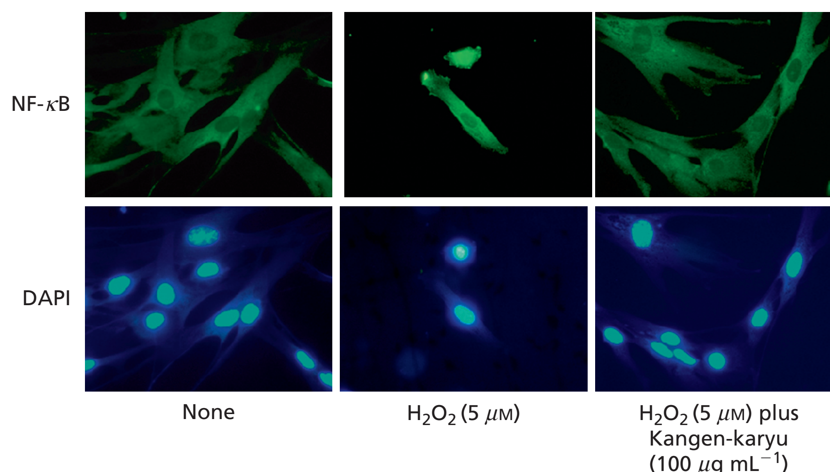

Figure 4 Translocation of nuclear factor-κB (NF-κB) on chronic low-dose H_2O_2-induced premature senescence. After treatment with 5 μM of H_2O_2 for 60 min a day for 3 days to TIG-1 human diploid fibroblasts, the cells were treated with vehicle or Kangen-karyu extract. Upper panel, nuclear NF-κB translocation; lower panel, DAPI staining.

capacity, suppression of antioxidant defence, and oxidant generation (Wolf et al 2002). HDFs, including WI-38 and TIG-1 human fibroblast cells, also showed an elevation in cellular oxidant production associated with RS. Furthermore, after exposure to several oxidative triggers, including H_2O_2, hyperoxia, or *tert*-butylhydroperoxide (*t*-BHP), the phenomena associated with RS are also observed in HDFs, showing the morphological phenotype of senescence (a decrease in cell saturation density, and increase in cell surface area and volume), a large increase in the population of cells showing SA-β-Gal activity, expression of senescence-associated genes, mitochondrial DNA deletion, and cell cycle regulation disorders (Toussaint et al 1995; Chen et al 1998; Dumont et al 2000). Consistent with these pieces of evidence, our results also showed that H_2O_2-treated WI-38 cells exhibited cellular senescence due to oxidative stress (Satoh et al 2005). Under the well-established cellular aging model for the evaluation of the anti-aging effect against oxidative stress, our previous study demonstrated the preventive efficacy of Kangen-karyu extract against H_2O_2-induced premature senescence (Satoh et al 2004, 2005). However, its therapeutic potential under H_2O_2-induced premature senescence was obscure. Therefore, the present study was carried out to study the effect of Kangen-karyu extract after the induction of premature senescence by acute oxidative stress via sublethal H_2O_2. Furthermore, we treated HDFs repeatedly with low-dose H_2O_2, in order to mimic chronic oxidative stress under pathophysiological conditions, and then the anti-aging activity of Kangen-karyu was evaluated.

It is well accepted that ROS generation in organs and cells is closely related to the aging process (Sohal et al 1994). Aged animals have defective mitochondria that can produce higher levels of ROS than those of their young counterparts, suggesting the elevation of mitochondrial ROS with aging. Furthermore, increased ROS generation under conditions of RS and SIPS due to several triggers, including H_2O_2 and *t*-BHP, leads to cell death (Teramoto et al 1999). Our previous study demonstrated that Kangen-karyu scavenged •OH, the most reactive and toxic radical produced by H_2O_2 degradation, in an in-vivo aging model (Satoh et al 2004). In addition, Kangen-karyu inhibited the generation of superoxide anion and nitric oxide. The present study also showed that SIPS caused by acute and repetitive oxidative stress increased intracellular ROS generation (Tables 1 and 4). However, treatment of TIG-1 cells with Kangen-karyu extract under conditions of SIPS decreased ROS generation, thereby attenuating oxidative stress. Oxidative stress-induced damage has several effects on intracellular biomolecules, such as lipids, proteins and DNA, and eventually

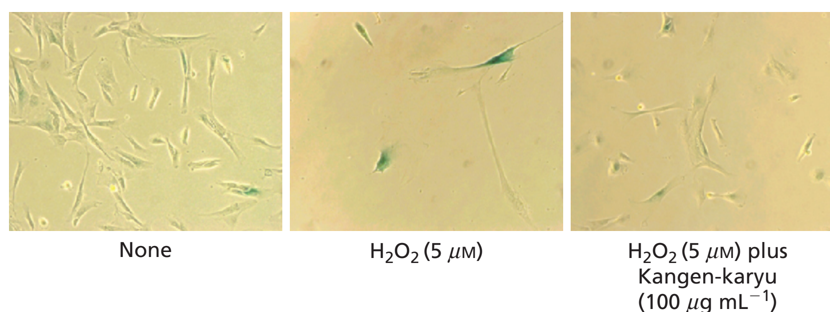

Figure 5 Senescence-associated β-galactosidase (SA-β-Gal) activity on chronic low-dose H_2O_2-induced premature senescence. After treatment with vehicle or Kangen-karyu extract followed by 5 μM of H_2O_2 for 60 min a day for 3 days, SA-β-Gal activity was determined by microscopy.

leads to cellular senescence. Morliere & Santus (1998) reported that the exposure of human skin fibroblasts to *t*-BHP, UV-A, and H_2O_2 resulted in the release of high levels of TBA-reactive substance into the media, an index of lipid peroxidation. The present study also showed that the TBA-reactive substance level in TIG-1 cells increased significantly in response to exposure to H_2O_2 (Tables 1 and 4). In contrast, treatment of TIG-1 cells with Kangen-karyu extract under SIPS inhibited lipid peroxidation and was associated with the attenuation of oxidative stress.

The mechanisms responsible for the aging process include NF-κB translocation to the nucleus for its activation. NF-κB is a powerful transcriptional factor that plays a pivotal role in the regulation of the immune system and inflammatory response with the modulation of several genes and 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, tumour necrosis factor-α, interleukin-1, UV light, hypoxia, lipopolysaccharide and H_2O_2, the NF-κB dimer dissociates from IκB and translocates to the nucleus, which is followed by NF-κB activation (Sen & Baltimore 1986; Stein et al 1989; Cordle et al 1993; DiDonato et al 1995; Baldwin 1996; Schmedtje et al 1997). Our present study demonstrated that HDFs under the condition of SIPS caused by sublethal H_2O_2 and a chronic low-dose of H_2O_2 showed NF-κB translocation to the nucleus from the cytosol (Figures 2 and 4), suggesting that cells undergoing SIPS would show NF-κB translocation following oxidative stress. Furthermore, our previous result on quantitative measurement of NF-κB protein expression showed that Kangen-karyu led to the decrease in the expression of nuclear NF-κB protein (Yokozawa et al 2006). Therefore, the prevention of NF-κB translocation resulting from oxidative stress shown by the present results suggest that Kangen-karyu extract exerts an anti-aging effect through NF-κB modulation.

The increase of SA-β-Gal activity and G_0/G_1 phase arrest of the cell cycle are the crucial characteristics of RS. Consistent with these findings, HDFs exposed to H_2O_2 showed an elevation in SA-β-Gal activity (Figures 3 and 5). However, Kangen-karyu extract exerted a protective effect against the increase in SA-β-Gal activity. These results imply that Kangen-karyu would exhibit a therapeutic role against H_2O_2-induced cellular senescence of HDFs. In addition, G_0/G_1 arrest in the cell cycle was observed in TIG-1 cells under SIPS by H_2O_2-induced acute and chronic oxidative stress (Tables 2 and 5). On the other hand, the increase in the percentage of cells in the G_0/G_1 phase was accompanied by a reduction in the percentage of cells in the S phase. Kangen-karyu extract did not exert an effect on cell cycle regulation under acute oxidative stress induced by sublethal H_2O_2 (Table 2), while the treatment with Kangen-karyu extract under SIPS by H_2O_2-chronic oxidative stress normalized the cell cycle by reducing the number of cells in the G_0/G_1 phase and elevating the proportion of cells in the S phase (Table 5), suggesting the effect of Kangen-karyu extract on cell cycle regulation.

It has been reported that the growth rate of HDFs is 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 (Table 3), suggesting that treatment with H_2O_2 inhibits the growth rate of HDFs and these premature replicative senescence phenomena were probably caused by the cell cycle arrest and oxidative stress by H_2O_2. However, Kangen-karyu extract improved cell viability via protection from H_2O_2-induced oxidative damage, through the decrease in ROS generation and TBA-reactive substance levels (Table 1). Furthermore, the treatment with Kangen-karyu extract prolonged the lifespan of TIG-1 cells under oxidative stress. 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 syndrome and Hutchinson-Gilford, also had a reduced lifespan in-vitro (Hayflick 1975; Allsopp et al 1992; Oshima et al 1995; Adelfalk et al 2001; Yegorov & Zelenin 2003). Therefore, the present findings suggest that Kangen-karyu might prolong not only the lifespan of cells in-vitro but also play a crucial role in the longevity of the organism.

Conclusion

The treatment of TIG-1 with H_2O_2 at a sublethal dose and repetitive low dose revealed the phenomena of RS in HDFs, accumulation of oxidative damage, increase of SA-β-Gal activity, NF-κB nuclear translocation from the cytosol, and disorder of cell cycle regulation. However, Kangen-karyu extract protected against cellular senescence as its therapeutic efficacy through attenuating oxidative damage with the inhibitions of ROS generation and lipid peroxidation, regulation of the cell cycle, and modulation of NF-κB nuclear translocation from the cytosol. In addition, Kangen-karyu led to prolongation of the cellular lifespan under oxidative stress. The present study suggests that Kangen-karyu might play a therapeutic role against the aging process caused by oxidative stress.

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