The antioxidative activity of Kangen-karyu extract delays senescence of human lung fibroblasts

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Replicative senescence (RS) of human diploid fibroblasts (HDFs) has become a classical model of aging and HDFs, such as WI-38 cells, display increased cellular oxidant production associated with RS. Several phenomena associated with RS are also observed in stress-induced replicative senescence (SIPS). In particular, SIPS of WI-38 cells caused by hydrogen peroxide (H_2O_2) is a useful and reasonable cellular aging model for evaluating the effects of potential anti-aging agents against oxidative stress. We used this well-established model to evaluate the anti-aging effect of Kangen-karyu, focusing on its antioxidant activity. Treatment of WI-38 cells undergoing SIPS caused by H_2O_2 with Kangen-karyu extract significantly reduced reactive oxygen species (ROS) generation and lipid peroxidation levels. In addition, the intracellular GSH levels, reflecting cellular ROS generation, were reduced by treatment with Kangen-karyu extract. These results suggest that Kangen-karyu attenuated the age-associated increase of cellular oxidative damage. Moreover, Kangen-karyu extract normalized the G_0/G_1 phase arrest and reversed the diminished cell viability resulting from exposure to H_2O_2. Furthermore, the extract prolonged the lifespan of WI-38 cells undergoing SIPS. This study suggests that Kangen-karyu may delay the aging process in cells undergoing SIPS by attenuating oxidative damage.

Key words Kangen-karyu, aging, WI-38, hydrogen peroxide, free radical.

Introduction

Aging is an inevitable physiological phenomenon caused by several factors and various theories on the aging process have been suggested. Among them, the most attractive and predominant theory, the free radical theory of aging, hypothesizes that various oxidative reactions occurring in an organism (mainly in mitochondria) generate free radicals which cause multiple lesions in macromolecules, leading to their damage and aging. Therefore, the oxidative damage induced by free radicals is believed to contribute to several age-associated disorders. On the basis of the free radical theory, research into aging has been carried out using *in vivo* models and cellular systems.

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[1] 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 they become senescent, showing cellular changes related to the aging process.[2-7] In addition, HDFs, including WI-38 cells, exhibit the stress-induced premature senescence (SIPS) phenotype after being subjected to many different sub-lethal stresses, including oxidative stress,[8] and this SIPS phenotype is almost identical to the phenotype associated with RS. Several studies have demonstrated that cells subjected to hydrogen peroxide (H_2O_2)-induced oxidative stress showed senescence, several changes typical of RS and oxidative damage of several components.[9-11] Therefore, we have used WI-38 fibroblast cells undergoing H_2O_2-induced cellular senescence to evaluate potential anti-aging compounds, focusing on the free radical theory of aging.

Antioxidants have been suggested to be promising agents for delaying the aging process associated with SIPS, as well as RS. Recently, great effort has been devoted to searching for antioxidants without toxicity and side effects, such as traditional crude drugs, Chinese medicinal prescriptions and functional foods. However, studies on the anti-aging potential and effects on cellular senescence of such preparations has not been carried out yet. Among the Chinese traditional prescriptions, Kangen-karyu, which is comprised of six crude drugs, has received much attention due to its numerous biological activities, such as inhibition of platelet aggregation and suppression of hypertension.[12-15] Furthermore, Takahashi *et al.*[16] demonstrated that Kangen-karyu affected the recovery of learning and memory impairment in the senescence-accelerated mouse by preserving the activities of choline acetyltransferase and superoxide dismutase (SOD) in the cerebellum. In addition, our previous study showed that Kangen-karyu inhibited the oxidative stress-related aging process in senescence-accelerated mice through enhancing antioxidative enzyme activity and scavenging reactive oxygen species (ROS) and eventually contributing to improvement of tissue dysfunction with age, suggesting that Kangen-karyu is a potential anti-aging compound.[17]

In this study, we used WI-38 human diploid fibroblasts

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with H_2O_2-induced oxidative damage to determine whether Kangen-karyu has anti-aging effects.

Materials and Methods

Materials. Basal medium of Eagle (BME) was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). 2',7'-Dichlorofluorescin diacetate (DCFH-DA), monochlorobimane (mBCl) and propidium iodide (PI) were purchased from Molecular Probes (Eugene, Oregon, USA). Calcium- and magnesium-free phosphate buffered saline (PBS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS), trypsin solution and ribonuclease A (RNase) were purchased from Life Technologies Inc. (Grand Island, NY, USA), Nakarai (Kyoto, Japan) and Funakoshi (Tokyo, Japan), respectively. Normal human lung diploid fibroblasts (WI-38) were purchased from the 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 (root of *Paeonia lactiflora* PALLAS), 2.25 g Cnidii Rhizoma (rhizome of *Cnidium officinale* MAKINO), 2.25 g Carthami Flos (petal of *Carthamus tinctorius* L.), 1.125 g Cyperi Rhizoma (rhizome of *Cyperus rotundus* L.), 1.125 g Aucklandiae Radix (root of *Aucklandia lappa* DCNE.) and 4.5 g Salviae Miltiorrhizae Radix (root of *Salvia miltiorrhiza* BUNGE). These herbs were extracted with 25 volumes 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.

Cell culture. WI-38 human diploid fibroblast cells were cultivated in 10-mm culture dishes containing BME medium 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. The end of their replicative lifespan was considered to have been reached when they were unable to complete one population doubling during a 4-week period which included three consecutive weeks of refeeding with fresh medium containing 10% FBS.[18]

Cellular senescence induced by oxidative stress. WI-38 cells were seeded at a density of 10^5 cells/ml in 6- or 96-well culture plates and incubated for 2 h, as described above. After treatment with Kangen-karyu extract (5-100 μg/ml) or equivalent volumes per ml of control vehicle (non-treatment), the cells were treated with 300 μM H_2O_2 for 60 min. This treatment was started when the population doubling level (PDL) of the fibroblasts was 37.2 and the comparison of each group was performed after calibrating by cell viability. In the experiment comparing the lifespans of Kangen-karyu extract-treated and non-treated cells, the media were changed after the addition of H_2O_2 and continued until the cultivated cells reached a crisis.

ROS generation. To measure ROS generation by cells, we used the methods of Wang and Joseph.[19] DCFH-DA is adapted to the detection of ROS and electron transfer processes. This assay measures the oxidative conversion of stable, non-fluorescent DCFH-DA to the highly fluorescent DCF in the presence of ROS. After treatment with Kangen-karyu extract in seeded WI-38 fibroblast cells, 100 μM (final concentration) 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, Switzerland) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

TBA-reactive substance levels. TBA-reactive substance levels in media were determined as described by Mihara and Uchiyama.[20] After treatment with Kangen-karyu extract, the cells were treated with 300 μM H_2O_2, the media were collected, treated with 0.67% TBA and 20% TCA and boiled at 100°C for 45 min. The mixtures were cooled with ice and extracted with n-BuOH. After centrifuging at 4,000 x 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).

Intracellular glutathione (GSH) levels. According to the method of Osseni *et al.*,[21] mBCl, which is a UV fluorogen bimane probe, reacts specifically with reduced GSH through glutathione transferase to form a fluorescent derivative. After treatment with Kangen-karyu extract and/or H_2O_2 in 96-well plates for 60 min, the cells were added to 100 μM (final concentration) mBCl solution and incubated for 60 min. The fluorescent reaction products were assayed on a microplate reader (Tecan, Switzerland) with an excitation wavelength of 360 nm and an emission wavelength of 480 nm.

Flow-cytometric cell cycle analysis. After treatment with Kangen-karyu extract and/or H_2O_2, the cells were collected by centrifugation, fixed for at least 30 min at 4°C in 3 ml 70% ice-cold EtOH, washed twice with PBS, incubated with RNase solution for 30 min at 37°C and then treated with PI at 4°C for 30 min. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) with laser excitation at 488 nm using a 639-nm band pass filter to collect the red PI 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.[22,23]

Cell viability. Cell viability was assessed using the MTT colorimetric assay.[24] To the cells treated with Kangen-karyu extract and/or H_2O_2 in each well of a 96-well culture plate, a 100-μl aliquot of MTT solution (1 mg/ml) was added, the cells were incubated for 4 h at 37°C and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 μl dimethyl sulfoxide and the absorbance at 540 nm of each well was read using a microplate reader (Model 3550-UV, Bio-Rad, Tokyo, Japan).

Lifespan evaluation. To evaluate the cellular lifespans

using the Cristofalo and Charpentier method,[18] the population doubling level (PDL) was determined as current PDL = last PDL + $\log_2$ (collected cell number/seeded cell number).

Statistics. Results are presented as means ± S.E. of five replicates for each of the independent experimental conditions. The effect of Kangen-karyu extract on each parameter was evaluated using Dunnett's test and differences at $p<0.05$ were considered to be statistically significant.

Results

ROS generation. Figure 1 shows the effect of Kangen-karyu extract on ROS generation by WI-38 cells treated with Kangen-karyu extract together with H_2O_2. Exposure of H_2O_2 to WI-38 cells led to marked ROS generation. However, treatment with 10, 50 and 100 μg/ml Kangen-karyu extract prior to exposure to 300 μM H_2O_2 decreased ROS generation significantly and concentration-dependently.

TBA-reactive substance levels. As shown in Fig. 2, the TBA-reactive substance level, a parameter of lipid peroxidation, of H_2O_2-treated WI-38 cells increased significantly compared with that of non-treated cells. When WI-38 cells were cultured with 5 μg/ml Kangen-karyu extract, the TBA-reactive substance level decreased dramatically to below the level of non-treated cells, but cells treated with concentrations over 5 μg/ml did not show a further decrease in TBA-reactive substance levels.

Intracellular GSH levels. The effect of Kangen-karyu extract on the intracellular GSH level of WI-38 fibroblasts treated with H_2O_2 is shown in Fig. 3. The intracellular GSH level of the H_2O_2-exposed cells increased significantly compared to that of non-treated control cells (from 100 to 150%, $p<0.001$). In contrast, Kangen-karyu extract pre-treated cells showed significant concentration-dependent decreases in the levels of intracellular GSH.

Cell cycle. Table 1 shows the effect of Kangen-karyu extract on the cell cycle disturbance induced by H_2O_2. After treatment with H_2O_2, the proportion of WI-38 cells in the G_0/G_1 phase increased significantly from 46.1 to 54.3%, but Kangen-karyu extract (100 μg/ml) pre-treated cells showed a significant decrease in this proportion from 54.3 to 49.5%. The percentage of S-phase cells was decreased by H_2O_2 treatment, but Kangen-karyu extract (100 μg/ml) pre-treated cells showed a higher percentage of S-phase cells than H_2O_2-treated control cells. Although the percentages of non-treated cells and H_2O_2-treated control cells in the G_2/M phase did not differ, Kangen-karyu extract (100 μg/ml) pre-treated H_2O_2-exposed cells had a lower percentage of cells in the G_2/M phase than the non-treated and H_2O_2-treated control cell cultures.

Cell viability. H_2O_2-exposed cells showed significantly decreased cell viability compared with non-treated cells ($p<0.001$). However, treatment with 5, 10 and 25 μg/ml Kangen-karyu extract prior to H_2O_2 exposure resulted in significant and concentration-dependent increases cell viability from 61.9% to 65.4, 67.0 and 74.6%, respectively. Treatment with more than 25 μg/ml Kangen-karyu extract did not improve cell viability further and viability reached a plateau.

Lifespan. To evaluate the effect of Kangen-karyu extract on the lifespan of cells undergoing H_2O_2-induced cellular senescence, we compared the PDLs. As represented in Fig. 5, H_2O_2-exposed cells had a reduced lifespan compared with non-treated cells (from PDL 44.2 to PDL 38.2). In contrast, treatment with 100 μg/ml Kangen-karyu extract dramatically extended the lifespan of WI-38 cells from PDL 38.2 to PDL 40.2.

Discussion

SIPS of HDFs has become a classical model for aging

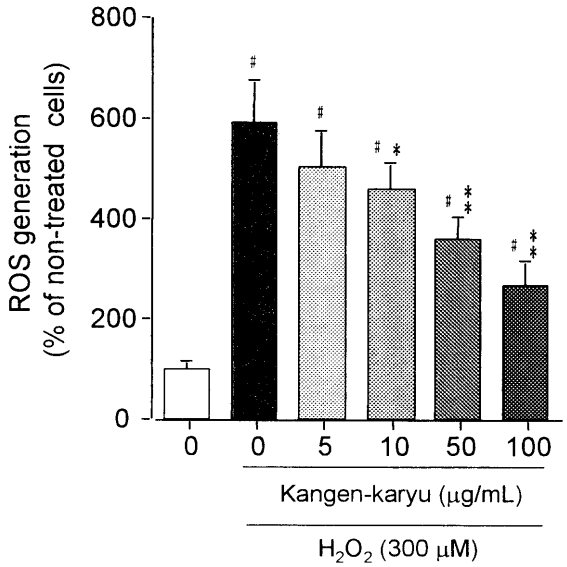

Fig. 1 Effect of Kangen-karyu extract on ROS generation. #$p<0.001$ vs. no treatment values; *$p<0.01$, **$p<0.001$ vs. H_2O_2 treatment values.

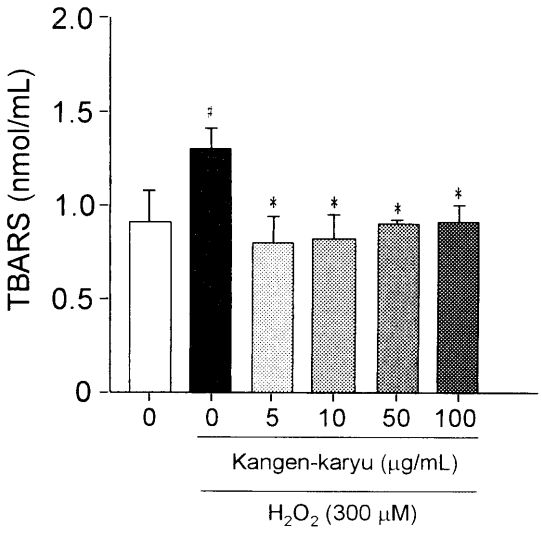

Fig. 2 Effect of Kangen-karyu extract on TBA-reactive substance levels. #$p<0.001$ vs. no treatment values; *$p<0.01$ vs. H_2O_2 treatment values.

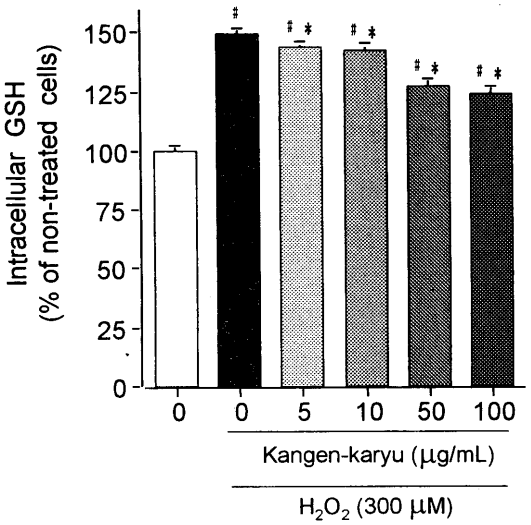

Fig. 3 Effect of Kangen-karyu extract on intracellular GSH levels. $^{\#}p<0.001$ vs. no treatment values; $^{*}p<0.001$ vs. H_2O_2 treatment values.

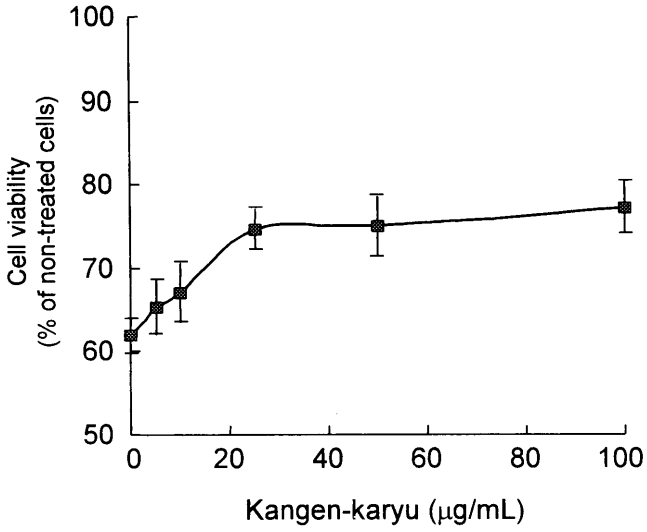

Fig. 4 Effect of Kangen-karyu extract on cell viability.

Table 1 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.

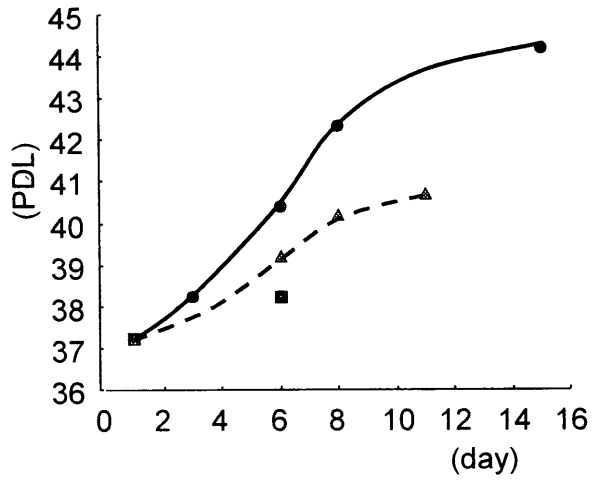

Fig. 5 Effect of Kangen-karyu extract on PDL (none, ●; H_2O_2 treatment, ■; H_2O_2 plus Kangen-karyu extract treatment, △).

research, as senescence is caused by exhaustion of the cellular proliferative potential, a change in electron transport potential, suppression of antioxidant defenses and oxidant generation.[9] WI-38 human fibroblast cells also displayed increased cellular oxidant production associated with RS. Furthermore, after exposure to several 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 (decreased cell saturation density and increased cell surface area and volume), a large increase in the population of cells showing senescence-associated β-galactosidase activity, expression of senescence-associated genes, mitochondrial DNA deletion and cell cycle regulation disorders.[10,25,26] In particular, Wolf *et al.*[11] 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 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 of this extract.

Against SIPS, antioxidants play crucial roles in the prevention of cellular senescence. Okada and Okada[27] reported that antioxidants with free radical-scavenging activity delayed cellular aging-dependent degeneration by increasing GSH and catalase activities in human fibroblasts. Therefore, we expected Kangen-karyu extract to show anti-

aging effects on H_2O_2-treated HDFs due to its antioxidative activity.

ROS, including O_2^- and H_2O_2, are produced by the biochemical pathways of all aerobic cells in which mitochondria are the major sites of aerobic energy.[28] Aged animals have defective mitochondria that can produce higher levels of ROS than those of their young counterparts, suggesting that mitochondrial ROS levels of some organs and cells increase with aging.[29] Furthermore, increased ROS generation under conditions of RS and SIPS caused by several triggers, including H_2O_2 and t-BHP, induced cell death.[30] Our study also showed that SIPS caused by H_2O_2 increased intracellular ROS generation. In contrast, Kangen-karyu extract pretreatment of WI-38 cells under conditions of SIPS decreased ROS generation, thereby reducing oxidative stress (Fig. 1).

Oxidative stress-induced damage has effects on intracellular biomolecules, such as lipids, proteins and DNA, that are attributed to cellular senescence. Morliere and Santus[31] reported that exposing human skin fibroblasts in culture to t-BHP, ultraviolet-A and H_2O_2 resulted in the release of high levels of TBA-reactive substances into the media, an index of lipid peroxidation. The present study showed that the TBA-reactive substance level detected in the medium of WI-38 cells also increased significantly in response to exposure to H_2O_2. In contrast, treatment with Kangen-karyu extract reduced the magnitude of the lipid peroxidation level elevation and this was associated with the attenuation of oxidative stress. However, it should be noted that the changes in the TBA-reactive substance levels of Kangen-karyu-treated cells we observed in the present study may not have been entirely concentration-dependent. Conversely, Kangen-karyu extract reduced ROS generation concentration-dependently. The difference between these effects may be due to the different sites of analysis: ROS generation was measured in cells in the endogenous oxidative state, whereas TBA-reactive substance levels were measured in the culture media and reflected exogenous oxidative damage.

Antioxidant defense systems that include antioxidant enzymes and antioxidant compounds play crucial roles in protecting against cellular senescence caused by free radicals. However, our study showed that intracellular GSH levels of HDFs treated with H_2O_2 increased compared with those of non-treated HDFs. We propose that exposing HDFs to high concentrations of H_2O_2 resulted in a strong oxidative status and consequently, the HDFs up-regulated the antioxidant defense system in order to resist cellular oxidative damage. The present results are supported by those of others who demonstrated that GSH levels of contact-inhibited quiescent HDFs increased following H_2O_2-treatment. In addition, these cells did not show the signaling of cell death induced by DNA damage, such as p38 MAP kinase activation or Bax over-expression, phenomena that are meant to resist oxidative stress.[32] However, Kangen-karyu extract prevented the GSH level increase, reflecting relatively low oxidative status and ROS generation after H_2O_2-treatment. Accordingly, the ability of Kangen-karyu extract to attenuate the oxidative status could mean this prescription has anti-aging potential.

The characteristics of RS of HDFs included G_0/G_1 phase arrest of the cell cycle.[11] 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 1), implying there is a correlation between a quiescent state of cells, such as growth arrest, and the antioxidant defense system. Our results clearly indicate that Kangen-karyu can prevent H_2O_2-induced growth arrest of HDFs by preventing G_0/G_1 phase arrest under cell cycle distribution as well as reducing ROS generation and oxidative damage.

The enhancement of oxidative stress by several factors, including ROS generation, results in diminished cell viability.[28] The viability of WI-38 cells was reduced by H_2O_2-induced oxidative damage. However, Kangen-karyu extract pre-treatment improved cell viability by protecting against H_2O_2-induced oxidative damage, reflected by decreased ROS generation and TBA-reactive substance levels (Fig. 4). H_2O_2 treatment induced apoptotic cell death and/or cell cycle arrest by contact inhibition and resistance to oxidative stress-induced apoptosis, phenomena which are irreversible.[32] The present results demonstrated that treating cells with 25 μg/ml or more Kangen-karyu extract did not improve cell viability in a concentration-dependent manner. These observations suggest that some of the H_2O_2-exposed cells were undergoing apoptosis, the associated oxidative stress of which is never protected, on account of the high concentrations of H_2O_2 to which they were exposed.

In addition, H_2O_2-induced HDFs lose the ability to proliferate and then reach, whereas Kangen-karyu extract treatment prolonged the lifespan of HDF cells (Fig. 5). 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, had a reduced lifespan *in vitro*.[33-37] Therefore, the present finding of prolongation of the lifespan of WI-38 cells by Kangen-karyu suggests that this prescription might prolong not only the lifespan of cells *in vitro* but also longevity of the organism.

In this study, we used H_2O_2-treated HDFs as an *in vitro* aging model and 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. In addition, our results suggest that Kangen-karyu extract may delay the aging process by virtue of its antioxidative effects.

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Japanese abstract

ヒト線維芽細胞の示す細胞老化は，老化モデルとして用いられており，老化とともに酸化傷害の増加が見られる。細胞老化に見られる種々の現象は，ストレス誘導性細胞老化にも同様に観察され，特に過酸化水素により引き起こされるWI-38の細胞老化は，酸化ストレスに着目した抗老化作用を検討するための有効な老化モデルとして用いられている。我々は，この確立された老化モデルを用い，冠元顆粒の抗老化作用を抗酸化作用に着目して検討した。WI-38を冠元顆粒で前処理し，その後過酸化水素を添加した場合，前処理しない細胞と比較して，活性酸素量と過酸化脂質量が有意に減少した。それに加え，細胞内活性酸素量を反映する細胞内グルタチオン量は，冠元顆粒の前処理により減少した。これらの結果から，冠元顆粒は加齢に伴う細胞内酸化傷害の増加を軽減していることが示唆された。さらに冠元顆粒は，過酸化水素添加による細胞周期のG_0/G_1期停止を改善し，細胞生存率を改善した。また，分裂寿命の有意な上昇が認められた。本研究により，冠元顆粒は酸化ストレスを調節することによって，細胞老化を遅延していることが示された。

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