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Full Length Research Paper

Anti-aging effect of black rice against H_2O_2-induced premature senescence

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This study was focused on the anti-aging effect of black rice under stress-induced premature senescence (SIPS) of WI-38 human diploid fibroblasts cells caused by hydrogen peroxide (H_2O_2), a well-established experimental model of cellular aging. The protective effect of the MeOH extract from black rice against H_2O_2-induced premature senescence was investigated using WI-38 cells by evaluating the cell viability, lipid peroxidation and cell life span. H_2O_2-treated WI-38 cells exhibited the phenomena of SIPS, the loss of cell viability, the increase of lipid peroxidation and shortening of the cell lifespan. However, the treatment with black rice attenuated cellular oxidative stress through increase in the cell viability and inhibition in lipid peroxidation. In addition, the life span of WI-38 cell showed extension of the population doubling level, suggesting that it would delay aging process. These results suggest that the MeOH extract from black rice may delay the aging process by attenuation of oxidative stress under SIPS cellular model.

Key words: Black rice, ageing, WI-38 cell, premature senescence, oxidative stress.

INTRODUCTION

Aging is an inevitable biological process that affects most living organisms. There are several theories of aging, error-catastrophe, protein modification, free radical (oxidative stress), mitochondrial DNA and some developmental-genetic theories, including the longevity gene (Kinight, 2000; Hipkiss, 2003; Troen, 2003; Oliveira et al., 2010; Rattan, 2010). The current view on free radical-induced oxidative damage is that most physiological and pathological changes related to aging are due to molecular and cellular damage caused by free radicals (Harman, 1983; Beckman and Ames, 1998). 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 accumulation of injuries caused by ROS is an important factor determining the life spans of living cells and the body. On the other hand, the antioxidative defense system that protects against the oxidative damage caused by ROS is suppressed with age, 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 is an important factor that counteracts aging and age-associated disorders. Many experimental studies support the suggestion that administration of antioxidant agents can prevent the development of age-associated disorders such as cancer (Kim et al., 1998), cardiovascular disorders (Inoue et al., 1990; Kondo et al., 1994; Yokozawa et al., 1998) and some neurodegenerative disorders (Sano et al., 1997).

Black rice has long been consumed in Korea, Japan and China (Ryu et al., 2000; Kowalczyk et al., 2003; Han et al., 2004). A recent report showed that black rice may have beneficial effects on various pathologies to reduce the risks of degenerative diseases such as cardiovascular

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disorder and cancer (Ling et al., 2001; Xu et al., 2001; Ling et al., 2002; Xia et al., 2003). It has anti-inflammatory (Chen et al., 2006), antioxidative (Ichikawa et al., 2001; Chiang et al., 2006; Park et al., 2008) and anti-hyperlipidemic activities (Guo et al., 2007). It contains bioactive anthocyanins such as cyanidin-3-glucoside, peonidin-3-glucosie, and its metabolites cyanidin and protocatechuic acid that are well-known to alleviate oxidative stress and inflammation (Hou et al., 2010; Min et al., 2010). Chiang et al. (2006) reported that anthocyanin from black rice attenuated oxidative stress by reducing ROS and increasing antioxidant enzyme activities both *in vitro* and *in vivo*. In addition, anthocyanin is associated with the reduction of cardiovascular diseases, cancer and liver damage (Chen et al., 2006; Mauray et al., 2010; Hou et al., 2010). On the other hand, the effect on aging and lifespan has not been studied yet.

Many investigators have developed several models for studying human aging (Kuro-o, 2001). To study aging-associated molecular changes, the cellular model using human diploid fibroblasts (HDFs) has become a classical experimental model of cellular aging (Chen and Ames, 1994; Chen, 2000, Toussaint et al., 2000). After serial passage, HDFs lose the ability to proliferate and become senescent, showing cellular changes related to the aging process (Hayflick, 1976; Linskens et al., 1995), so-called replicative senescence (RS). In addition, HDFs exhibit the stress-induced premature senescence (SIPS) phenotype after being subjected to different sub-lethal stresses, including oxidative stress, and this SIPS phenotype is almost identical to the phenotype associated with RS (Dumont et al., 2000). In the present study, we investigated the effects of black rice on anti-aging activity and its life span from aging under SIPS using WI-38 human fibroblast cell.

MATERIALS AND METHODS

Basal medium of eagle (BME), 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-2thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT), dimethyl formaldehyde, dimethyl sulfoxide (DMSO), potassium ferrocyanide and potassium ferricyanide, Nonidet P-40 (NP-40) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and trypsin solution were obtained from Life Technologies Inc. (Grand Island, NY, USA), and Nakarai (Kyoto, Japan), respectively.

Preparation of methanol (MeOH) extracts of black rice

Black rice was obtained from Jeon-ju, Korea. Whole black rice was washed, dried, and ground to powder. 10 g of black rice powder was refluxed with 200 ml of MeOH water for 24 h at room temperature and vacuum filtered through Whatman No.4 filter paper. This was repeated three times, and the extract was concentrated using a rotary evaporator at 34°C. The yield of MeOH extract from black rice was 3.7% (w/w). This dried extract was kept in a deep freezer at -80°C until used. The extract was dissolved in PBS for the experiments.

Cell culture and treatment protocols

WI-38 cells (human embryonic lung-derived diploid fibroblasts) were originally obtained from ATCC (Manassas, VA, USA). They were cultured in complete medium (Modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) in a humidified incubator at 37°C and 5% CO_2. The cells were subcultured with 0.05% trypsin-EDTA in PBS. Cells at early passages (between 26 and 33 passages) were used in all experiments to avoid complications of replicative senescence, since WI-38 cells have a mean life span about 45 to 60 passages. WI-38 cells were seeded at 1×10^5/well in 96-well plates and preincubated for 2 h. The cells were pretreated with/without black rice extracts followed by 300 µM H_2O_2 for 60 min. 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/without each concentration (5 to 100 µg/ml) of black rice extract. Chronic oxidative stress was induced by adding 5 µM H_2O_2 for 60 min in a day for 3 days.

Thiobarbituric acid-reactive substance levels

Thiobarbituric acid (TBA)-reactive substance levels were determined as described by Mihara and Uchiyama (1978). After treatment with black rice and/or H_2O_2, 0.67% TBA and 20% trichloroacetic acid were added and 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.

Cell viability

Cell viability was assessed using the MTT colorimetic assay (Mosmann, 1983). The cells were pretreated with/without black rice extracts followed by H_2O_2 and then incubated in fresh medium for 60 min. A 100-µl aliquot of MTT solution (1 mg/ml) 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.

Cell lifespan

The population doubling level (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 and Charpentier (1980).

Statistical analysis

All statistical analysis was performed by SAS software (SAS Institute, Cary, NC, USA). $P < 0.05$ was determined as statistically significant. Measurement data (n = 5) were expressed as mean ± standard deviation.

RESULTS

Treatment of black rice extract prior to exposure of H_2O_2

Figures 1 and 2 show the effect of black rice extract on

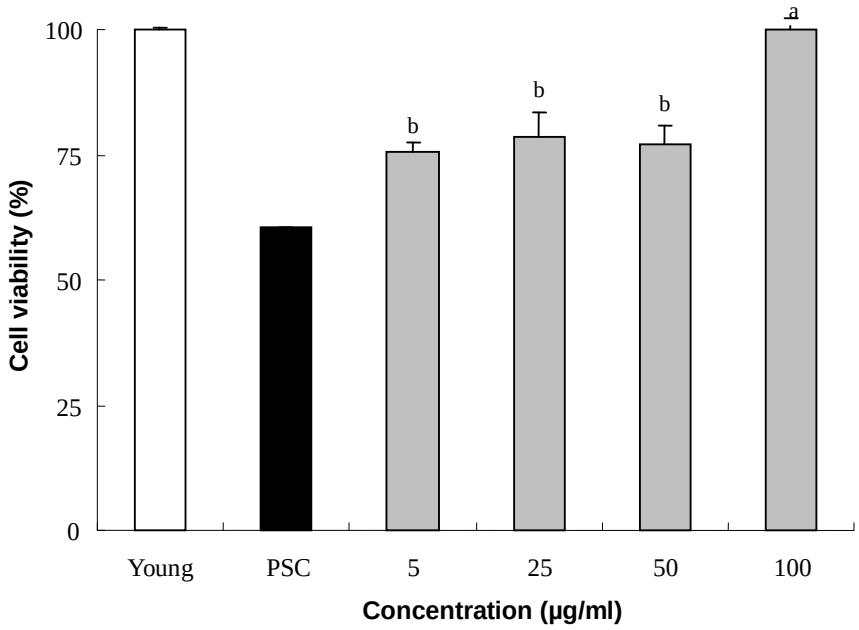

Figure 1. Effect on viability of treatment of black rice extract prior to H_2O_2-induced premature senescence in WI-38 cells. PSC, premature senescence control. Values are mean ± SD. a - b, means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

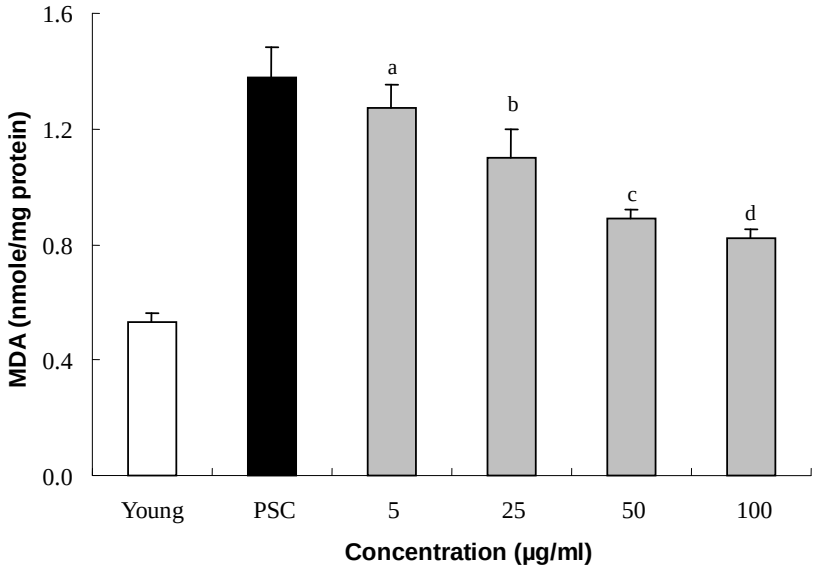

Figure 2. Protective effect on TBARS generation of treatment of black rice extract prior to H_2O_2-induced premature senescence in WI-38 cells. PSC; premature senescence control. Values are mean ± SD. a – d, means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

cell viability and MDA level under premature senescence in WI-38 cell induced by H_2O_2 treatment. The treatment of 300 μM H_2O_2 for 60 min to WI-38 cells decreased significantly the cell viability to 60.3% (Figure 1). However, treatment of MeOH extract from black rice showed dose-dependent increase in cell viability with significance. At the treatment of 5 and 100 μg/ml concentrations, the cell viability was elevated to 75.5 and 99.9%, respectively. In

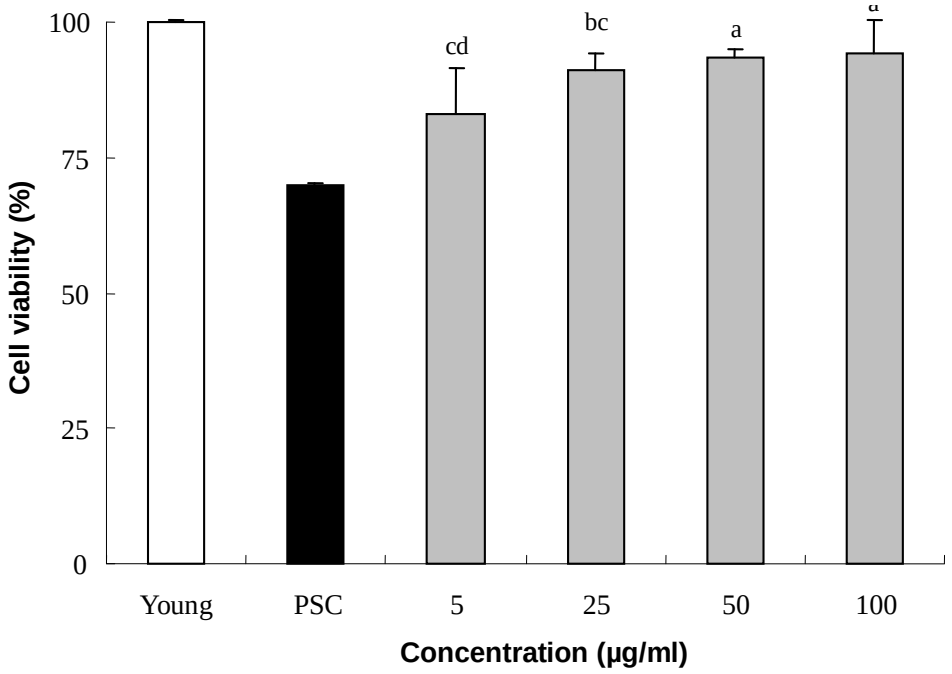

Figure 3. Effect on viability of treatment of black rice extract after H_2O_2-induced premature senescence in WI-38 cells. PSC; premature senescence control. Values are mean ± SD. a-d, means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

addition, the malondialdehyde (MDA) level, a parameter of lipid peroxidation, of H_2O_2-treated premature senescence cells was markedly increased from 0.53 to 1.38 nmole/mg protein (Figure 2). When black rice extract to premature senescence cells were treated, the MDA level was decreased dramatically as a concentration-dependent manner. The MDA production was inhibited to 0.82 nmole/mg protein from 1.38 nmole/mg protein at 100 μg/ml concentration (decrease of 40.6%).

Treatment of black rice extract after exposure of H_2O_2

The effect of black rice extract on cell viability and MDA levels in WI-38 cells under sublethal dose of H_2O_2 treatment was shown in Figures 3 and 4. The treatment of 50 μM H_2O_2 for 60 min to WI-38 cells decreased significantly the cell viability to 69.8% (Figure 3). However, black rice extract led to significant increase in cell viability as dose-dependent manner. At the treatment of 5 and 100 μg/ml concentrations, the cell viability was elevated to 83.0 and 94.3%, respectively. In addition, the MDA level of H_2O_2-treated premature senescence cells increased significantly from 0.45 to 1.15 nmole/mg protein that was elevated up to 2.6 folds (Figure 4). However, when WI-38 cells were cultured with black rice extract, the levels were decreased dose-dependent manner. The MDA production was inhibited to 0.61 and 0.48 nmole/mg protein from 1.15 nmole/mg protein by the treatment of black rice extract at 50 and 100 μg/ml concentration (decreases of 47.0 and 58.3%, respectively).

Effect of black rice extract on chronic oxidative stress by repeating treatment of H_2O_2

The effects of black rice extract on cell viability and TBA-reactive substance levels in WI-38 cells treated repeatedly with low-dose H_2O_2 were investigated (Figures 5 and 6). The treatment of 50 μM H_2O_2 for 60 min during 3 days to WI-38 cells decreased significantly the cell viability to 64.5% (Figure 5). However, black rice extract-treated cells showed dose-dependent increase in cell viability with significance. At the treatment of 100 μg/ml concentration, the cell viability was elevated to 82.8%. On the other hand, TBA-reactive substance levels of H_2O_2-treated control WI-38 cells increased markedly compared with non-treated cells, while it was decreased by the treatment of black rice extract. The treatment with 100 μg /ml black rice extract led to a decrease in TBA-reactive substance levels from 0.49 to 0.21 nmole/mg protein.

The effect of black rice extract on the lifespan of WI-38 fibroblast

As shown in Table 1, the treatment of black rice extract prolonged cellular lifespan through all age groups. The group of middle- and old-age extended the PDL from 62 to 64 and 60 to 62, respectively. In particular, the group of

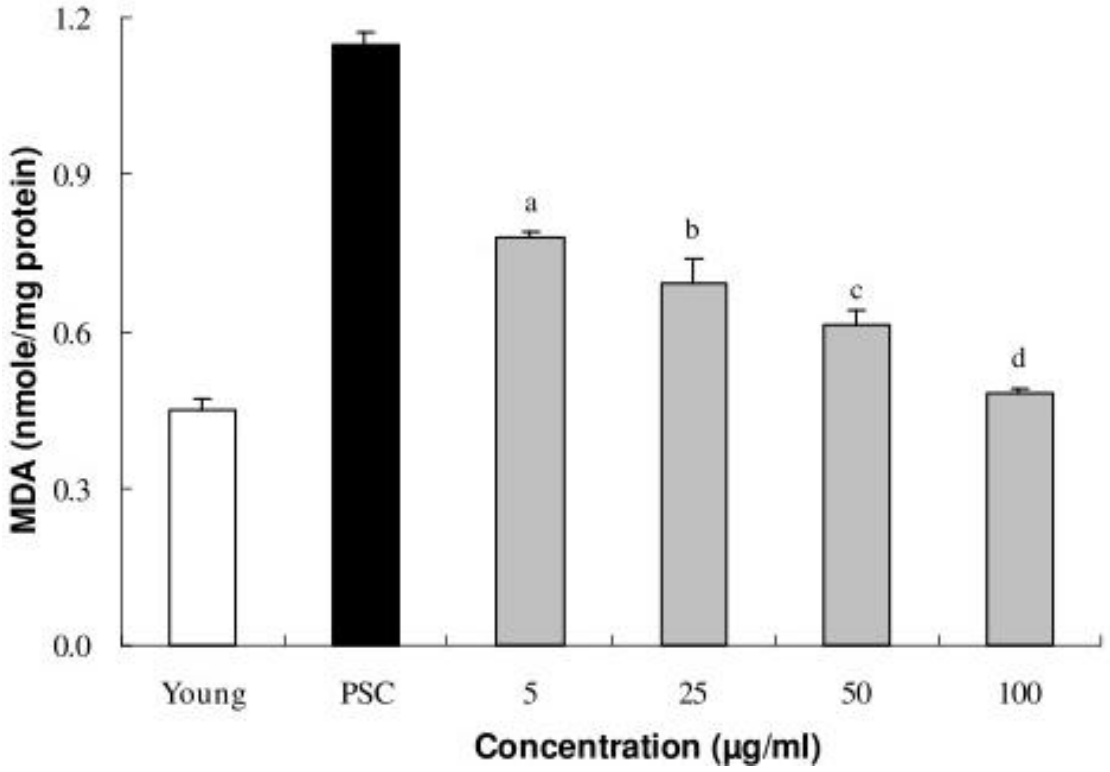

Figure 4. Protective effect on TBARS generation of treatment of black rice extract after H_2O_2-induced premature senescence in WI-38 cells. PSC, premature senescence control. Values are mean ± SD. a – d, means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

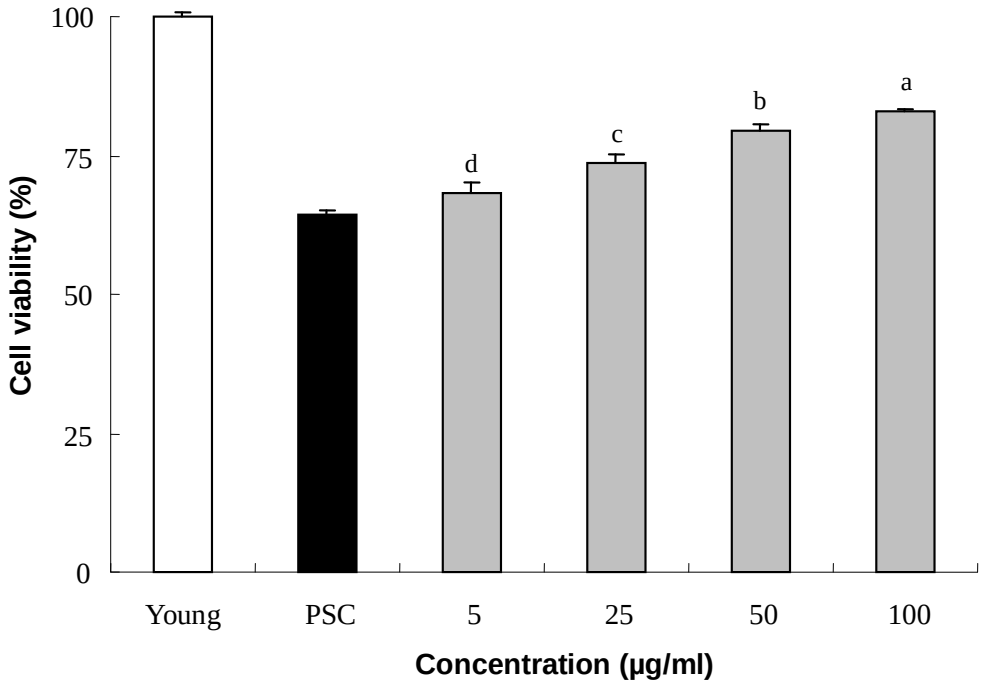

Figure 5. Effect of treatment of black rice extract on viability against premature senescence induced by repetitive and low dose of H_2O_2 treatment to WI-38 cells. PSC, premature senescence control. Values are mean ± SD. a – d, means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

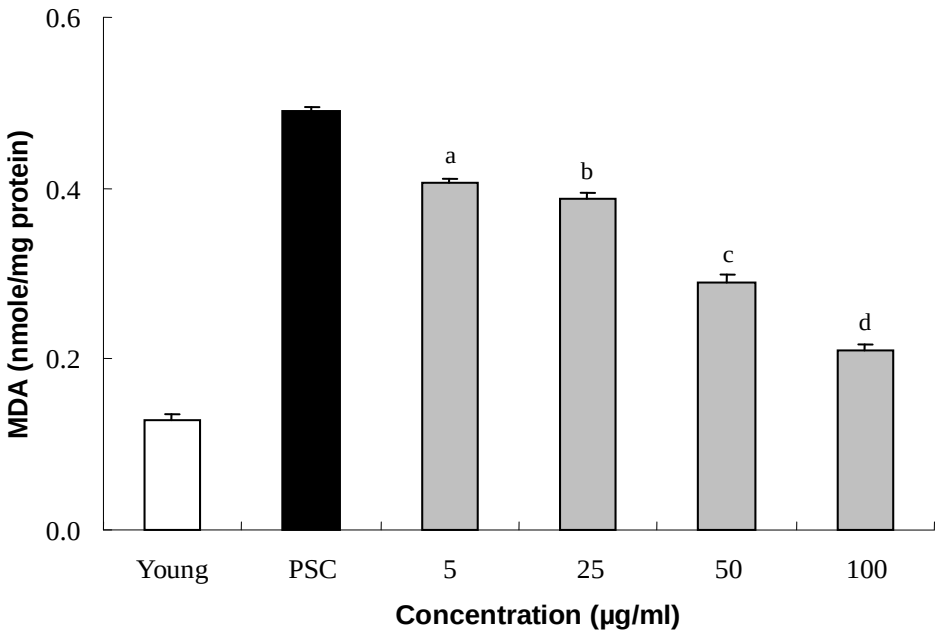

Figure 6. Effect of treatment of black rice extract on TBARS generation against premature senescence induced by repetitive and low dose of H_2O_2 treatment to WI-38 cells. PSC; premature senescence control. Values are mean ± SD. a – d, means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

Table 1. The effect of black rice extract on the lifespan of WI-38 fibroblast cell.

Group	Life span
Young age	PDL
None	26 → 64
Plus black rice	26 → 66
Middle age	PDL
None	42 → 62
Plus black rice	42 → 64
Old age	PDL
None	58 → 60
Plus black rice	58 → 62

PDL; Population doubling level PDL = Last PDL + $\log_2$ (collected cell number/seeded cell number).

Table 2. The effect of black rice extract on the lifespan of H_2O_2-induced WI-38 fibroblast cell.

Group	Life span
Young age	PDL
H_2O_2	26 → 28
H_2O_2 plus black rice	26 → 30
Middle age	PDL
H_2O_2	42 → 42
H_2O_2 plus black rice	42 → 44

PDL; Population doubling level PDL = last PDL + $\log_2$ (Collected cell number/seeded cell number).

young age showed extension of PDL the most effectively, from 64 to 66. Furthermore, the effect of black rice extract on the lifespan of cells undergoing H_2O_2-induced cellular senescence was also investigated (Table 2). The young age group of H_2O_2-treated premature senescence showed the decrease of lifespan to PDL of 28, while the treatment of black rice extract increased the PDL to 30. Moreover, the lifespan was prolonged to 44 from 42 of PDL in middle age group.

DISCUSSION

Aging is a complex and multifactorial phenomenon with molecular, cellular, and organizational aspects. The mechanisms of aging can be broadly categorized into either accumulation of damage or genetic control. Among the several theories of aging that have been suggested, the free radical theory of aging, various oxidative reactions occurring in organisms (many within mitochondria) generate free radicals, which cause multiple lesions in macromolecules, leading to their damage and the aging process (Knight, 2000; Oliveira et al., 2010). In addition, the decline in antioxidant defenses, as well as the progressive increase in oxidative stress, is commonly associated with aging and age-related degenerative diseases.

HDFs exhibit finite proliferative potential under *in vitro* (Hayflick and Moorhead, 1961). They undergo a limited number of population doubling before entering a state of

permanent growth arrest, referred to as "replicative senescence", "cellular senescence" or "cellular aging " (Campisi, 1996; Campisi et al., 2001), in which they remain alive and metabolically active but are completely refractory to mitogenic stimuli. HDFs offer the typical model for studying the process of aging under *in vitro*. Various oxidative stresses have been used to study the onset of cellular senescence, such as H_2O_2 (Frippiat et al., 2001; Frippiat et al., 2001), hyperoxia (Honda et al., 2001), tert-butylhyfroperoxide (t-BHP) (Toussaint et al., 1992) and UV (Rodemann et al., 1989).

Exposure of normal HDFs to H_2O_2 has been widely used as a model to study cellular aging phenomena. The exposure of HDFs to a sublethal concentration of H_2O_2 induces permanent cell cycle arrest and phenotypic changes that mimic replicatively senescent cells (Chen, 2000).

The SIPS of HDFs is caused by the exhaustion of cellular proliferation potential, a change in electron transport potential, the suppression of antioxidant defenses, and oxidant generation (von Zglinicki et al., 1995). WI-38 HDFs are a classical experimental model of cellular aging and are used to study aging-associated molecular changes in human cells after serial passage (Hayflick, 1976; Linskens et al., 1995).

After serial passage, WI-38 cells lose the ability to proliferate and become senescent, showing cellular changes related to the aging process termed RS. WI-38 human fibroblast cells also display elevations in cellular oxidant production associated with RS. Based on these facts, the SIPS of WI-38 cells caused by H_2O_2 is a useful and reasonable cellular aging model to evaluate the anti-aging effects of agents that counteract oxidative stress. Therefore, we used the SIPS of WI-38 cells caused by oxidative stress to evaluate the anti-aging effects of black rice.

WI-38 human fibroblast cells also display elevations in cellular oxidation production associated with RS. Therefore, an aging model of H_2O_2-induced cellular senescence was employed to investigate the protective effect of black rice extract on oxidative stress by prior treatment of H_2O_2. Therefore, the present study was carried out to study the effect of black rice extract after the induction of premature senescence by acute oxidative stress via sublethal H_2O_2. In addition, we treated HDFs repeatedly with low dose H_2O_2, in order to mimic chronic oxidative stress.

Black rice, as a special anthocyanin-rich cultivar of rice, has been regarded as a health-promoting food. The previous studies supported beneficial effects on health and it was associated with anthocyanin (Chen et al., 2006; Mauray et al., 2010, Hou et al., 2010). It includes cyanidin-3-glucoside, peonidin-3-glucoside, cyanidin-3, 5-diglucoside and cyanidin-3 rutinoside (Hou et al., 2010). In addition, the metabolites of anthocyanin, such as cyanidin and protocatechuic acid, were also demonstrated to have protective effects from oxidative stress and inflammation (Min et al., 2010; Hou et al., 2010). We also reported in the previous study that cyanidin protected cellular senescence induced by oxidative stress through the inhibition of nuclear factor-κB activation and down-regulation of inducible nitric oxide synthase and cyclooxygenase-2 (Choi et al., 2010). The growth rate of HDFs was reported to be delayed under condition of cellular senescence (Satoh et al., 2004; Matuoka et al., 2001). Consistent with these evidences, our results also showed that SIPS due to H_2O_2 treatment led to the loss of cell viability (Figure 1), suggesting that treatment with H_2O_2 led to premature replicative senescence (Chen, 2000; Toussaint et al., 2000). Phenomena were probably caused by the cell cycle arrest triggered by H_2O_2 and the effect of oxidative stress on cellular aging (Wolf et al., 1989; Wanga et al., 2004). The treatment of black rice extract improved cell viability by protecting against H_2O_2-induced oxidative damage.

Furthermore, in this study we have shown that SIPS caused by acute and repetitive oxidative stress decreased cell viability (Figures 3 and 5). However, treatment of WI-38 cells with black rice extract under conditions of SIPS increased cell viability, thereby attenuating oxidative stress.

Oxidative stress in cell has harmful effects on intracellular biomolecules, such as lipids, proteins and DNA, and eventually leads to cellular senescence. Morliere and Santus (1998) reported that the exposure of 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, an index of lipid peroxidation. MDA is used as a biological maker on lipid peroxidation. In the present result, MDA level was increased significantly in response to exposure to H_2O_2 (Figures 2, 4 and 6). In contrast, treatment of WI-38 cells with black rice extract under SIPS inhibited lipid peroxidation, suggesting the protective role of black rice extract against oxidative stress.

The attenuation of oxidative stress results in the extension of cellular lifespan (Tables 1 and 2). In case of WI-38 fibroblast cell, the lifespan of fibroblasts decreased with aging, however, the treatment of black rice extract prolonged the cell lifespan on all age groups (Table 1). In addition, the lifespan of fibroblasts cell induced by H_2O_2 premature senescence was also effectively prolonged by the treatment of black rice extract (Table 2). 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 had reduced lifespan (Hayflick, 1975; Yegorov and Zelenin, 2003). Therefore, the present finding of prolongation of lifespan in HDFs cell by black rice extract suggests the possibility that black rice extract might prolong not only the lifespan of cells *in vitro* but also longevity of organism, although further studies has to be supported. These findings suggest that black rice would play the beneficial role against aging process and lifespan.

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