Screening Methods for Anti-senescence Activity in Dermal Fibroblasts under Pyruvate-deprivation Conditions

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Abstract: The identification of compounds with anti-senescence activity in cell culture system is a first step in aging research. Given that pyruvate can be used energy source by conversion to acetyl-CoA in mitochondria, and protects cultured cell from various stress-induced cell damage and cell death, synthetic media (e.g., DMEM) often includes 1 mM pyruvate, which is very higher than the pyruvate concentration in human blood (approximately 30 μM). However, the use of medium containing high concentration of pyruvate is not suitable for screening anti-senescence compounds, because pyruvate also protects against the cellular senescence of primary human dermal fibroblasts (NHDFs) through NAD+ generated during conversion to lactate. In this study, four extracts, i.e., Sprouted seed and fruit complex, *Poncirus trifoliata* fruit extract, Jaum balancing complex, and *Prunus mume* extract were used for evaluation of different anti-senescence effect in the absence or presence of 0.1 mM pyruvate, similar to the physiological pyruvate concentration. The senescence in NHDFs cultured with DMEM in the presence of 0.1 mM pyruvate (approximately the physiological concentration in human blood) is accelerated, as observed in pyruvate deprivation conditions. The cytotoxicity of the *Poncirus trifoliata* fruit extract was protected by pyruvate, and Jaum balancing complex and *Prunus mume* extract had anti-senescence activity in the presence of 0.1 mM pyruvate, but not in the absence of pyruvate. Given that pyruvate is a powerful protector against both cytotoxicity and cellular senescence, the screening of candidate agents for anti-senescence in high pyruvate conditions using an in vitro cell culture system is not valid. Therefore, we recommend the use of a low concentration of pyruvate to evaluate the anti-senescence effects of candidates, which is more similar to *in vivo* aging conditions than excessive stress-induced senescence models, to exclude the effect of excessive pyruvate *in vitro*.

Keywords: Pyruvate, Senescence, Aging, Screening, Normal human dermal fibroblasts

1. Introduction

Aging is associated with a gradual loss of the cellular replicative capacity and specific tissue functions, resulting in an increase in aging-related diseases, such as metabolic diseases, neurodegenerative diseases, or cancer[1]. Thus, the identification of aging mechanisms and the development of anti-aging drugs are major focuses of research[2-4]. During aging, cellular senescence is a complex biological process induced by many extrinsic factors, including reactive oxygen species (ROS), inflammation, or sun exposure, as well as by intrinsic factors, including spontaneous replication or telomere shortening, resulting in mitochondrial and lysosomal dysfunction, cell cycle arrest, and increased senescence-associated secretory phenotypes (SASP)[1,5]. To identify the key molecular regulators of the human lifespan, the development of a primary screening method is necessary for the identification of the anti-senescence activity of new compounds or extracts with human diploid fibroblasts. Senescence in primary human diploid cells is accelerated by spontaneous replication, treatment with H_2O_2 or mitochondrial inhibitors, ionizing or UV irradiation, or transfection of a constitutively active oncogene[6-10]. However, these stress-induced senescence

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models, excluding spontaneous replication, are induced by excessive stimuli that do not occur *in vivo*, and the spontaneous replicative senescence model takes a lot of time to establish, generally several months.

We previously showed that pyruvate deprivation induces cellular senescence in primary human dermal fibroblasts (NHDFs) after 2 weeks[11]. Pyruvate, a crucial metabolite in energy generation, is derived by glycolysis from glucose or by direct importation from the extracellular region[12]. Although pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex as an energy source in the TCA cycle, it is also converted to lactate by lactate dehydrogenase and generates NAD+, a potent anti-aging molecule, from NADH[13-15]. Therefore, fibroblasts cultured in the absence of pyruvate for 12 days exhibit a premature cellular senescence under normal conditions by mitophagy defects via decrease in NAD+ generation and increase in senescence-associated problems, such as cell cycle arrest and the up-regulation of SASP[11]. Given that NHDFs cultured in Dulbecco's Modified Eagle's Media (DMEM) in the presence of 1 mM pyruvate are protected from replicative senescence compared with pyruvate deprivation conditions, they are unsuitable as an *in vitro* model for the screening of anti-senescence reagents.

Based on pyruvate deprivation-induced senescence, the anti-senescence effect of four extracts (Sprouted seed and fruit complex, *Poncirus trifoliata* fruit extract, Jaum balancing complex, and *Prunus mume* extract) were studied under a low concentration (0.1 mM or 0 mM) of pyruvate to minimize its protective role against senescence in the culture media. We found that the culture system with 0.1 mM pyruvate, but not with 1 mM pyruvate or with no pyruvate, is appropriate for evaluating the anti-senescence activity of novel reagents or extracts in NHDFs.

2. Material and Methods

2.1. Isolation and Preparation of Four Extracts

Sprouted seed and fruit complex

The Sprouted seed and fruit complex was composed of five crude herb materials, i.e., the *Prunus mume* fruit, *Pinus koraiensis* seed, *Chaenomeles sinensis* fruit, germinated *Rosa multiflora* fruit, and germinated black *Sesamum indicum* seed. Equal amounts of the dried herbs were extracted with four - or five - fold volumes (v/w) of an ethanol solution [50% (v/v) ethanol-water] at 60 – 70 °C for 6 h. After filtering the insoluble matter, the filtrate was evaporated under reduced pressure to obtain a dried powder.

Poncirus trifoliata fruit extract

Dried *Poncirus trifoliata* fruit was mixed with a salt solution with a salt concentration of 0.5%. Then, the mixture was fermented at 1 - 4 °C for about 7 days. Then, five volumes (v/w) of 70% ethanol aqueous solution was added and the resulting solution was extracted three times under reflux. The settled solution was filtered through a filter cloth and centrifuged to obtain a residue and a filtrate. The separated filtrate was concentrated under reduced pressure to obtain a dried powder, thus yielding a salt-fermented extract of *Poncirus trifoliata* fruit.

Jaum balancing complex

Five fresh herbs, i.e., the roots of *Paeonia lactiflora*, flowers of *Nelumbo nucifera*, roots of *Rehmannia glutinosa*, bulbs of *Lilium candidum*, and rhizomes of *Polygonatum officinale*, were mixed at the same weight ratio and extracted with an ethanol solution [10% (v/v) ethanol-water] for longer than 18 h. The solution was filtered and the filtrate was evaporated under a vacuum to obtain a dried powder, referred to as the Jaum balancing complex sample.

Prunus mume extract

Dried flowers of *Prunus mume* were extracted with ten volumes (v/w) of an ethanol solution [70% (v/v) ethanol-water] at room temperature for longer than 2 h. The solution was filtered and the filtrate was evaporated under a vacuum to obtain a dried powder.

2.2. Cell Culture

NHDFs were purchased from Lonza (Switzerland), and maintained in DMEM (WelGene, South Korea) supple-

mented with 10% fetal bovine serum, 1% penicillin/ strepto-mycin mixture, and 4 mM L-glutamine in the absence or presence of 1 or 0.1 mM pyruvate (Sigma, USA).

2.3. Proliferation Rate and Cell Viability Analysis

NHDFs were plated in triplicate in 12 well plates at 1 - 5 × 10^4 cells per well. After incubation for the indicated number of days in the presence or absence of pyruvate or extracts, the cells were trypsinized, stained with trypan blue, and counted using a hemocytometer. The proliferation rate was determined as described previously using the following formula: Proliferation rate (doubling/day) = log2 (final cell count (day 12)/initial cell count (day 0)/12 (days).

2.4. Quantitative Reverse Transcription (RT) and Real-Time PCR Analysis (qRT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, USA) and was subjected to RT with random-hexamer primers using the ABI cDNA Synthesis Kit (Applied Biosystems, USA). The resulting cDNA was subjected to qRT-PCR using TaqMan PCR technology (Applied Biosystemsm USA) and an ABI PRISM 7700 system (Applied Biosystems, USA) to determine the expression of the following loci: *MMP-1*, Hs00899658_m1; *Col1A1*, Hs00164004_m1; *Col3A1*, Hs00943809_m1; *GAPDH*, Hs02786624_g1.

2.5. SA-*β* gal Staining

SA-βgal staining was performed using commercially available kits (Abcam, UK). For the quantification of senescent cells, a minimum of 200 cells were counted to determine the number of SA-βgal-positive cells using MetaMorph (Molecular Devices, USA).

2.6. Measurement of Mitochondrial ROS Generation

Mitochondrial ROS levels were analyzed using a flow cytometer after staining with MitoSOX (5 μM for 30 min). The stained cells were evaluated using a FACSCalibur (Becton Dickinson, USA) and analyzed using FlowJo (Tree Star, USA).

2.7. Statistical Analysis

Quantitative data are presented as means ± standard deviation (SD) and were analyzed using Student's t tests. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Pyruvate is a Potent Anti-Senescence Metabolite in the *In Vitro* Cell Culture Systems

For the reexamination of the anti-senescence effect of pyruvate, NHDFs were cultured in DMEM in the absence or presence of 0.1 or 1 mM pyruvate for 12 days. Consistent with previous results[11], a significantly lower rate of NHDF proliferation from day 4 and doubling rate at day 12 were observed in pyruvate deprivation conditions (0 mM) than in the presence of pyruvate (Figure 1A and B). Interestingly, the inhibition of cell proliferation and doubling rate were not recovered by treatment with 0.1 mM pyruvate, which is similar to the physiological concentration. Moreover, stronger positive staining for senescence- associated β-galactosidase (SA-βgal) was observed in both 0 and 0.1 mM pyruvate conditions than in 1 mM pyruvate conditions (Figure 1C). According to the increase in SA-βgal activity, mitochondrial ROS levels and the expression of MMP-1 were increased ~ 1.5 - and ~ 5 - fold, respectively, in both 0 and 0.1 mM pyruvate conditions (Figure 1D). In addition, the expression levels of collagen 1A1 (Col1A1) and 3A1 (Col3A1) were decreased by ~ 50% in both 0 and 0.1 mM pyruvate conditions compared with levels in 1 mM pyruvate conditions (Figure 1E). These results indicate that a high concentration of pyruvate (1 mM) not only protects NHDFs from cellular senescence, but also maintains skin elasticity, whereas a physiological concentration of pyruvate (0.1 mM) has no influence on these parameters.

3.2. Cytotoxicity by the *Poncirus trifoliata* Fruit Extract is Prevented by Treatment with Pyruvate

We evaluated the anti-senescence activity of four candidate extracts in pyruvate deprivation conditions. The Sprouted seed and fruit complex was composed of five crude herb materials, including the *Prunus mume* fruit,
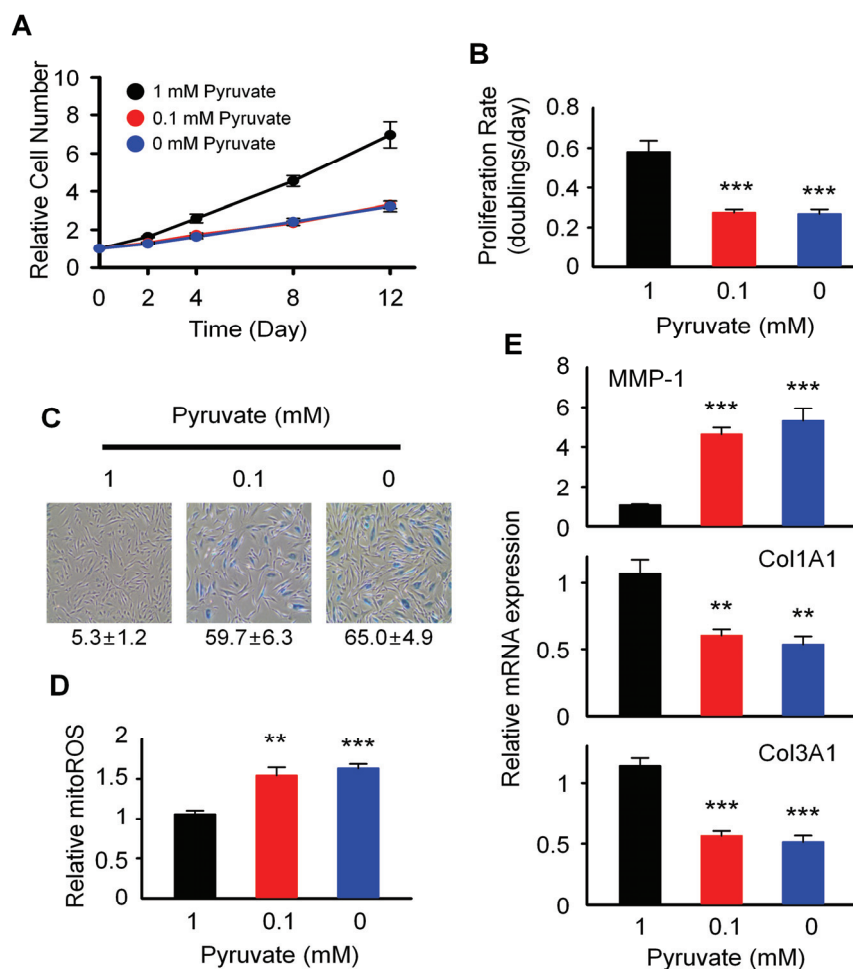
Figure 1. Induction of senescence of NHDFs by pyruvate deprivation.
(A) NHDFs (passage 4) were cultured in DMEM with the indicated concentration of pyruvate and then counted at the indicated days. The relative cell number compared to day 0 is presented as the mean ± SD of one representative experiment (N = 3 wells), with three independent replicates. (B) The proliferation rate of NHDFs studied in A was assayed at 12 days. Means ± SD (N = 3). **$p < 0.01$ versus the corresponding value for 1 mM pyruvate-treated cells. (C) NHDFs cultured as descried in A for 12 days were subjected to SA-βgal staining. The percentages of SA-βgal–positive cells were quantified using MetaMorph image-analysis software. Means ± SD (N = 3). (D) NHDFs cultured as in A for 12 days were stained with MitoSOX for the detection of mitochondrial ROS and analyzed by flow cytometry. Mean ± SD (N = 3). **$p < 0.01$, ***$p < 0.001$ versus the corresponding value for 1 mM pyruvate-treated cells. (E) Total RNA of NHDFs cultured as in A for 12 days were subjected to qRT-PCR to evaluate the mRNA levels of MMP-1, Col1A1, and Col3A1. Means ± SD (N = 4). **$p < 0.01$, ***$p < 0.001$ versus the corresponding value for 1 mM pyruvate-treated cells.

Pinus koraiensis seed, Chaenomeles sinensis fruit, germinated Rosa multiflora fruit, and germinated black Sesamum indicum seed. Jaum balancing complex was also composed of five herbs, i.e., the roots of Paeonia lactiflora, flowers of Nelumbo nucifera, roots of Rehmannia glutinosa, bulbs of Lilium candidum, and rhizomes of Polygonatum officinale. The *Poncirus trifoliata* fruit extract and *Prunus mume* extract were isolated from the *Poncirus trifoliata* fruit or flowers of *Prunus mume*, respectively.

Although some natural extracts isolated from plants

have anti-senescence effects, they often show cytotoxicity. Given that pyruvate is not only the major source of energy through the TCA cycle but is also a cellular protector against oxidative stress or mitochondrial dysfunction[16, 17], we analyzed cell viability in the presence of absence of pyruvate to identify the toxicity of the four extracts to NHDFs. In pyruvate deprivation conditions, the viability of NHDFs was significantly decreased only by treatment with the *Poncirus trifoliata* fruit extract at 2 days (Figure 2A). However, the decrease in viability by treatment with 100 μg/mL *Poncirus trifoliata* fruit extract was recovered in a pyruvate concentration-dependent manner (Figure 2B). These data indicate that the *Poncirus trifoliata* fruit extract has a toxic effect, and pyruvate protects against this toxicity.

3.3. Extracts do not Show Anti−Senescence Activity under Complete Pyruvate−Deprivation Conditions (No Added Pyruvate).

Pyruvate has an anti-senescence effect in normal conditions or stress-induced senescence models[10, 11]. Therefore, the anti-senescence effects of extracts were evaluated in pyruvate deprivation conditions to exclude the effect of pyruvate. Because *Poncirus trifoliata* fruit extract-treated NHDFs exhibited the decrease in viability under pyruvate deprivation, we analyzed NHDFs treated with the three alternative extracts, i.e., the Sprouted seed and fruit complex, Jaum balancing complex, and *Prunus mume* extract. In pyruvate deprivation conditions, the cell proliferation rates of NHDFs were not affected by treatment with the three extracts at 20 or 50 μg/mL for 12 days
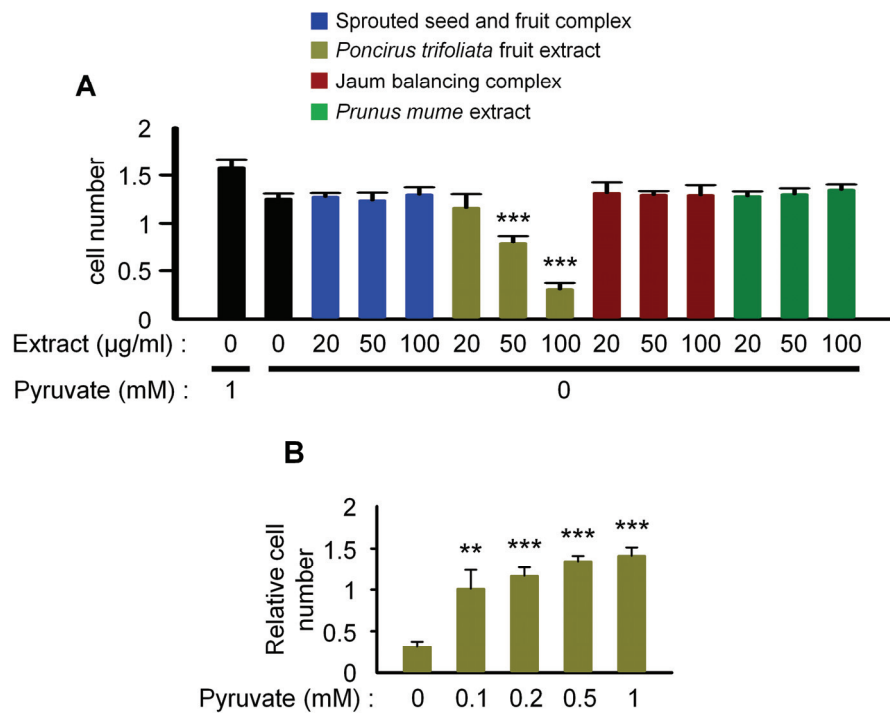

Figure 2. Pyruvate attenuates the cytotoxicity of the *Poncirus trifoliata* fruit extract in NHDFs. (A) NHDFs were cultured in DMEM with or without 1 mM pyruvate in the presence of the indicated concentrations of four extracts for 2 days. The relative cell number compared to day 0 is reported as the mean ± SD of one representative experiment (N = 3 wells), with three independent replicates. ***$p < 0.001$ versus corresponding value for NHDF cultured without pyruvate and extracts. (B) NHDFs were treated with 100 μg/ml *Poncirus trifoliata* fruit extract in the indicated concentration of pyruvate for 2 days. The relative cell number compared to day 0 is reported as the mean ± SD of one representative experiment (N = 3 wells), with three independent replicates. **$p < 0.01$, ***$p < 0.001$ versus the corresponding value for NHDF cultured without pyruvate.

(Figure 3A). In addition, SA-βgal-positive cells (Figure 3B), mitochondrial ROS levels (Figure 3C), and the levels of MMP-1, Col1A1, and Col3A1 mRNA (Figure 3D) did not differ between NHDFs cultured in pyruvate deprivation conditions treated with the three extracts at 20 or 50 μg/mL for 12 days and NHDFs cultured in the absence of pyruvate and extracts. These results suggested that the three extracts do not affected NHDF senescence induced by pyruvate deprivation.

3.4. Jaum balancing Complex and *Prunus mume* Extract Attenuate the Senescence of NHDFs under Low Pyruvate Conditions.

Although NHDF senescence was not affect by treatment with the extracts under pyruvate deprivation conditions, the anti-senescence activity of the four extracts were re-examined in the presence of 0.1 mM pyruvate. Unlike in pyruvate deprivation conditions, the cell proliferation rate of NHDFs were slightly increased by treatment with 20 μg/mL Jaum balancing complex or *Prunus mume* extract, but not by treatment with the Sprouted seed and fruit com-
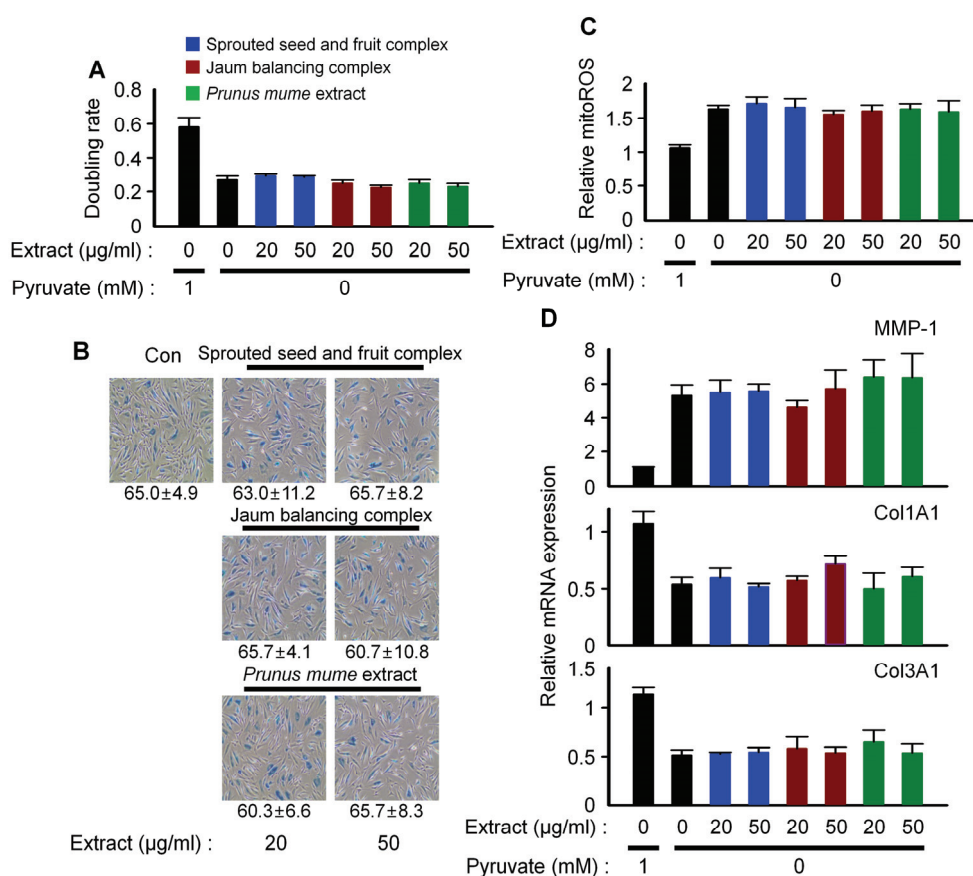
Figure 3. Effects of several plant extracts on the senescence of NHDFs under complete pyruvate-deprivation conditions (no added pyruvate). (A) The proliferation rate of NHDFs cultured in DMEM with or without the indicated concentration of pyruvate and extracts was assayed at 12 days. Means ± SD (N = 3). (B) NHDFs cultured as described in A for 12 days were subjected to SA-βgal staining. The percentages of SA-βgal–positive cells were quantified using MetaMorph image-analysis software. Means ± SD (N = 3). Con, NHDF cultured without pyruvate and extracts. (C) The NHDFs cultured as in A for 12 days were stained with MitoSOX for the detection of mitochondrial ROS and analyzed by flow cytometry. Means ± SD (N = 3). (D) Total RNA of NHDFs cultured as described in A for 12 days were subjected to qRT-PCR to determine the mRNA levels of MMP-1, Col1A1, and Col3A1. Means ± SD (N = 3).

plex and *Poncirus trifoliata* fruit extract (Figure 4A). In an analysis of anti-senescence activity, strong positive staining for SA-βgal in 0.1 mM pyruvate was substantially decreased by treatment with 20 or 50 μg/mL Jaum balancing complex or *Prunus mume* extract (Figure 4B). Moreover, the increase in mitochondrial ROS in 0.1 mM pyruvate conditions was decreased by ∼ 50% by treatment

with the Jaum balancing complex or *Prunus mume* extract (Figure 4C). Consistent with the decrease in the senescence of NHDFs by treatment with the Jaum balancing complex or *Prunus mume* extract, the mRNA levels of MMP-1 and Collagens (Col1A1 and Col3A1) decreased by 50% and increased ∼ 1.3 fold compared with those in NHDFs cultured only in the presence of 0.1 mM pyruvate (Figure 4D).
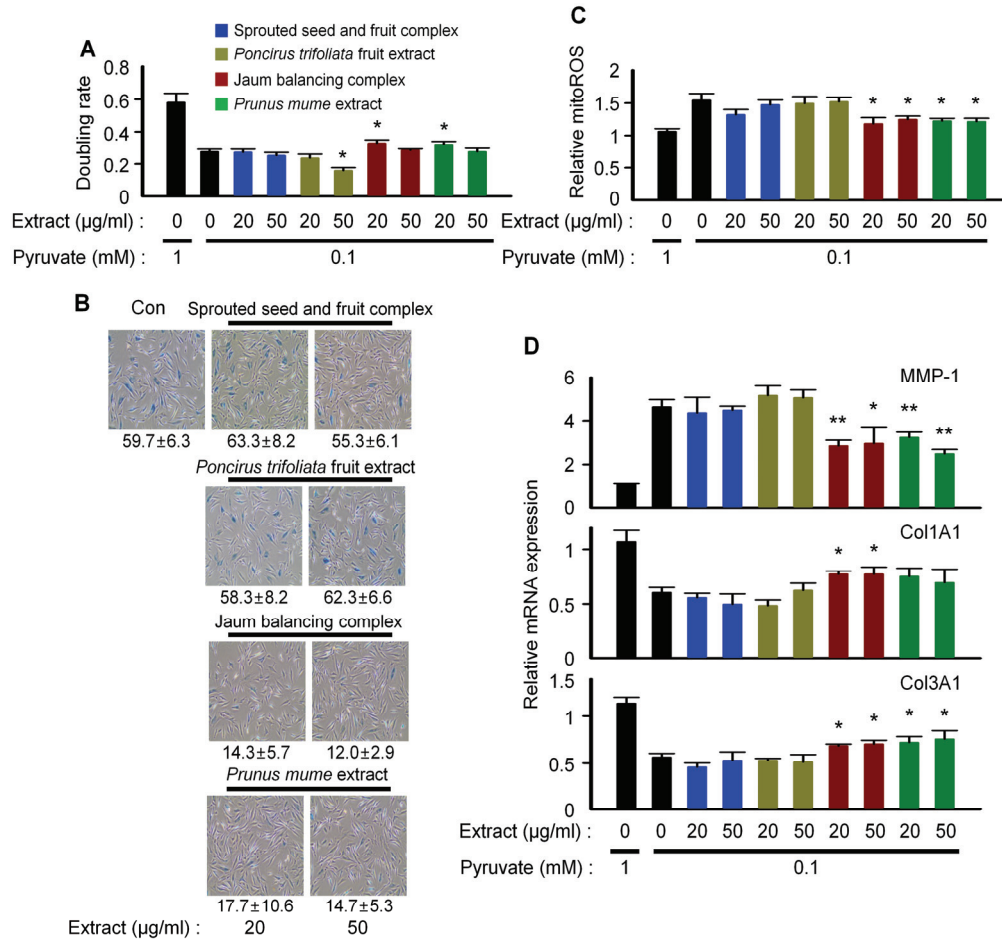
Figure 4. Jaum balancing complex and *Prunus mume* extract attenuate the senescence of NHDFs under low pyruvate conditions. (A) Proliferation rate of NHDFs cultured in DMEM with or without the indicated concentration of pyruvate and extracts was assayed at 12 days. Means ± SD (N = 3). $^{*}p < 0.05$ versus the corresponding value for NHDF cultured with 0.1 mM pyruvate in the absence of extracts. (B) NHDFs cultured as described in A for 12 days were subjected to SA-βgal staining. The percentages of SA-βgal–positive cells were quantified using MetaMorph image-analysis software. Means ± SD (N = 3). Con, NHDF cultured with 0.1 mM pyruvate in the absence of extracts. (C) NHDFs cultured as described in A for 12 days were stained with MitoSOX for the detection of mitochondrial ROS and analyzed by flow cytometry. Mean ± SD (N = 3). $^{*}p < 0.05$ versus the corresponding value for NHDF cultured with 0.1 mM pyruvate in the absence of extracts. (D) Total RNA of NHDFs cultured as described in A for 12 days were subjected to qRT-PCR to determine the mRNA levels of MMP-1, Col1A1, and Col3A1. Means ± SD (N = 3) $^{*}p < 0.05$, $^{**}p < 0.01$ versus the corresponding value for NHDF cultured with 0.1 mM pyruvate in the absence of extracts.

4. Discussion

The pyruvate concentration in human blood, approximately 30 μM, is lower than that in synthetic media (e.g., DMEM) used for *in vitro* cell culture systems [18]; accordingly, we expected the high concentration of pyruvate in synthetic media (1 mM) to not only induce a change in cellular metabolism but also to protect against cellular senescence or oxidative damage. Therefore, the anti-senescence effect of candidates may be masked by the strong anti-senescence activity of pyruvate at high conditions. Although the senescence of NHDFs was not affected by treatment with the four extracts under pyruvate deprivation conditions, Jaum balancing complex and *Prunus mume* extract synergistically protected against pyruvate deprivation-induced senescence using 0.1 mM pyruvate, resulting in a decrease in mitochondrial ROS and the maintenance of skin elasticity. It has recently been reported that pyruvate protects against cellular senescence induced by pyruvate deprivation or treatment with mitochondrial inhibitors[10, 11]. Therefore, we hypothesized that pyruvate in culture media can explain the anti-senescence effect of Jaum balancing complex and *Prunus mume* extract.

Although the *Poncirus trifoliata* fruit extract did not show anti-senescence activity in the absence or presence of 0.1 mM pyruvate, it is associated with skin whitening[19]. Therefore, a reduction in the cytotoxicity of *Poncirus trifoliata* fruit extract is very important to enhance whitening, without skin problems. Pyruvate not only shows anti-senescence activity but also protects against the cytotoxicity of extracts, suggesting that it is one of the most important supplements for skin aging and associated problems. Given that pyruvate protects against cellular senescence through generation of NAD+. it may be necessary to consider whether connection of Jaum balancing complex and *Prunus mume* extract with NAD+ metabolism controls the cellular senescence under low pyruvate conditions or *in vivo*.

Consequently, we recommend that studies of the anti-senescence effects of candidate agents use 0 or 0.1 mM pyruvate, which is more similar to *in vivo* aging conditions than excessive stress-induced senescence models, to exclude the effect of excessive pyruvate *in vitro*.

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