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Unripe pear fruit extract induces the transcriptional activity of sirtuin-related genes to extend the chronological lifespan of *Saccharomyces cerevisiae*

Wakae Murata[1,2], Marina Hasegawa[2], Seiru Sakasegawa[2], Ken-ichi Fujita[1], Toshio Tanaka[1] and Akira Ogita[1,3]*

[1]Graduate School of Sciences, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan.
[2]Department of Materials Science, National Institute of Technology, Yonago College, 4448 Hikona, Yonago, Tottori 683-8502, Japan.
[3]Research Center for Urban Health and Sports, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan.

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ABSTRACT

Calorie restriction is the main intervention known to effectively extend the lifespan through anti-aging effects in eukaryotic cells. However, the identification of nutrients with benefits similar to those of calorie restriction for the development of anti-aging agents has been gaining increasing research interest. To this end, in the present study, we evaluated the potential anti-aging effects of unripe pear fruit extract by employing a chronological lifespan assay in the yeast *Saccharomyces cerevisiae*. Exposure of unripe pear fruit extract at 1.0% (v/v) significantly prolonged the chronological lifespan of the yeast compared with that of untreated yeast cells ($P < 0.05$). Furthermore, the underlying mechanism was explored through the analysis of the expression of silent information regulator genes by reverse transcription-polymerase chain reaction, which demonstrated that the expression levels of sirtuin family genes-known regulators of the aging process-were significantly increased upon treatment with unripe pear fruit extract ($P < 0.05$). These results suggest that unripe pear fruit extract might contribute to anti-aging effects in mammalian cells *in vivo*, supporting further exploration of the benefits of a human homolog corresponding to the aging regulator gene of yeast.

Keywords: Unripe pear fruit extract, chronological lifespan, sirtuin, *Saccharomyces cerevisiae.*

*Corresponding author. E-mail: ogita@sports.osaka-cu.ac.jp. Tel/Fax: +81-6-6605-3164.

INTRODUCTION

Global demographics are rapidly changing, whereby the proportion of the older population is growing at an unprecedented rate. Given the increase in the aged population, aging-related diseases are on the rise. Cellular senescence, the decline in cellular function due to aging, causes the gradual loss of physiological functions, leading to several cellular senescence-related chronic diseases such as metabolic syndrome, cardiovascular disease, cancer, osteoporosis, diabetes, and hypertension, which negatively affect the quality of human life (Fontana et al., 2010; Hall et al., 2013; Jin et al., 2014; Reeve et al., 2014). Thus, appropriate intervention of the cellular senescence process may help to reduce the incidence and slow the progression of these aging-related diseases, while contributing to the longevity and quality of human life.

Despite differences in the characteristics and manifestations of aging among species (Kirkwood, 2005; Shmookler, 2012), some molecular processes related to aging are conserved, even across disparate taxa. Arguably, research on the budding yeast *Saccharomyces cerevisiae* has directly or indirectly contributed to the identification of more mammalian genes affecting aging than any other model organism has (Longo et al., 2012). Therefore, the chronological aging of yeast is a complementary model to a replicative model, which

simulates the cellular aging of non-dividing, albeit metabolically active, mammalian cells such as those of the brain and heart (Longo et al., 2012; MacLean et al., 2001). Because *S. cerevisiae* is a simple model system that can provide significant insights into the human genetics and molecular biology of senescence, it is considered to be a suitable cellular model for research on mammalian cells.

Caloric restriction, defined as a reduction in nutrient availability, is well known to increase the lifespan of an organism, and it can delay the onset of age-associated diseases in yeast, worms, flies, and rodents (Masoro, 2005). Caloric restriction was shown to extend the lifespan of *S. cerevisiae* cells by increasing the activity of the most well-known aging-related gene, *SIR2*, a member of the conserved sirtuin family of NAD-dependent protein deacetylases (Guarente and Picard, 2005). Although the effect of caloric restriction on aging suppression is well known, extreme caloric restriction is an unrealistic and dangerous condition for various organisms. Therefore, substantial research attention has been paid to the identification and development of natural products such as fruits and vegetables with anti-aging properties. To date, targeted and conserved longevity mechanisms have been revealed for a few natural products such as resveratrol (Howitz et al., 2003; Baur and Sinclair, 2006) and rapamycin (Harrison et al., 2009), which have been proposed to act as caloric restriction mimetics to slow aging in multiple model organisms (Steinkraus et al., 2008).

The Asian pear (*Pyrus pyrifolia*), also known as the “Japanese” or “Oriental” pear or “Nashi” (the Japanese word for pear), is widely grown in Asia, but it has been gaining popularity worldwide over the last 20 years. Pears have been used in traditional medicine in China for more than 2,000 years because of their anti-inflammatory, anti-hyperglycemic, diuretic, and antitussive effects (Li et al., 2014). Pears are also a good source of phenolic acids, chlorogenic acid, arbutin, ferulic acid, and citric acid. In particular, chlorogenic acid is of interest in terms of its nutritive value, owing to its role in regulating glucose and lipid metabolism. A recent review (Meng et al., 2013) on chlorogenic acid identified 27 studies, including three human clinical trials and 18 animal studies, which demonstrated improvements in a range of metabolic biomarkers related to diabetes, cardiovascular health, and obesity, including serum lipids, insulin resistance, glucose tolerance, and obesity-related hormones. Ferulic acid has also been reported to have a range of anti-inflammatory, anti-atherogenic, antidiabetic, neuroprotective, and hepatoprotective effects, which are attributed to its potent antioxidant activity. However, at present, it is not clear whether and how much dietary intake of ferulic acid is adequate to generate the biological effects observed in laboratory studies (Srinivasan et al., 2007; Zhao and Moghadasian, 2008).

Given these well-documented health benefits of the pear, in the present study, we aimed to investigate the potential lifespan-extending effects of pear extract. To this end, we used *S. cerevisiae* as a model and treated cells with unripe and mature pear extract to determine the effects on cell longevity in comparison to untreated control cells. We further investigated the mechanisms contributing to these effects by evaluating the changes in the expression of aging-related genes with reverse transcription-polymerase chain reaction (RT-PCR). These results can contribute to the scientific basis for the application of pear extract as a calorie restriction mimetic to achieve anti-aging effects.

MATERIALS AND METHODS

Preparation of pear fruit extract

Unripe and mature fruits of the Japanese pear (*Pyrus pyrifolia* Nakai) cultivar 'Nijisseiki' were obtained from a manufacturing farmhouse and a supermarket, respectively, in Yonago, Tottori, Japan. The pear fruit at approximately a month after pollination is considered unripe, and its weight and diameter are approximately 2.5 g and 2 cm, respectively. Unripe pears are small and are usually thinned out without being used for edible purposes. A mature pear is formed around 5 months after pollination, and the fruit at this stage is edible. The weight of a mature pear is around 300 to 400 g, with a standard diameter of approximately 9 to 10 cm.

Whole unripe and mature pear fruit, containing peels and seeds (30 g), were homogenized. The homogenized component of unripe or mature pears was mixed with distilled water (10 ml). After centrifugation at 5,000 × *g* for 3 min, the supernatant was obtained and used as the unripe pear fruit extract (UPE) or mature pear fruit extract (MPE), respectively.

Yeast strains and growth conditions

S. cerevisiae W303-1A cells were used in this study. In preparation for measuring the chronological lifespan, yeast cells were pre-incubated in YPD culture medium, consisting of 1% yeast extract (Difco Laboratories, Detroit, MI, USA), 2% peptone (Difco Laboratories), and 2% glucose, with shaking (140 rpm) at 30°C for 16 h. For the preparation of agar plates, 1.8% agar (INA agar, INA Food Industry, Nagano, Japan) was added to the YPD culture medium. The cells were grown at 30°C unless otherwise indicated.

Cell viability and chronological lifespan

After pre-incubation in YPD culture medium for 16 h, *S. cerevisiae* cells were inoculated at 10^6 cells/ml into 1.5 ml of YP culture medium, consisting of 1% yeast extract and 2% peptone, supplemented with 5% glucose in the presence or absence of pear extract at various concentrations, and incubated for 4 days for growth till the late stationary phase. These grown cells were further incubated with shaking (140 rpm) at 30°C in the initial culture medium without the addition of further nutrients. Aliquots of cells were then sampled every third day of continued incubation and spread onto YPD agar plates. Viable cell numbers were determined after 48 h of incubation at 30°C on YPD agar plates. The chronological lifespan is measured as the duration for which cells can remain viable in a stationary culture. Viability is calculated as the fraction of the culture that is able to reenter the cell cycle after an extended state of quiescence (Kaeberlein et al., 2007a). Therefore, the chronological lifespan of cells was evaluated by the determination of the viable cell number according to the count of

colony-forming units after the late stationary phase of cell growth.

RT-PCR analysis

In the gene expression profiling for entire organisms, mRNA is the only intermediate between DNA and protein (Lucchini et al., 2001). However, it is indicated that there is a significantly high correlation between mRNA expression and protein abundance in the yeast nucleolus (Greenbaum et al., 2003; Marchfelder et al., 2003). Therefore, relative gene expression levels were quantified using RT-PCR in *S. cerevisiae* W303 cells grown on YPD culture medium in the presence or absence of 1.0% UPE and MPE with shaking at 30°C for 4 h. Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and 0.5 to 5 µg was used for cDNA synthesis using ReverTra Ace (Toyobo, Osaka, Japan). PCR was carried out using a standard quantitative amplification protocol (Murata et al., 2016). The primers used in this study are listed in Table 1. The expression levels of each gene were calculated on the basis of densitometry using Fujifilm Multi Gauge Version 2.1.

Table 1. Primers used in RT-PCR analysis.

Name	Sequences
TDH1-forward	5′-GGTGCCAAGAAGGTTGTCAT-3′
TDH1-reverse	5′-CCTTAGCAGCACCGGTAGAG-3′
SIR1-forward	5′-GGAAAAGCCAATTGTGGAGA-3′
SIR1- reverse	5′-CTCAGGATTCGCCTGAAAAG-3′
SIR2-forward	5′-ATTTATGCACGACCCCTCTG-3′
SIR2- reverse	5′-CGACATTGAACCCTGTGATG-3′
SIR3-forward	5′-CAGTGAGTGGGCAGAAGACA-3′
SIR3- reverse	5′-TGGCTTTGACCCAGGTTTAC-3′
SIR4-forward	5′-CTGGAATTGCAAAACCGAAT-3′
SIR4- reverse	5′-GCTGTTTCTACGCCCTTGAG-3′

Statistical analysis

Group means were compared using Student's *t*-test, and $P < 0.05$ was considered statistically significant.

RESULTS

Pear fruit extract extended the lifespan of *S. cerevisiae*

As shown in Figure 1A, yeast cell viability was gradually reduced, and complete loss of viability was observed on day 9 of incubation, after the late stationary phase of cell growth was attained in the culture medium without the addition of further nutrients to the initial growth environment. In the presence of MPE, the decrease in cell survival was slightly delayed from 3 to 6 days after the stationary growth phase. However, the presence of MPE at concentrations from 1.0 to 10.0% (v/v) did not exert an influence on the definitive chronological lifespan of the cells (Figure 1A). In contrast, in the presence of UPE, the chronological lifespan of cells was significantly extended compared to the control cells, in a dose-dependent manner from 1.0 to 10.0% ($P < 0.05$) (Figure 1B). Notably, UPE at concentrations of 5.0 and 10.0% (v/v) improved cellular survival, with increases of >20% and >40%, respectively, in the definitive lifespan of the cells compared to that of control cells in the absence of UPE.

Transcription activity of sirtuin-related genes in the presence of UPE

In RT-PCR analysis, the expression level of *TDH1*, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as a positive control. The expression levels of *SIR1* in cells treated with UPE or MPE were slightly increased compared to that of the control, but the difference was not statistically significant. The expression levels of *SIR2*, *SIR3*, and *SIR4* of MPE-treated cells showed no significant change compared to control cells. However, the expression levels of *SIR2* and *SIR4* were significantly increased ($P < 0.05$), and the expression level of *SIR3* also showed a noteworthy increase, although not quite reaching significance ($P < 0.06$), in treated cells compared to that of control cells in the absence of UPE at 1.0% (Figure 2).

DISCUSSION

For maintaining a healthy and active life, it is important to determine the beneficial effects of daily foods consumed, such as fruit and vegetables. Japanese pear is consumed in a large part of the world, and its numerous health benefits have been reported; however, these studies have thus far been limited to the mature pear fruit, since unripe fruit is not usually available in the market. Therefore, the beneficial health effects of unripe pear fruit, particularly with regard to the extension of cell lifespan, have not yet been investigated directly. Therefore, we examined whether extracts of the Japanese pear fruit 'Nijisseiki' have the capacity to extend the chronological lifespan of yeast *S. cerevisiae* cells. Although MPE did not have a significant effect on cell longevity, UPE improved the lifespan of *S. cerevisiae* cells in the stationary growth phase by more than 40% compared to that of controls.

Chronological lifespan can be increased by calorie (dietary) restriction, which is accomplished by reducing glucose concentrations or by exhausting available nutrients from the initial growth medium (Fabrizio and Longo, 2007; Smith et al., 2007). Moreover, the conserved NAD-dependent histone deacetylase Sir2p promotes replicative longevity in *S. cerevisiae* by

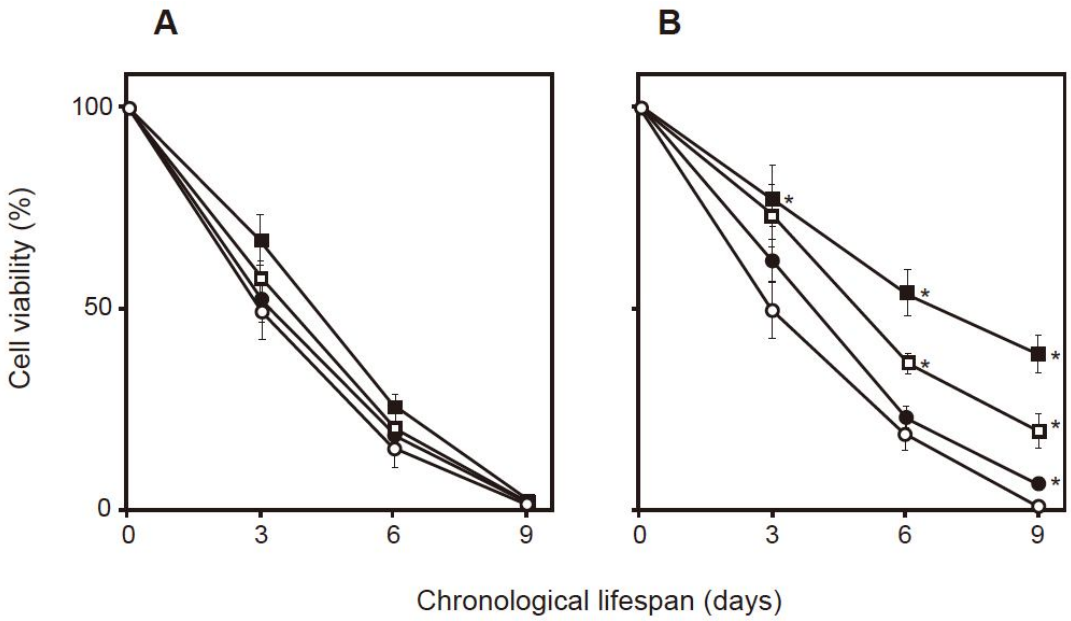

Figure 1. Extension of the chronological lifespan in *S. cerevisiae* W303 cells by the addition of pear extract. Cells (10^6/ml) were grown to the late stationary phase in YP medium initially containing 5% glucose with (A) mature pear extract (MPE) or (B) unripe pear extract (UPE) at 0 (○), 1 (●), 5 (□), or 10% (■) (v/v). After growth till the late stationary phase, the cells were further incubated with shaking at 30°C in the initial culture medium without the addition of further nutrients. The cells were sampled every 3 days during continued incubation and spread onto YPD agar plates. The chronological lifespan was evaluated by the determination of the viable cell number based on the count of colony-forming units (CFUs). Data represent the means ± standard deviations of triplicate assays. *P < 0.05 vs. control cells not treated with pear extract.

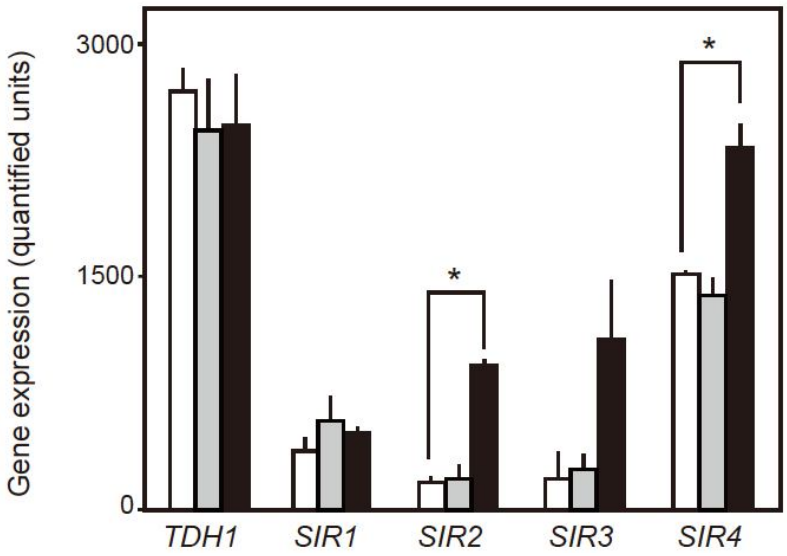

Figure 2. Effects of mature pear extract (MPE) and unripe pear extract (UPE) on the expression of anti-aging-related genes. *S. cerevisiae* cells were treated with no extract (white columns), 1% MPE (gray columns), or 1% UPE (black columns) for 4 h. *TDH1*, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as a positive control. Levels of *TDH1*, *SIR1*, *SIR2*, *SIR3*, and *SIR4* were quantified using Fujifilm Multi Gauge Version 2.1. Data represent the means ± standard deviations of triplicate assays. *P < 0.05.

suppressing recombination within the ribosomal DNA (rDNA) locus (Imai et al., 2000; Landry et al., 2000; Tanner et al., 2000), and it has thus been proposed to mediate the effects of calorie restriction on chronological aging (Smith et al., 2007). *SIR2* is necessary for transcriptional silencing near the telomeres (Aparicio et al., 1991), HM loci (Ivy et al., 1986; Rine and Herskowitz, 1987), and rDNA (Bryk et al., 1997; Smith and Boeke, 1997). Thus, in *S. cerevisiae* cells, calorie restriction extends the lifespan by increasing the activity of *SIR2*, and it is also connected to the regulation of the chronological lifespan (Medvedik et al., 2007). Therefore, the transcription activities of the sirtuin-related genes of UPE-treated cells were further investigated to elucidate the relevance of the observed extension in chronological lifespan and gene expression. Although the expression of *SIR1* was not affected, the transcript levels of *SIR2* and *SIR4* were remarkably increased, and the levels of *SIR3* also increased markedly in cells treated with UPE. The increasing gene transcription activity by UPE treatment might be principally involved in an increase in the relevant protein levels (Greenbaum et al., 2003; Marchfelder et al., 2003).

The activation of the Sir2 family of protein deacetylases has been shown to reduce the rate of aging and increase the lifespan in response to calorie restriction (Guarente and Picard, 2005; Masoro, 2005; Sinclair, 2005). Moreover, *SIR2* is involved in lifespan determination and calorie restriction in yeast mother cells, and its activity is increased by calorie restriction in yeast (Lin et al., 2002). Considering that the lifespans of the strains lacking *SIR2*, *3*, and *4* are short, the activity associated with *SIR2* is known to be essential for longevity in yeast (Kaeberlein et al., 1999; Schumpert et al., 2016). It remains uncertain whether UPE has activity other than SIR action. However, it was established that UPE could extend yeast lifespan via SIR activity. Thus, our results indicate that UPE extended the chronological lifespans of yeast cells by modulating the transcriptional activity of *SIR2* family genes. UPE treatment might be similar to the effects of calorie restriction in yeast. The silencing proteins Sir2p, Sir3p, and Sir4p are physically associated in the cell and directly interact with each other, and these proteins elicit transcriptional silencing by forming repressive chromatin structures that are confined to specific chromosomal domains. Therefore, transcriptional silencing is initiated either by the recruitment of a preassembled silent information regulator (Sir) protein complex to the silencer or by the stepwise assembly of this complex at the silencer (Gartenberg, 2000). The noteworthy high levels of *SIR2*, *SIR3*, and *SIR4* transcriptional activity following the addition of UPE indicated that the gene transcriptional silencing associated with the extension of chronological lifespan was possibly caused by the complexation of these Sir family proteins.

In mammalian cells, there are seven sirtuins, *SIRT1–SIRT7*, which are evolutionarily united by their highly conserved *S. Cerevisiae SIR2* family members (Frye, 2000), with *SIRT1* being the most closely related to *S. cerevisiae SIR2*. Meanwhile, according to recent reports, *SIRT6* is an important regulator of mammalian longevity, and the feasibility of manipulating *SIRT6* levels to treat age-related diseases has been demonstrated (Kanfi et al., 2012; Mostoslavsky et al., 2006). Our results suggested that UPE activity might have anti-aging effects on such mammalian sirtuins, that is, human homologs corresponding to the aging regulator gene of yeast.

Currently, calorie restriction is the only intervention known to extend the lifespan of eukaryotic cells, including human cells (Kaeberlein and Powers, 2007b), and there has been growing interest in the development of compounds that might replicate these calorie restriction-related benefits without having to restrict food intake (Ng et al., 2018). Although natural products with anti-aging properties have attracted great attention recently, examples of such compounds remain exceedingly scarce. Thus, UPE might have the same effect as calorie restriction on the chronological lifespan of cells in YP culture medium including 5% glucose by increasing the expression of Sir family genes.

CONCLUSION

Although the detailed molecular mechanism by which UPE extends the chronological lifespan of yeast remains unidentified, the present results suggest that *Sir* genes play important roles in the UPE-regulated chronological lifespan extension of yeast. Furthermore, this activity of UPE suggests similar anti-aging effects for mammalian cells via *SIRT1*, which is a human homolog corresponding to the yeast sirtuin gene. In addition, UPE might have the ability to extend cellular lifespan through a pathway similar to calorie restriction. Therefore, further exploration of the molecular mechanism of the UPE-induced lifespan extension in the cells of organisms other than yeast might provide useful insights and targets for improving human health and delaying the onset or progression of age-related diseases.

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