Inhibitory effect of *Lycium barbarum* polysaccharides on cell apoptosis and senescence is potentially mediated by the p53 signaling pathway

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Abstract. *Lycium barbarum (L. barbarum)* fruit or extract has been regarded as a superior-grade Chinese medicine, used to modulate body immunity and for anti-aging purposes. However, the underlying molecular mechanisms behind these effects remain unclear. In the present study, *L. barbarum* polysaccharides (LBPs), considered a major contributor of *L. barbarum* effects, were used to elucidate its mechanism of action by phenotypic and senescence associated-β-galactosidase (SA-β-gal) assays, evaluation of survival rates *in vivo* and expression profiling of genes related to the p53 signaling pathway in a zebrafish model. Zebrafish embryos were continuously exposed to various concentrations of LBPs (1.0, 2.0, 3.0 and 4.0 mg/ml) for 3 days. The results of fluorescent acridine orange and SA-β-gal staining indicated that cell apoptosis and senescence mainly occur in the head at 24 hours post fertilization (hpf) and 72 hpf. In addition, resistance to replicative senescence was observed at low doses of LBPs, especially at the 3.0 mg/ml concentration. Furthermore, the expression of genes that relate to aging, such as *p53*, *p21* and *Bax*, was decreased, while that of *Mdm2* and *TERT* genes was increased after treatment with LBPs. The results demonstrated that the effects of LBPs on cell apoptosis and aging might be mediated by the p53-mediated pathway.

Introduction

Aging is defined as the accumulation of diverse deleterious changes in cells and tissues (1). It is commonly associated with a reduction in physiological functions, and is closely related to apoptosis. In biology, the state or process of aging is known as senescence. It occurs at the level of the organism (organismal senescence), as well as at the level of individual cells (cellular senescence). At the individual cell level there are two types of senescence, replicative and stress-induced premature senescence (SIPS). Genetic and environmental manipulations have revealed that aging is regulated by specific pathways, involved in hormonal signaling, nutrient sensing and signaling, mitochondria and ROS signaling and genome surveillance (2). However, the question of whether all these signaling pathways exert their effects separately, or involve one or more junction co-regulators among these signaling pathways in the aging process remains to be determined.

Chinese herbs have been widely applied in the field of medicine as anti-aging drugs because of their few side-effects (3,4). The plant species *Lycium barbarum* (*L. barbarum*) belongs to the family of *Solanaceae*, and its fruit or extract has been regarded in Chinese pharmacopoeia as a superior-grade medicine for the modulation of body immunity (5-7). The effects of *L. barbarum* are attributed to the polysaccharides (LBPs) it contains, which can upregulate both innate and adaptive immune responses (8,9). It has been shown that LBPs can protect neurons from β-amyloid peptide neurotoxicity (10,11), and from neuronal death, glial activation and oxidative stress in a murine retinal ischemia/reperfusion model (12,13).

The tumor suppressor gene *p53* has been known to play an important role in the induction of cell cycle arrest and apoptosis (14). In a recent study, LBPs were found to stimulate the proliferation of MCF-7 cells via the ERK pathway, which may be associated with the p53 pathway (15). Although findings of previous studies have demonstrated the beneficial effects of LBPs on the health of humans and animals (16,17), to the best of our knowledge, there have been scarce studies that systematically investigate the signaling pathway(s) via which LBPs exert these beneficial effects, especially in zebrafish, an excellent model for studying angiogenesis, senescence, and toxicity responses (18-21). In this study, we examined senescence during the early development stages of zebrafish, cell apoptosis, and senescence-associated gene expression upon treatment with LBPs from the Chinese herb *L. barbarum* in the zebrafish model, in order to investigate the effects of this

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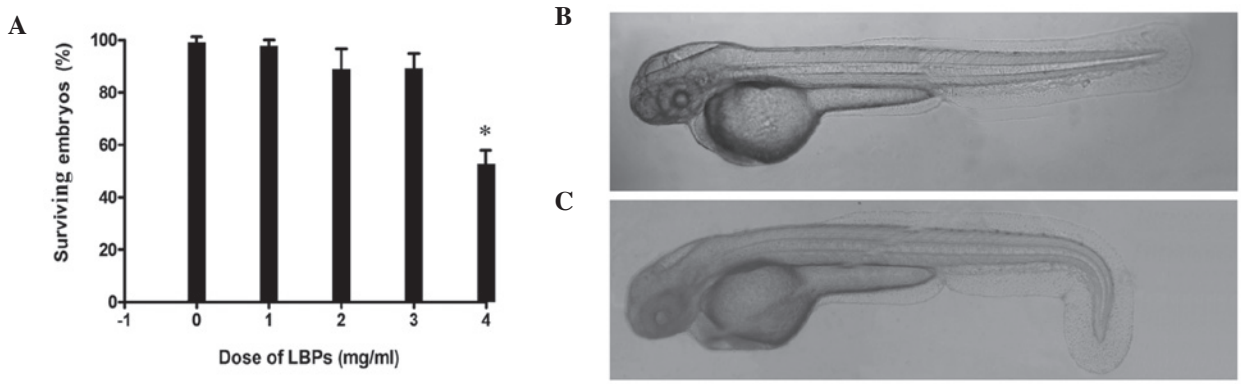

Figure 1. Effect of *Lycium barbarum* polysaccharides (LBPs) on the growth and development of zebrafish embryos at 48 hours post-fertilization (hpf). (A) The percentage of surviving embryos after treatment with different doses of LBPs. Data are presented as mean ± SD (n=30). (B) Phenotype of 48 hpf embryos not treated with LBPs and (C) treated with 4 mg/ml LBPs.

herb and identify the related signaling pathway(s) potentially involved in anti-aging processes.

Materials and methods

Materials and animals. Purified *L. barbarum* polysaccharides (LBPs) were purchased from Shanxi Undersun Biomedtech Co., Ltd. (Xi'an, Shaanxi, China) and an extract was prepared using the method of Luo *et al* (22). Zebrafish were maintained in standard fish facility conditions with a 14:10 h light/dark cycle and fed with living brine shrimp twice per day. The water temperature was maintained at 28˚C. The study was approved by the ethics committee of Ocean University of China (Qingdao, China).

Embryo treatment and image collection. Embryos at 8 hours post-fertilization (hpf) were placed into a 24-well microplate (Millipore Co., Bedford, MA, USA). Thirty embryos were continuously exposed to varying concentrations of LBPs (0, 1, 2, 3 and 4 mg/ml) for 3 days (d). LBPs were renewed daily. Each treatment with an LBP concentration was repeated three times and the standard deviation was calculated. To collect images of embryo phenotypes, treated and non-treated embryos were anesthetized with 0.2 mg/ml tricaine (3-aminobenzoic acid ethylester; Sigma-Aldrich, St. Louis, MO, USA) and embedded in methylcellulose.

Senescence associated-β-galactosidase (SA-β-gal) assay. Embryos of 72 hpf were fixed in 4% paraformaldehyde in phosphate-buffered saline, at 4˚C overnight. Staining of SA-β-gal and quantifications were performed according to the method of Kishi *et al* (23).

In vivo detection of cell death. For the *in vivo* detection of cell death, 2 day-old embryos were incubated in 5 mg/ml acridine orange stain (AO; Sigma-Aldrich) in zebrafish embryo medium [NaCl, 5.03 mM; KCl, 0.17 mM; $CaCl_2 \cdot 2H_2O$, 0.03 mM; $MgSO_4 \cdot 7H_2O$, 0.03 mM; methylene blue, 0.1% (w/v)] and kept in the dark for 15-30 min at 28˚C, then rinsed thoroughly 8 times with egg water. Stained embryos were anesthetized with MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na_2HPO_4) and mounted in a depression slide for observation using methylcellulose. They were then visualized using a fluorescence microscope (AZ100, Nikon, Tokyo, Japan) and images were captured for <60 sec (the signal is quenched after 60 sec of exposure to fluorescence). Embryos that were not stained with AO were used to determine baseline fluorescence.

Semi-quantitative RT-PCR. Total RNA was extracted from 72 hpf embryos using the TRIzol reagent. Semi-quantitative PCR was performed with an initial incubation for 5 min at 94˚C, followed by 22 cycles of incubation at 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec. The primers for amplification of *p53* and *p21* were the same as in (24): *p53* forward/reverse: CTCTCCCACCAACATCCACT/ACGTCCACC ACCATTTGAAC; p21 forward/reverse: CGGAATAAA CGGTGTCGTCT/CGCAAACAGACCAACATCAC. The primers for amplification of *Bax*, *Mdm2*, *TERT* and *β-actin* were: *Bax* forward/reverse: GGAGATGAGCTGGAT GGAAAT/ATGACGTGCTCCTGAATGTAG; *Mdm2* forward/reverse: GACTACTGGAAGTGTCCCAAAT/ GTCCACTCCATCATCTGTTTCT; *TERT* forward/reverse: GTGTGTGTGTCCTGGGTAAA/CAGCCTGAGGTCTAA GAAGATG; *β-actin* forward/reverse: CCCAGACATCAG GGAGTGAT/TCTCTGTTGGCTTTGGATT. Subsequently, 10 μl PCR products were loaded into gels for agarose gel electrophoresis.

Statistical analysis. Data are presented as mean ± SD (n=30). Differences between groups were assessed by analysis of variance (ANOVA) and Student's t-tests. Statistical analyses was carried out using SPSS for Windows, Version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Zebrafish embryo bioassay for assessing the toxicity of LBPs. The dose of LBPs that was lethal for embryos was determined by incubating embryos in different concentrations of LBPs. The results indicated that the growth and development of embryos were significantly inhibited at the concentration of 4.0 mg/ml (Fig. 1A). The number of surviving embryos was not significantly different among the lower concentrations (0, 1, 2 and 3 mg/ml). Half of the embryos died in this experiment, and

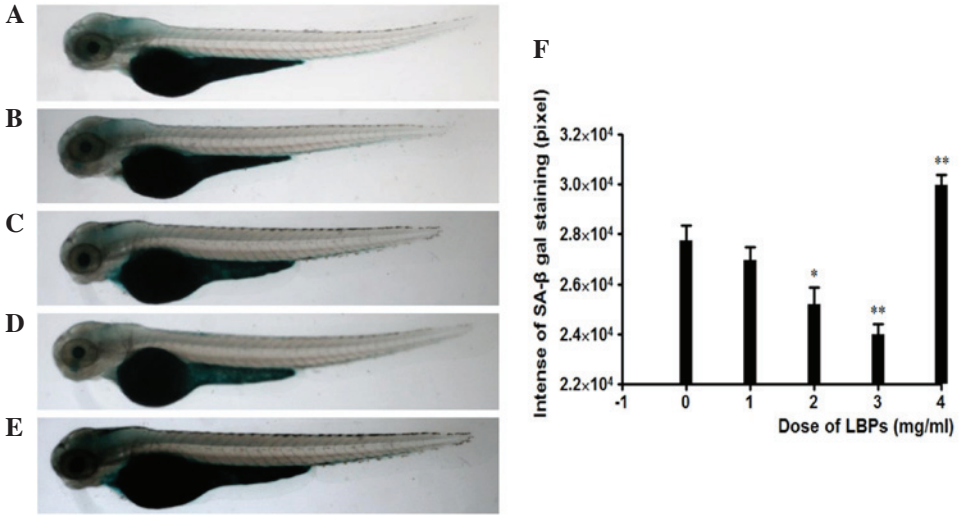

Figure 2. Senescence associated-β-galactosidase (SA-β-gal) activity changes in 72-hour post fertilization (hpf) embryos treated with *Lycium barbarum* polysaccharides (LBPs). Phenotype of (A) non-treated and (B-E) treated embryos with 1, 2, 3 and 4 mg/ml of LBPs, respectively. (F) Relative intensities of SA-β-gal staining expressed as pixels from computer scanning analysis. **P<0.05 vs. (0 mg/ml); **P<0.01 vs. control (0 mg/ml).

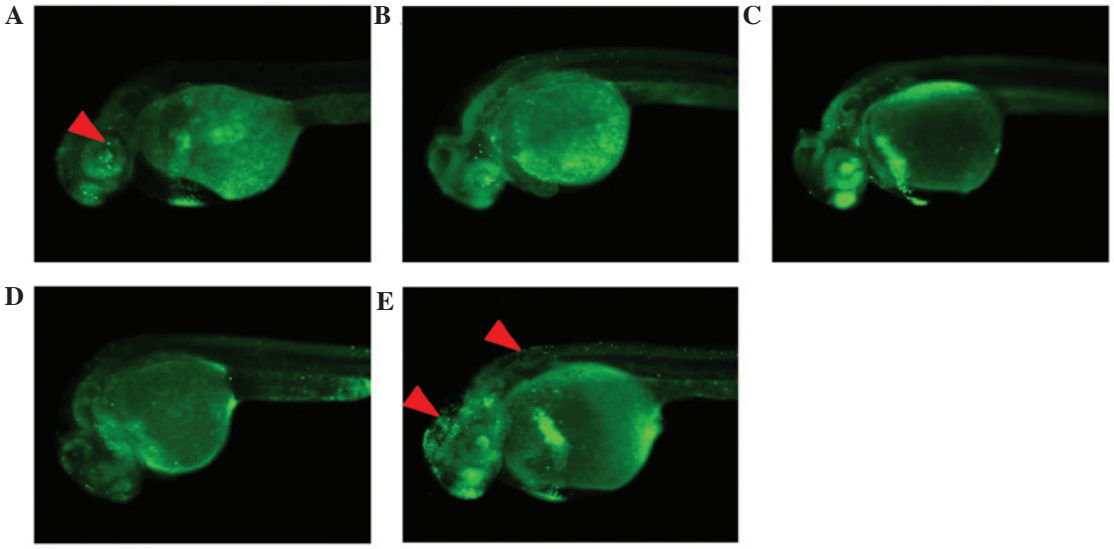

Figure 3. Representative cellular death phenotypes detected at 24 hours post-fertilization (hpf). (A-E) Microscopic images of acridine orange (AO) staining on embryos treated with 0, 1, 2, 3 and 4 mg/ml of *Lycium barbarum* polysaccharides (LBPs), respectively. Quantitative analysis revealed that apoptosis decreased in a dose-dependent manner and that the 4 mg/ml concentration of LBPs induced cell apoptosis. Red arrowheads denote the main areas of positive staining.

the embryos showed an abnormal phenotype (Fig. 1B and C) when treated with the highest concentration of LBPs tested in the present study (4 mg/ml).

Assessing senescence in zebrafish embryos

SA-β-gal staining. Cytochemically and histochemically detectable SA-β-gal at pH 6.0 has been shown to increase during the replicative senescence of cells *in vitro* and in tissue samples (25), and has subsequently been widely used as an *in vivo* and *in vitro* marker of cellular senescence in a number of vertebrate animal systems (26-28). To assess the effect of LBPs on replicative senescence, the SA-β-gal activity was histochemically detected at pH 6.0 in the 72 hpf embryos. The untreated embryos exhibited low background staining, especially in the head (Fig. 2A), while embryos treated with LBPs showed faint background staining, which was reduced with

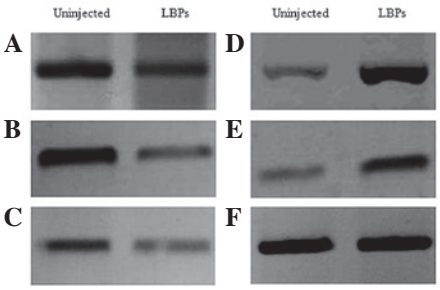

Figure 4. Expression profiles of key genes related to senescence at 72 hours post fertilization (hpf) in zebrafish treated with 3 mg/ml *Lycium barbarum* polysaccharides (LBPs) and in non-treated animals (uninjected). (A-C) The expression levels of genes encoding the regulatory protein p53, the cell cycle checkpoint protein p21 and the apoptotic- and senescence-related protein Bax respectively, increased upon treatment with LBPs, while those of genes encoding the (D-E) p53 regulator Mdm2 and the apoptotic- and senescence-related protein TERT decreased. (F) β-actin was used as a control of baseline expression.

treatment with increased concentrations of LBPs (Fig. 2B-D). Only at the 4 mg/ml concentration were embryos more strongly stained compared to untreated ones and to those treated with 1-3 mg/ml LBPs (Fig. 2E). Quantification of SA-β-gal staining was performed using high-resolution digital imagery. SA-β-gal staining at 1, 2 and 3 mg/ml LBPs was estimated to correspond to 88.3, 81.7 and 68.3% of the staining observed in the control (treated with 0 mg/ml LBPs), while it was 112.7% of the control for the 4 mg/ml LBPs ,as shown in Fig. 2F. The less amount of staining was equivalent to that of the less senescent mass.

Acridine orange (AO) staining. To explore the effect of LBPs on apoptosis, AO staining was performed. Treatment with 1-3 mg/ml LBPs reduced cell apoptosis in a dose-dependent manner (Fig. 3A-D), while at the 4 mg/ml LBPs concentration, cell apoptosis was induced, as observed from the comparison to control animals and to those treated with lower LBPs concentrations (Fig. 3E). Embryos treated with 4 mg/ml LBPs showed high levels of AO staining (red arrowheads) in the brain and the neural tube, compared to non-treated embryos (Fig. 3A) at 24 hpf.

The expression levels of genes related to the p53 signaling pathway after LBPs treatment. It was reported that in MCF-7 breast cancer cells, LBPs can activate ERK, which may be associated with the p53 signaling pathway (15). There is also the hypothesis that different types of intrinsic and extrinsic stress signals likely converge on the activation of the p53 protein, the Rb protein, or both during senescence (29). In this context, we examined whether zebrafish and mammals share similar mechanisms for the response to LBPs treatment. To further investigate p53-dependent transcriptional responses in the treated embryos, we examined the expression of *p53,* along with that of response genes such as *p21*, *Mdm2* and *Bax,* using semi-quantitative RT-PCR and the β-actin gene as a control of baseline expression. The results showed that the expression level of *p53*, *p21* and *Bax* were decreased in treated embryos compared to non-treated ones (Fig. 4A-C), while the levels of *Mdm2* and even, that of the cellular senescence-related (30-32) telomerase reverse transcriptase (*TERT*), which allows to maintain the telomere ends, were increased (Fig. 4D and E).

Discussion

L. barbarum fruits (berries) or extract have been widely used for centuries to balance the 'Yin' and the 'Yang' in the body (33). The major ingredient of the liquid fraction of these berries, LBPs, has been the object of research focus in studies aiming to identify anti-aging remedies (34) or agents alleviating cellular damage (12,35).

p53, as a central mediator of cellular responses induced in various processes including apoptosis (36), was shown to exert similar effects to ERK during stress induced by DNA damage (37). In MCF-7 cells, LBPs increased the activity of ERK (15). p21 as a regulator of cell cycle progression controlled by the tumor suppressor protein p53, inhibited tumor progression, thus preventing cell proliferation, while inducing cell apoptosis (38). In our study, cell apoptosis was inhibited following treatment with non-toxic doses of LBPs (1-3 mg/ml), as shown by AO staining, the reduced expression of *p53* and the increased expression of its negative regulator, *Mdm2*. A potential explanation for these findings is that the cellular responses triggered by the activated p53 protein (cellular senescence) act as potent barriers against the effects of LBPs.

In this study, we assessed the relevance of one molecular mechanism potentially underlying the effect of LBPs on cellular senescence in a zebrafish model by phenotypic and SA-β-gal assays, *in vivo* detection of survival rates and expression profiling of genes that related to the p53 signaling pathway during senescence. Zebrafish senescence was alleviated by LBPs via inhibition of cell death and apoptosis in the early development, a reduction in the expression level of *p53*, *p21* and *Bax* genes and an increase in the expression of *Mdm2* and *TERT* genes. In conclusion, our findings suggest that the anti-aging effects of LBPs are mediated by the p53 signal pathway. The specific targets of LBPs in this pathway may be identified in future studies.

Acknowledgements

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