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Yulangsan polysaccharide improves redox homeostasis and immune impairment in D-galactose-induced mimetic aging

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ABSTRACT

Yulangsan polysaccharide (YLSP) is a traditional Chinese medicine used in long-term treatment as a modulator of brain dysfunction and immunity. In this study, we evaluated the protective effect of YLSP against D-galactose-induced impairment of oxidative stress and the immune system and evaluated its possible mechanism of action. D-galactose was subcutaneously injected into the dorsal necks of mice daily for 8 weeks to establish the aging model. YLSP was simultaneously administered once daily. The results indicate that YLSP significantly improves the general appearance of the aging mice. YLSP significantly increased the levels of antioxidant enzymes, such as super oxide dismutase, glutathione peroxidase, catalase and total anti-oxidation capability, while decreasing the content of malondialdehyde in different tissues, including the liver, brain, and serum. YLSP also increased the interleukin-2 level while decreasing the interleukin-6 level. Moreover, YLSP significantly inhibited advanced glycation end product formation. Furthermore, YLSP decreased p21 and p53 gene expressions in the liver and brain of D-galactose-treated mice. These results suggest that YLSP may have a protective effect suppressing the aging process by enhancing antioxidant activity and immunity, as well as modulating aging-related gene expression.

Key words: Yulangsan polysaccharide; anti-aging; D-galactose; immunity; gene

1. Introduction

Aging is a continuously deleterious process and also associated with the development of neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease.1, 2 Thus far, the mechanisms underlying the process of aging remain uncertain. One widely acknowledged theory is referred to as the oxidative stress theory.3 It proposes that the progressive decline in physiological function is due to an imbalance between oxidative damage and anti-oxidative defense, eventually leading to neuronal death.4, 5 In addition, immunity decreases with aging.6 The aging
process is associated with a reduction in functioning T cells, B cells and mononuclear macrophage. Interleukin-2 (IL-2) is an important cytokine for lymphocyte reproduction. By contrast, advanced glycation end products (AGEs) are a feature of aging, and AGEs increase oxidative stress and inflammation. It has been reported that D-galactose(D-gal) can exert oxidative and glycative stress to the entire body, including the brain, liver and immune system. Thus the D-gal-induced senescent mouse model mimics aging in vivo.

Yulansan polysaccharide (YLSP) is a major component of the root of Yulansan (Millettia pulchra Kurz). It has been commonly used as a natural herb in Guangxi province of China for centuries to increase memory and immunity. Our previous studies demonstrated that YLSP has neuroprotective effects that include improving learning and memory, reducing oxidative stress, and anti-apoptosis effects. Recently, it was found that YLSP exhibited a protective effect on cognitive impairment in SAMP8 mice by modulating oxidative stress, neurotransmitters, such as norepinephrine, dopamine and 5-hydroxytryptamine, and gene expression. Moreover, YLSP has hepatoprotective effects against isoniazid and rifampicin-induced liver injury in mice.

Based on the data from our laboratory, we hypothesize that YLSP may be effective in attenuating oxidative stress in D-gal induced aging mice. In the present study, we investigated the effect of YLSP on oxidative and immune impairment induced by D-gal in mice, and the underlying anti-aging molecular mechanisms were also assessed.

2. Materials and methods

2.1. Chemicals

D-gal was obtained from Sigma Aldrich Co. Ltd (Germany) and dissolved in physiological saline. Vitamin E was purchased from Guilin Pharma Co., Ltd (Guilin, China). Super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), total anti-oxidation capability (T-AOC) and malondialdehyde (MDA) kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). IL-2,
interleukin-6 (IL-6), and AGEs were purchased from Wuhan Boster Bio-engineering Co., Ltd. (Wuhan, China). Primary antibodies against p21 (ab7960) and p53 (ab1431) were purchased from Abcam (Shanghai, China). β-actin (AP-0060) was purchased from Bioworld technology, co., Ltd (Nanjing, China). Horseradish peroxidase (HRP)-labeled secondary antibodies were from Keygen Biotechnology Development Co., Ltd. (Nanjing, China).

2.2. Preparation of YLSP extract

YLSP was prepared as described previously. The root of Yulangsan was dried, powdered, and extracted three times with boiling water. The polysaccharide in the filtrate was precipitated fractionally with alcohol. The protein in the product was removed using the Savage method and further purified using DEAE ion exchange cellulose (DEAE-52). The components of the saccharide were identified by gas chromatography. The results showed that YLSP consists of D-glucose and D-arabinose in a molar ratio of 90.79% and 9.21%, with an average molecular weight of 14,301 Da.

2.3 Animals and treatment

Male Kunming mice, weighing 18-22 g, were provided by the Experimental Animal Center of Guangxi Medical University (Certificate No. SYXK 2009-0002). The research was conducted according to protocols approved by our institutional ethical committee (approval no.: 20110501202). The animals were housed under controlled conditions at 25 ± 2 °C, with a relative humidity of 60 ± 10%, and a 12-hr light/dark cycle (light turned on from 8:00 AM to 8:00 PM). Food and water were available ad libitum.

After 1 week of acclimatization to laboratory conditions, the animals were randomly divided into six groups of 10 mice per group as follows: group 1 (normal control group); group 2 (D-gal model group); group 3 (D-gal + vitamin E); and group 4 to group 6 (D-gal + YLSP). For the establishment of the aging models according to the method of , D-gal (in physiological saline, 150 mg/mL) was subcutaneously injected into the dorsal necks of mice once daily at a dose of 200 mg/kg per day for 8
weeks in groups 2 to 6. In addition to D-gal, group 3 was intragastrically administered
vitamin E (Vit E, in 0.1% Tween 80, 0.2 g/kg), and groups 4 to 6 were intragastrically
administered YLSP (in 0.1% Tween 80, 0.15, 0.30, and 0.60 g/kg, representing low,
medium and high doses, respectively) at the same time. Groups 1 and 2 were
administered equivalent vehicle using the same method.

2.4 Observation of general appearance, body weight measurement, and organ index

During the entire experimental process, the general appearance was observed
daily. After 8 weeks of administration, the mice were weighed and sacrificed. Blood
and tissues were immediately collected and assayed as described below. The spleens
and thymus glands were cleared of residual blood with filter paper and weighed. The
organ index was calculated using the following equation: organ index (mg/g) = (organ
weight (mg)/body weight (g)).

2.5 Antioxidant Measurements in serum, brain, and liver

Blood samples were collected from the retro-bulbar venous plexus, allowed to clot
for 1 h, and then centrifuged at 3,000 rpm for 10 min at 4 ºC, and the supernatant was
collected as serum for assays.

Both the liver tissue and brain homogenates were prepared in ice-cold
physiological saline (10% w/v) using a homogenizer (Ningbo, China). After
centrifugation at 10,000 rpm at 4 ºC for 10 min, the supernatant was used immediately
for the biochemical analysis.

The activities of SOD, CAT, T-AOC and GSH-Px, as well as the levels of MDA
in the serum, brain, and liver were determined according to the manufacturer’s
instructions.

2.6 Detection of IL-2 and IL-6 in the serum by ELISA

The levels of IL-2 and IL-6 in the sera from each group were measured by ELISA
following the manufacturer’s instructions.

2.7 Detection of AGEs in the serum by ELISA
The production of AGEs was measured with a mouse AGEs ELISA kit, and all procedures were according to the manufacturer’s instructions.

2.8 Western Blot Analysis

The tissue proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with the primary antibodies diluted with primary antibody diluent. The secondary antibody was diluted 1:10,000 in TBST. The immune-reactive bands were detected with HRP staining. β-Actin was the protein loading control. The gray intensity of the bands was quantified using the Odyssey V 3.0 software.

2.9 Data Analysis

All data listed in the figures or tables are expressed as the mean ± SE and evaluated by one-way analysis of variance (ANOVA) with Tukey’s test for multiple posthoc comparisons (SPSS 13.0). Differences were considered statistically significant for $P$-values < 0.05.

3. Results

3.1. Effect of YLSP on general appearance, body weights and organ indexes

During the entire experimental process, there was no inflammation at the injection sites of the mice. The mice in the D-gal model group exhibited clear symptoms of aging, such as mood changes, decreased activity, and loss of hair. However, these changes in the general appearance were improved in the D-gal plus YLSP or vitamin E treatment groups.

As shown in Table 1, after 8 weeks of administration, the mean food intake, body weight and organ indexes, including thymus and spleen, of the model group were significantly lower than the normal control group ($P < 0.05$). However, these parameters were reversed following YLSP and vitamin E administration.

3.2. Effect of YLSP on MDA, SOD, GSH-Px, CAT and T-AOC content in liver, brain and serum of aging mice induced by D-gal
The MDA contents for the D-gal model group were significantly higher \((P < 0.05)\) compared to the normal control group in the liver, brain and serum (Fig. 1). Moreover, the increase of MDA contents induced by D-gal in these tissues was attenuated by YLSP \((0.15, 0.30 \text{ and } 0.60 \text{ g/kg})\) administration. As shown in Fig. 1, the MDA contents were highest in serum in each group. The last was brain, followed by liver.

The activities of SOD, GSH-Px and CAT, as well as the levels of T-AOC in the D-gal model group were significantly lower compared to the normal control group in the liver, brain and serum \((P < 0.05, \text{ Fig 1})\), whereas YLSP treatment \((0.30 \text{ and } 0.60 \text{ g/kg})\) significantly and dose-dependently alleviated the reduction induced by D-gal in the above tissue \((P < 0.05)\). In addition, YLSP \((\text{D-gal} + 0.15 \text{ g/kg YLSP})\) oral administration increased the levels of GSH-Px and T-AOC in the liver, brain and serum. As shown in Fig. 1, Trends of the SOD, GSH-Px and T-AOC activities in the serum and tissue (liver and brain) were the same as MDA. However, the CAT contents were highest in liver in each group and closer in serum and brain.

3.3. Effect of YLSP on IL-2 and IL-6 contents in sera of aging mice induced by D-gal

In the present study, the effects of PSG-1 on the IL-2 level in serum were determined using ELISA. As shown in Fig 2, compared to the normal control mice, the IL-2 level in the D-gal model mice was significantly reduced, whereas it was significantly increased after the aging model mice were treated with D-gal plus YLSP.

The level of the proinflammatory cytokine IL-6 was significantly increased in the D-gal-treated group compared to the saline-treated control group. However, increased levels of IL-6 in the serum induced by D-gal were significantly reduced for all three doses of D-gal plus YLSP \((0.15, 0.30 \text{ and } 0.60 \text{ g/kg})\) treatment groups, relative to the D-gal-administration group \((P < 0.05)\).

3.4 Effect of YLSP on AGEs content in sera of aging mice induced by D-gal

As shown in Fig 2, our results revealed that D-gal administration significantly increased by 2.2-fold the AGEs levels in the serum compared to the control group. Interestingly, D-gal plus YLSP administration at 0.30 and 0.60 g/kg per day decreased the AGEs expression by 23.4 - 39.0% compared to the D-gal treatment alone \((P < 0.05)\).
3.5. Effect of YLSP on the Aging-Related protein level

The western blot analysis shown in Figure 3 demonstrates that the protein expression of p53 and p21 in the D-gal administration group was significantly higher than the normal control group in the brain. However, in the D-gal plus YLSP (0.60 g/kg) treatment group, the expression of p53 and p21 was significantly lower than the D-gal model group ($P < 0.05$).

4. Discussion

D-gal subcutaneous injection has been widely used to establish an aging model for anti-aging research. Accumulating experiments supported that the underlying mechanisms of D-gal-induced mimetic aging related to the mitochondrial dysfunction caused by complex I deficiency, formation of high concentration of AGEs, and the increase in the osmotic stress resulting from the reduction of galactose to galactitol. Accelerated senescence mice induced by D-gal, their age are closer to two years and exhibited neurological impairment, cognitive deficits and poor immune responses. The present study clearly demonstrated that administration of D-gal at a dose of 200 mg/kg per day for 8 weeks caused severe aging related appearance changes and a significant decrease in food intake and body weight. Intriguingly, YLSP partially reversed these adverse effects.

Recent study showed that chronic administration of D-gal induces a mimetic aging effect in various tissues of rodents, such as liver and brain. Hepatocytes are very rich in mitochondria and have a high respiratory rate, so these cells may be exposed to large amounts of ROS. Brain tissues are particularly vulnerable to deteriorated age-related redox homeostasis. The activities of brain mitochondrial respiratory chain (complex I, II, III and IV) were reduced in D-gal-induced mimetic ageing. Based on the free radical theory of aging, senescence is the result of oxidative stress. SOD, GSH-Px, and CAT are the most important antioxidant enzymes that inhibit free radical formation, which is regarded as the first line of defense against the ROS generated during oxidative stress. T-AOC reflects the capacity of the non-enzymatic antioxidant defense system. MDA is a major marker of endogenous lipid peroxidation, a well-known paradigm of the oxidative damage of membranes.
under conditions of oxidative stress.\textsuperscript{30} Due to prevent oxidative stress-related neurological alteration and enhance the ability of immune cells,\textsuperscript{31} Vitamin E was chose as control. Growing evidence supports the hypothesis that increasing SOD, CAT, and GSH-Px activities, as well as T-AOC level, and decreasing MDA content, are beneficial in the prevention of oxidative damage.\textsuperscript{32, 33} Our recent studies have demonstrated that YLSP has the antioxidization capacity, possibly by eliminating oxygen radical and reducing lipid peroxidation damage.\textsuperscript{12} Consistent with previous studies, the activities of SOD, GSH-Px, CAT and T-AOC were significantly lower, whereas the content of MDA was higher in sera of D-gal-treated mice than in normal mice. Similar findings were also observed in the liver and brain tissue of D-gal-treated mice. However, the increased SOD, GSH-Px, CAT and T-AOC content and decreased MDA content showed that oxidative stress is attenuated by YLSP by restoring antioxidant enzymes and the non-enzymatic antioxidant defense system in D-gal-induced aging mice. The results suggested that YLSP can be used as an antioxidant medicine for the prevention of aging-related diseases.

AGEs, a heterogeneous group of non-enzymatic glycation products of proteins, interact with the receptor for AGE (RAGE) to promote ROS production,\textsuperscript{28} which may trigger the early phases of age-related diseases.\textsuperscript{34} Additionally, the engagement of AGES-RAGE also activates the MAPK-NF-\kappa B signaling pathways to express proinflammatory cytokines, such as TNF-\alpha and IL-6.\textsuperscript{35, 36} D-gal also reacts readily with the free amines of amino acids in proteins to form AGEs,\textsuperscript{37} then the aging process is exacerbated.\textsuperscript{38} Consistent with our previous study, our results demonstrate that D-gal injection significantly increases AGEs. Importantly, YLSP administration markedly decreased AGEs expression in D-gal-treated mice.

Immune senescence indicates an impaired immune response\textsuperscript{39} and altered immune organ structure.\textsuperscript{40} The thymus and spleen are important immune organs that show senescent signs first.\textsuperscript{41} The manipulation of cytokines is a powerful approach for the regulation of immune functions in aging. IL-2 is an important, powerful T cell growth factor and plays an important role in the immune response, immunoregulation and anti-tumor effects.\textsuperscript{42} IL-6 exhibits various bioactivities and is involved in the inflammatory and immune response.\textsuperscript{25, 43} Moreover, aging is associated with increased
circulating levels of pro-inflammatory cytokines, which at least partly results from increased AGEs. It has been reported that oxidative stress can cause activation of NF-kB mediated signaling then regulate the pro-inflammatory genes, including TNF-α, IL-6 and IL-1β. We observed that the aging mice induced by D-gal after treatment with YLSP showed significantly increased thymus and spleen indices, IL-2 production, and decreased IL-6 production. These results support the hypothesis that YLSP enhances immune functions to prevent the decrease of immune functions and proinflammatory cytokines.

Many genes are closely related to mammalian aging. The cell cycle is regulated by important regulators, such as p53 and p21, which impair the cellular regeneration of an aging organism via the P19-MDM2-p53-p21 pathway. The activation of p53 can activate downstream p21 to maintain cell cycle arrest for DNA repair. If the damage cannot be completely repaired, the cell will progress into apoptosis to maintain homeostasis. In this study, we found that p53 and p21 expression was significantly higher in the aging model than normal control. Moreover, YLSP significantly decreased p53 and p21 expression. These results indicate that YLSP regulates the expression of genes to delay liver and brain senescence.

In conclusion, the present study provides evidence that YLSP has a protective effect against D-gal-induced aging in mice, and its anti-aging mechanism may be related to enhancing antioxidant activity, improving immune functions, and modulation of aging-related gene expressions. Therefore, our experimental results suggest that YLSP may be useful in developing functional food for the anti-aging purposes.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

**Acknowledgments**
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References

Tab 1. Effect of YLSP on food intake, body weight and organ indices in aging mice induced by D-gal.

The results are presented as the mean ± SE, (n = 10). *<p>0.05 compared to the normal control group. *<p>0.05 compared to the D-gal model group. The organ index is the organ weight/body weight.

Figure legends

Fig 1. Effect of YLSP on MDA, SOD, GSH-Px, CAT and T-AOC content in the liver, brain, and serum of aging mice induced with D-galactose. The results are presented as the mean ± SE, (n = 10). *<p>0.05 compared to the normal control group. *<p>0.05 compared to the D-gal model group.

Fig 2. Effect of YLSP on IL-2, IL-6 and AGEs content in the serum of aging mice induced with D-galactose. The results are presented as the mean ± SE, (n = 10). *<p>0.05 compared to the normal control group. *<p>0.05 compared to the D-gal model group.

Fig 3. Effect of YLSP on the expression of p53 and p21 in the liver and brain of aging mice induced with D-galactose. The relative protein level between the tested target protein and internal standard β3actin was calculated and labeled on the Y axis. The data values are expressed as the mean ± SE, (n = 10). *<p>0.05 compared to the normal control group. *<p>0.05 compared to the D-gal model group. The bands are from a representative blot. Lane-1: normal control group; lane-2: D-gal-treated control group; lane-3: 0.2 g/kg Vit-treated group; lane-4: 0.6 g/kg YLSP-treated group.
<table>
<thead>
<tr>
<th>Group</th>
<th>Food intake (g/d)</th>
<th>Body weight (g)</th>
<th>Thymus index /mg.g⁻¹</th>
<th>Spleen index /mg.g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.2 ± 0.2</td>
<td>36.04 ± 2.91</td>
<td>1.9 ± 0.1</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>D-gal</td>
<td>3.8 ± 0.3</td>
<td>26.53 ± 4.31ᵃ</td>
<td>1.1 ± 0.4ᵃ</td>
<td>2.6 ± 0.2ᵃ</td>
</tr>
<tr>
<td>D-gal + 0.2 g/kg Vit E</td>
<td>4.7 ±0.1ᵇ</td>
<td>30.12 ± 2.56</td>
<td>1.3 ± 0.3</td>
<td>3.2 ± 0.1ᵇ</td>
</tr>
<tr>
<td>D-gal + 0.15 g/kg YLSP</td>
<td>4.3 ± 0.2</td>
<td>29.78 ± 3.69</td>
<td>1.5 ± 0.4</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>D-gal + 0.30 g/kg YLSP</td>
<td>4.6 ±0.2ᵇ</td>
<td>35.32 ± 3.21ᵇ</td>
<td>1.6 ± 0.1ᵇ</td>
<td>4.0 ± 0.4ᵇ</td>
</tr>
<tr>
<td>D-gal + 0.60 g/kg YLSP</td>
<td>5.1 ±0.1ᵇ</td>
<td>37.15 ± 1.34ᵇ</td>
<td>1.8 ± 0.2ᵇ</td>
<td>4.3 ± 0.2ᵇ</td>
</tr>
</tbody>
</table>
D-galactose-induced mimetic aging model

General appearance
Organ indexes
MDA, SOD, GSH-Px, CAT, T-AOC
IL-2, IL-6
AGEs
p53 and p21