Effects of purple yam polysaccharides (PYPs) on the liver and brain in a D-galactose-induced model of aging in rats

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Abstract: Purple yam polysaccharides (PYPs) are natural polysaccharides extracted from purple yams, a Chinese yam with bright purple flesh used as a traditional Chinese medicine (TCM) and functional food. This study aimed to verify the anti-aging activity of PYPs and explore their mechanism of action at the molecular level. Purple yam powder water extracts were precipitated with ethanol at a final concentration of 80% (v/v). PYPs were obtained after the precipitate lyophilized. A model of aging in rats by injecting D-galactose for 45 d with the simultaneous administration of PYPs (20, 100, and 500 mg/kg bw) was constructed. The results showed that PYPs administration reduced damage to the liver and brain cells of rats and attenuated the production of malondialdehyde (MDA) induced by D-galactose. In addition, PYPs administration significantly restored the activities of superoxide dismutase, catalase, glutathione, and glutathione peroxidase and the total anti-oxidant capacity. The expression levels of the p16 and p21 genes in the liver and brain tissues of rats treated with PYPs were also found to be significantly down-regulated compared with their expression in the D-galactose group. Based on these results, the conclusions can be drawn that PYPs protect against the oxidative damage caused by D-galactose and can delay the process of senescence by affecting the p16/p21 pathway, and PYPs could be a beneficial ingredient for inclusion in healthcare products.
Keywords: purple yam polysaccharides, D-galactose, p16/p21 genes, aging

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1 Introduction

The Chinese yam (*Rhizoma dioscoreae, Dioscorea opposita*) has been used as a TCM for more than 2000 years for treating diabetes (Zhang et al., 2011; Marie et al., 2006), diarrhea (Park et al., 2013; Huang et al., 2012), pneumopathy (Yang et al., 2015), and other ailments (Han et al., 2014; Liu et al., 2007; Chiu et al. 2013). Chinese yam polysaccharides are the main functional components of this medicine and in the past decade, a number of studies on these polysaccharides have reported that the results showed the Chinese yam polysaccharides have anti-aging effects (Hsuet et al. 2009; Ju et al. 2014). The purple yam (*Dioscorea alata* L) is a type of Chinese yam with purple flesh and is considered to have a greater nutritional value than other Chinese yams due to its anthocyanin content (Kumi 1991a; Kumi 1991b; Lubag 2008). However, as a novel functional raw material, there are few studies on purple yam that focused on the breeding of this plant and analyzing its nutritional composition, such as the levels of anthocyanin, saponin, and polysaccharides. So far, no specific research has been conducted on the extraction and function of PYPs.

D-galactose (D-gal) is a reducing sugar that can disturb the body's metabolic process, cause cognitive declines, reduce the activity of anti-oxidant systems, and even cause cell senescence if fed to rats in excessive

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amounts. A great deal of research has been performed on D-gal-induced models of aging in rats to evaluate antioxidant capacities (Jin 2012), memory repair (Wang 2016), cognitive improvements (Otilia 2014; Wang 2010), protection against the effects of aging (Mao 2010) and other topics.

Currently, more and more researchers are focusing their studies on the molecular mechanism of senescence to evaluate the anti-aging activities of compounds and identify materials that may be valuable in preventing age-related diseases (Flachsbart 2010; Murielle 2009; Ryu 2008). In recent years, studies have shown that there are senescence-associated genes (SAGs) in the human body, which can lead to senescence when overexpressed (Yoon 2004; Park 2011). P16 and p21 are two of the most important senescence genes. A large number of studies have indicated that the main pathways of cellular senescence induced by oxidative stress involve the expression of p16 and p53/p21 (Lim 2011; H. Christian Reinhardt 2012). Therefore, p16 and p53/p21 likely play key roles in regulating pathways leading to cellular senescence.

In this paper, the D-gal-induced model of aging in rats was established. The activity of anti-oxidant enzymes and the expression of p16 and p21 mRNA were then measured and the histological changes in liver and brain tissues of aging rats were observed to evaluate the anti-aging effects of PYPs.

2 Material and methods

2.1 Preparation of PYPs

Purple yam rhizomes (*Dioscorea alata* L), which is a tuber of Dioscorea opposite Thunb of Dioscorea plants, are mainly produced in Jiangxi, Fujian, Guangxi provinces of China. In this study, the purple yam rhizomes were purchased from *Wanzai Huiming Organic Agriculture Science and Technology Co., LTD.* in Jiangxi province of China.

The rhizomes were washed with water, then sliced and freeze-dried and crushed to obtain the lyophilized purple yam powder. The powder (100 g) was soaked in 80% ethanol (8 times the volume of the powder) and stirred for 2 h at ambient temperature to remove some of the colored and organic materials. The extraction mixture was then centrifuged, and the supernatant was discarded. Crude PYPs were extracted from the precipitate under the following optimized extraction conditions: ratio of water to raw materials, 20:1; extraction temperature, 80°C; extraction time, 2 h; extractions were performed twice. Then, the solution was centrifuged at 8000 r/min for 30 min and concentrated with a rotary evaporator at 55°C under a vacuum. After this step, ethanol was added at a final concentration of 80% (v/v), and the solution was kept at 4°C overnight. The crude PYPs samples were obtained via centrifugation (8000 r/min, 30 min), and the precipitate was lyophilized.

2.2 Chemicals

The following chemicals were of analytical grade and were obtained commercially: D-galatose (dissolved in 0.9% saline at a concentration of 400 mg/mL; Beijing Chemical-Reagent Company, Beijing, China); Trizol (Invitrogen, USA), DNase I, 6×Loading Buffer (Fermentas (MBI), USA), M-MLV, dNTP, 2×Ex TaqMix (Takara, Dalian, China), and Eva_green (Biotium, USA). Assay kits for measuring superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) were purchased from Jiancheng Institute of Biotechnology, Nanjing, China.

2.3 Animals and models

A D-galactose-induced model of aging in rats was established as previously described (Fu, 2011). Fifty healthy male SD rats (average weight, 150±2.4 g; provided by the experimental animal center, Academy of Military Medical Sciences, Permit No. scxk (Army) 2012004) were randomly divided into five groups: a control group (Cont), a D-gal-treated group (D-gal), a low-dosage group (PYP-L, 20 mg/kg bw), a medium-dosage group (PYP-M, 100 mg/kg bw), and a high-dosage group (PYP-H, 500 mg/kg bw). The Cont group was injected with normal saline daily for 45 d, while the other groups were injected with D-gal (400 mg/kg bw) over the same period. In this period, the low, medium and high-dosage group was administrated with PYPs, while the Cont group and the D-gal group were administrated with normal saline. All groups were

housed in an air-conditioned animal facility maintained at a constant temperature of 23°C±1°C, a relative humidity of 60%±5%, and with a 12 h day/night cycle. After 45 d, all the rats were sacrificed, and the liver and brain tissues were collected.

2.4 Biochemical assays

The liver and brain tissue samples were ground (1:10 w/v) in a cold saline solution (0.86% NaCl). Homogenates was centrifuged at 3000 g for 10 min at 4°C, and the supernatant was collected for the detection of antioxidant enzymes. The T-AOC, the SOD, GPx and CAT activities and the GSH and MDA contents were measured using commercial kits according to the manufacture's protocol.

2.5 Histological analysis

Liver and brain tissue samples were rinsed with distilled water and fixed with 10% neutral buffered formalin. They were then dehydrated in an increasing series of ethanol, cleared in xylene and embedded in paraffin wax. Slices (4 μm thick) where collected and stained with hematoxylin-eosin (HE). Histological assessments were performed using a Leica camera (Germany).

2.6 RNA isolation and real-time PCR

Total RNA was extracted separately from liver and brain tissue using a Trizol solution following the manufacturer's protocol, and the samples were preserved at −70°C. Total RNA was quantified via denaturing agarose gel electrophoresis (1.5% agarose gel). A 40-μL reaction system was used for the reverse transcription, which contained 4 μg RNA, 4 μL reverse transcription primer T18 (50 μM), 8 μL5×first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 2 μL dNTP mix (10 mM), 1 μLRNase inhibitor (20 Uμ/L, MBI), 2 μLM-MuLV (MBI), and DEPC water. The reaction mixture was incubated at 42°C for 60 min and then heated at 70°C for 10 min to spot the reaction.

Quantitative RT-PCR reactions were conducted in a volume of 25 μL using an ABI-2720 PCR system (ABI). Each reaction contained 1.0 μL template (cDNA), 10 mM forward/reverse primer (0.75 μL), 12.5 μL 2xEx TaqMix, 0.75 μLEVA green, and 10 μLddH_2O. All primers are shown in Table 1. All PCR reactions were performed under the same conditions: an initial denaturation at 95°C for 5 min, a denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and termination of the reaction at 4°C.

Table 1 PCR primers

Primer	Sequence, 5′ to 3′	Base number	Length of product bp
Actin (forward)	GAAGTGTGACGTTGACATCCG	21	282
Actin (reverse)	GCCTAGAAGCATTTGCGGTG	20	282
p16 (forward)	CTACTCTCCTCCGCTGGGAA	20	116
p16 (reverse)	GGCCTAACTTAGCGCTGCTT	20	116
p21 (forward)	CTGGTGGCGTAGGCAAGA	18	201
p21 (reverse)	AGCCCTCCCCAGTTCTCAT	19	201

2.7 Statistical analysis

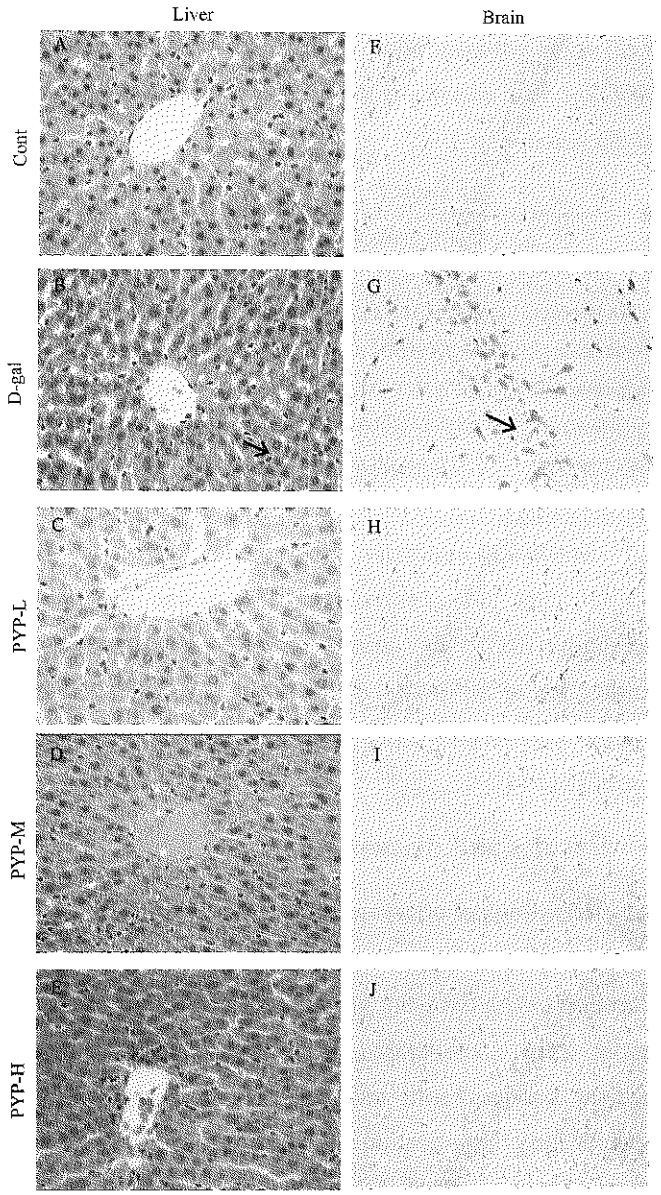
All data are expressed as the means ± standard deviation (SD), and analyses were performed using Turkey test in SPSS 19.0 (SPSS China, Shanghai, China). Significant between groups was determined using $p<0.05$ confidence limits.

3 Results and discussion

3.1 Histological observations

Typical histological images are shown in Figure 1. Compared with the Cont group, liver cells from the D-gal group were characterized by a column cells decrease, nuclear condensation, enhanced staining, and obvious cellular fatty degeneration. However, these characteristics were improved in PYP-treated rats, especially in the PYP-M and PYP-H groups, with the cells showing a tight and orderly organization and a no fatty degeneration. These results indicate that PYPs can prevent the liver damage induced by D-galactose.

The images of the CA1 region of the hippocampus in the Cont rats showed a normal appearance: 3-4 layers of pyramidal neurons arranged closely and neatly, all with a clearly visible nucleus, nucleolus, and cytoplasm. However, this arrangement of neurons became loose and disorganized following D-gal-induced injury: the structure of neurons was changed and some neurons died or even disappeared. PYP-treatment group appeared to obviously stimulate the recovery of neuronal damage caused by D-gal. As can be observed in Figure 1, the neurons in the samples from the PYP-M and PYP-H groups generally appeared normal.



B: Liver of D-gal rats. Tissue is marked by the appearance of obvious cellular fatty degeneration (black arrow), which vanishes in D (PYP-M, 100 mg/kg bw) and E (PYP-H, 500 mg/kg bw). G: CA1 region of the hippocampus of a D-gal rat brain. Pyramidal cell morphology is changed entirely, with cell deformation and death (black arrow). H (PYP-L, 20 mg/kg bw), I (PYP-M, 100 mg/kg bw), and J (PYP-H, 500 mg/kg bw) showed relatively normal pyramidal cell morphologies.

Figure 1 Effects of PYPs on the histological characteristics of liver and brain tissues of rats

3.2 Antioxidant enzyme activities and GSH and MDA contents in the liver and brain

Many studies have shown that bioactive polysaccharides have antioxidant properties that can enhance the activity of the body's antioxidant enzyme system. GSH, GPx, T-AOC, CAT, and SOD are the main antioxidant enzymes and can effectively scavenge ROS in the body. As is shown in Figure 2, compared with the activities in the Cont group, the antioxidant enzyme activities were obviously reduced in the D-gal group, while PYP treatment counteracted this D-gal-induced decline in antioxidant capacity and even significantly increased GPx activity ($p<0.01$), T-AOC ($p<0.01$) and the GSH content ($p<0.05$) in the liver of rats. PYP treatment also protected the antioxidant enzyme system in the brains of rats, but this influence was significantly weaker than that observed in the liver.

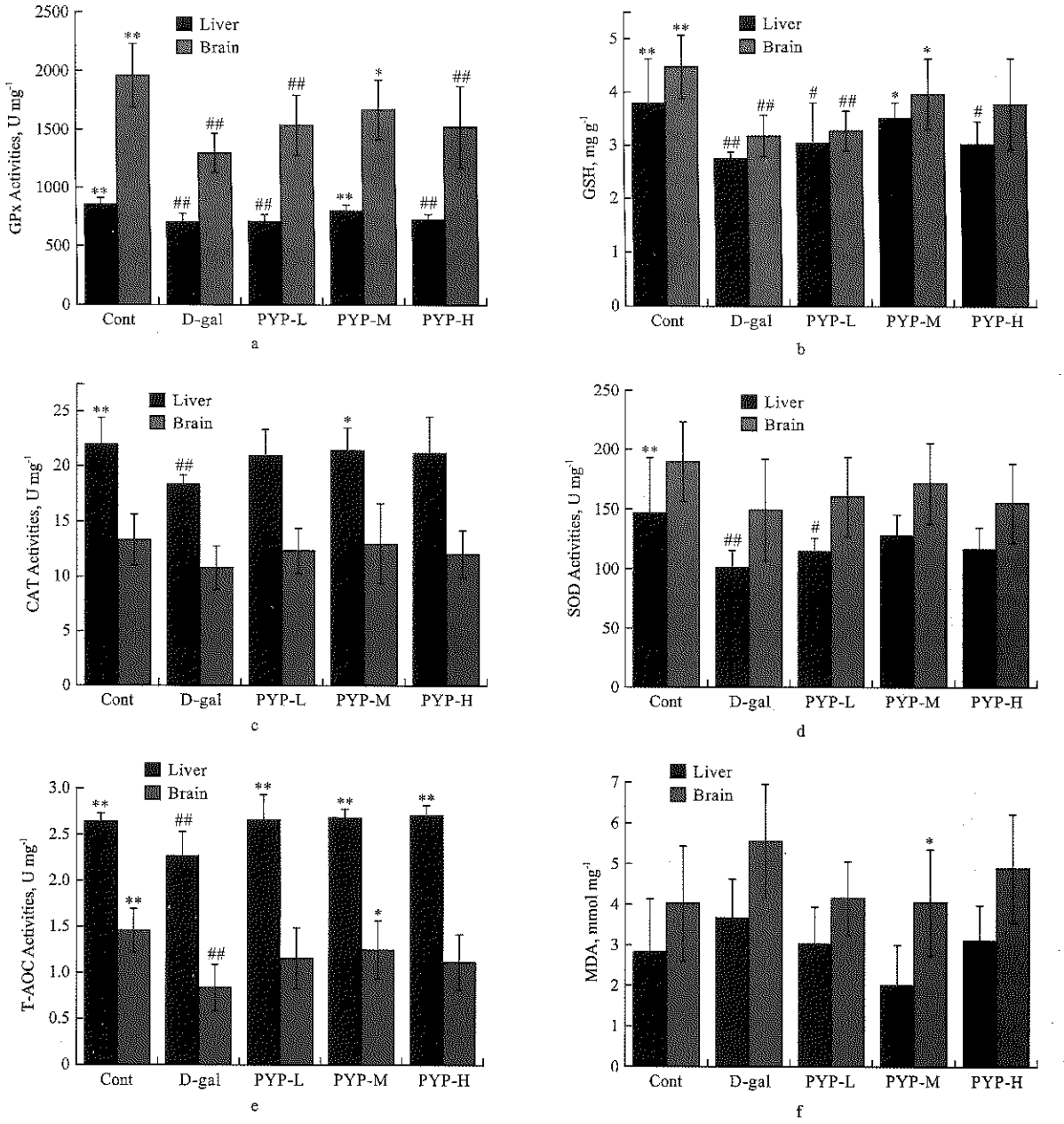
MDA is a by-product of the membrane lipid peroxidation caused by free radicals and is widely used as a biomarker of oxidative stress. The results in Figure 2f show that D-gal treatment led to higher MDA levels in the liver and brain compared with levels in the Cont group. However, the MDA content was decreased compared with levels in the D-gal group after PYP treatment.

3.3 Expression of p16, p21 mRNA in rat liver and brain tissues

Compared with their expressions in the Cont group, p16 and p21 mRNA expression in the D-gal group was significantly increased ($p<0.01$) in rat liver and brain tissues, indicating that the D-gal-induced aging model was successful. As Figure 3a and b show, the mRNA expression of p16 and p21 in the PYP-treated groups was nearly the same as that in the Cont group, with the low-, medium- and high-dose PYP-treated groups showing significantly lower expression levels than were observed in the D-gal group ($p<0.01$). This indicates that PYPs function to delay age-associated effects by reducing the expression of the senescence-associated genes p16, p21.

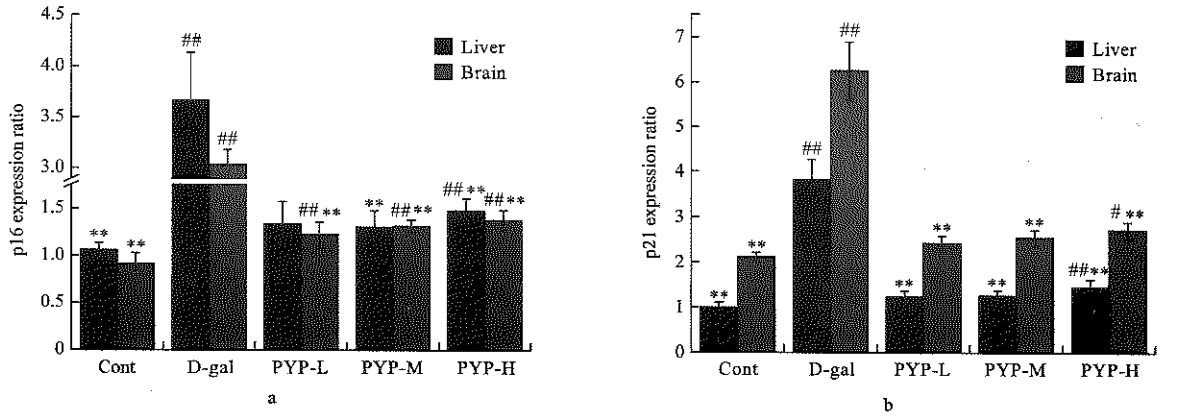
4 Discussion

An increasing number of studies have demonstrated that rats receiving subcutaneous injections of D-gal (50-500 mg/kg) for 6-8 weeks showed a loss of neurons, oxidative damage and mitochondrial dysfunction, resulting in cognitive dysfunction, memory impairment and other signs of aging, which are processes that are similar to those that normally occur in aging brains (Gong, 2016; Gong, 2015; Zhou, 2013). This study proved that long-term injections of D-gal can successfully establish an animal model of aging.



Note: Values are expressed as the means ±S.D. (n=10). $^{\#}p$<0.05 compared with the Cont group, $^{\#\#}p$<0.01 compared with the Cont group, $^{*}p$<0.05 compared with the D-gal group, $^{**}p$<0.01 compared with the D-gal group.

Figure 2 Effect of PYPs on the activities of antioxidant enzymes and the GSH and MDA content in liver and brain samples from rats



Note: Values are expressed as the means ±S.D. (n=10). $^{\#}p$<0.05 compared with the Cont group, $^{\#\#}p$<0.01 compared with the Cont group, $^{*}p$<0.05 compared with the D-gal group, $^{**}p$<0.01 compared with the D-gal group.

Figure 3 Effect of PYP treatment on p16 and p21 mRNA expression in the liver and brain tissues of rats

After 45 d of treatment, the rats in Cont group and the PYP-treated groups showed normal dietary habits, were lively, and had good skin elasticity, while the D-Gal group showed weakness, slowed activity, a dull hair color, and even depilation. Moreover, the antioxidant indices of D-gal-treated rats were significantly different than those of the control group, which shows that the D-gal-induced aging model was successful.

The overproduction of free radical and the chain reactions they can cause are essential factors leading to age-related effects in the body. Excessive free radicals can attack the lipids and proteins located in the inner mitochondrial membrane, resulting in mitochondrial dysfunction, cell damage, aging and disease.

SOD, GSH-Px, GSH and CAT exist in the body as an endogenous antioxidant defense system. GSH is a tripeptide and is involved in a number of crucial cellular functions, such as free-radical scavenging, metal binding and detoxification. GSH-Px is an enzyme specialized for protecting glutathione from peroxide reactions, and it plays a significant role in maintaining cell membrane integrity. In this study, the GSH content and GSH-Px activity were shown to be simultaneously and significantly increased, relative to the levels in the D-gal group, in the liver and brain tissues of PYP-treated rats, particularly in the PYP-M group, and both reach 80% of the levels in control group. These data indicated that PYPs has enhancing effects on the activity of antioxidant enzymes in removing ROS and resisting oxidative damage and oxidative stress. In addition, PYPs can recover the activities of CAT and SOD in D-gal-induced aging rats. MDA is an important product of lipid peroxidation, and it is a sensitive indicator that can reflect the level of free radicals and the extent of oxidative damage occurring under conditions of oxidative stress. The MDA content was sharply increased in the liver and brain tissues of the D-gal group, which demonstrates that the excessive intake of galactose leads to a disruption of lipid metabolism. MDA levels were reduced in the PYP-treated groups. All the results above suggest that PYPs can delay the aging-induced effects of oxidative stress in the liver and brain by improving the ability of the body to scavenge oxygen free radicals.

Recent research has found that aging is controlled by specific genes, such as the p16 gene (Shen, 2006). P16 is a significant Ink4 (inhibitor of CDK4) protein and can inhibit the effect of the cyclin-D1 CDK6 and cyclin-D1 CDK4 complex. Excessive p16 expression is considered a signal of cell senility. Although a few researchers suggest that the abnormal expression of some aging-related genes are just a small component of the factors that influence cell senescence, numerous experiments have confirmed that the overexpression of p16 or p53/p21 can specifically cause cell senescence (Victor, 2010; Brigitte, 2008; Wang, 2014; Tozawa, 2007; Feng, 2008; Guterres, 2013). The expression of p16, based on an immunohistochemical evaluation, is consistent with a good prognosis of some cancers, and the expression of the p16 gene can be used as a predictor for the aging-related diseases (Yang, 2014; Su, 2014; Tsygankov, 2009). In this study, the expression of the p16 gene was significantly upregulated in the liver and brain tissues of the D-gal group compared with its expression in the control group ($p< 0.01$) and was maintained at a level close to that of control group by PYPs treatment. This suggests that the capacity of PYPs to delay senility is related to a decrease in p16 gene expression.

P21 is another important biomarker of cell senescence, and the expression of p21 is significantly increased in aging cells. P21 is an important member of the cell cycle inhibiting group of proteins, which can inhibit the activity of the cyclin-dependent kinase (CDK) complex, regulating the relationship between the cell cycle and DNA repair. Many of the studies on p21 have mainly focused on the function of p21 in relation to tumor cells, and all the results showed that the expression level of p21 can influence many types of cancer (Koo 2015). Currently, p21 expression is used as an indicator in the diagnosis and prognosis of tumors (Masson, 2015; Varshney, 2015; Jawanjal, 2015). P21-induced cellular senescence is also related to reactive oxygen species (ROS). It has been found that p21 can increase the level of ROS in normal fibroblasts and p53-negative tumor cells, while ROS inhibitors can delay p21-induced senescence (Yang 2014). PYPs significantly reduced the overexpression of the p16 and p21 genes induced by

D-galatose ($p<0.01$), suggesting that PYPs function in delaying cellular senescence by reducing the expression of senescence genes.

5 Conclusions

In this paper, D-gal-induced model of aging in rats was successfully established and these rats with PYPs were treated. The results showed that PYPs can improve the activity of antioxidant enzymes in the body, helping to prevent or repair the damage caused by D-gal in the livers and brains of rats. Furthermore, PYPs can alter the expression levels of the senescence genes p16 and p21 in livers and brains of aging rats. These results suggest that PYPs can play an important role in preventing the aging-related effects caused by oxidative stress and that they could be a new type of raw material for use in health foods, acting as a potential functional antioxidant.

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