Pharmacogn. Mag.
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ORIGINAL ARTICLE

The Effects of Polysaccharides from *Rehmannia glutinosa* on *Caenorhabditis elegans*

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Submitted: 03-12-2018 Revised: 14-01-2019 Published: 16-05-2019

ABSTRACT

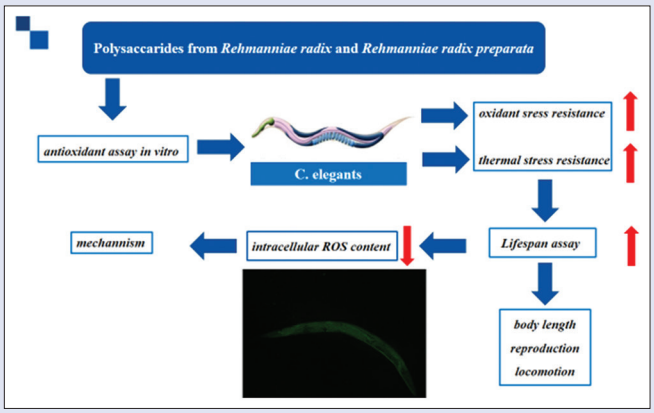
Background: Polysaccharides are isolated from *Rehmannia glutinosa* (RG), a well-known traditional Chinese medicine starting from ancient China. However, their effects on anti-aging activity have not yet been evaluated. **Objective:** To evaluate the stress resistances and anti-aging effects of polysaccharides from RG by *Caenorhabditis elegans* N2 wild type. **Materials and Methods:** Preparing the polysaccharides from Rehmanniae radix preparata (PRRP) and Rehmanniae radix (PRR), we determined the antioxidant activity *in vitro*, stress resistance, lifespan, fertility, physical growth, locomotion, and impact on the formation of reactive oxygen species (ROS) on *C. elegans*. **Results:** The results showed that both polysaccharides have little scavenging ability of free radicals *in vitro*. After PRRP and PRR treatment, the stress resistance and body bending frequencies of nematodes were significantly increased. PRRP was shown to extend the lifespan and promote physical growth of *C. elegans*. Both polysaccharides had little effect on fertility and locomotion ability of *C. elegans* but can reduce excessive intracellular ROS. **Conclusion:** For *C. elegans* organism model, both PRRP and PRR are heat resistant and antioxidant; PRRP not only can extend lifespan but can also promote growth and development. PRRP had an anti-aging effect on *C. elegans* without affecting their reproductive capacity. Based on the scavenging capacity of ROS, we hypothesized that the mechanism of PRRP prolonging lifespan may be related to increase resistance and remove excess free radicals in time.

Key words: *Caenorhabditis elegans,* lifespan, polysaccharides, *Rehmannia glutinosa*, stress resistance

SUMMARY

- The purity and antioxidant activity of polysaccharides from *Rehmannia glutinosa* (RG) were determined after extraction, separation, and purification
- Polysaccharides from RG were confirmed to be anti-aging and antistress on *Caenorhabditis elegans*
- The mechanism of polysaccharides from RG prolonging lifespan was preliminarily research.

Abbreviations used: RG: *Rehmannia glutinosa*; *C. elegans: Caenorhabditis elegans*; PRRP: Polysaccharides from Rehmanniae radix preparata; PRR: Polysaccharides from Rehmanniae radix; ROS: Reactive oxygen species; RR: Rehmanniae radix; RRP: Rehmanniae radix preparata; PRG: Polysaccharides from *Rehmannia glutinosa*; DPPH: 1,1-diphenyl-2-picrylhydrazyl; NGM: Nematode growth medium; *E. coli* OP50: *Escherichia coli* OP50.



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DOI: 10.4103/pm.pm_618_18

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INTRODUCTION

Rehmannia glutinosa (RG), a genus of plants in the family of *Scrophulariaceae Rehmannia* Libosch, is a traditional Chinese medicine recorded in *Shen Nong's Herbal Classics*. It has been used in China for >2200 years. According to the traditional Chinese medicine theory and clinical needs, Rehmanniae radix (RR) and Rehmanniae radix preparata (RRP) are two different Chinese herbal slices. As is recorded in the *Chinese pharmacopoeia*,[1] their preparation methods and efficacies are different. For example, RRP needs to be steamed or boiled but RR does not. RR has the effect of clearing heat, cooling blood, nourishing Yin, and producing fluid. However, RRP has the effect of nourishing blood, enriching Yin, and boosting essence. Among them, polysaccharides from *Rehmannia glutinosa* (PRG), as one of the most effective components in RG, play an important pharmacological role in regulating blood glucose and lipids and improving immunity and being anticancer. For example, Zhou *et al.*[2] studied the antihyperglycemic and antihyperlipidemic effect of oral administration of a purified PRG and its underlying mechanisms in streptozotocin-induced diabetic

Cite this article as: Yuan Y, Kang N, Lu Y, Miao X, Zhang X, Liu Y, *et al*. The effects of polysaccharides from *Rehmannia glutinosa* on *Caenorhabditis elegans*. Phcog Mag 2019;15:385-91.

mice, which illustrated that RGP may become a potential treatment option for type 1 diabetes. Zhang *et al.*[3] proved that PRG promote the maturation of dendritic cells and provided evidence and theoretical basis for the use of PRG to enhance host immunity under different clinical conditions. Huang *et al.*[4] investigated the effect of PRG treatment on dendritic cells using "3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide method and found that it can significantly promote the proliferation of lymphocytes, while the growth rate of T-cells is more significant. PRG was also found to have certain anticancer activity. Wang *et al.*[5] demonstrated that PRG can induce human dendritic cells to mature and have an anticancer effect on mice. Xu *et al.*[6] found that PRG can induce Toll-like type 4 receptor-dependent splenic dendritic cell maturation and inhibit the growth of cancer and melanoma in mice. Xu *et al.*[7] confirmed that PRG activate natural killer cells and inhibit tumor growth. However, there have been almost no experimental studies on the anti-aging effect of PRG until now.

Polysaccharides from *Rehmanniae* radix preparata (PRRP) and *Rehmanniae* radix (PRR) were extracted from PRR and RR, respectively. Since RRP and RR have different efficacies, the pharmacological effects of PRRP and PRR may also be different. For example, it may be the preparation methods of PRR that changes its pharmacodynamic components and results in different pharmacological effects. Tan *et al.*[8] studied the antifatigue effects of PRRP in mice and confirmed that PRRP may be responsible for the pharmacological effects of antifatigue of radix Rehmanniae preparata. Cui *et al.*[9] investigated mechanism-based antianxiety effects of PRRP through two-dimensional electrophoresis analysis with mass spectrometry of the hippocampus proteins in rats treated with monosodium L-glutamate. This study aims to elucidate the pharmacological effects and differences of PRRP and PRR.

In terms of researches on the antioxidant and anti-aging effects of PRG, there is a lack of a biological evaluation model with simple operation, low cost, and time-saving. Because of its short life cycle, easy reproduction, clear genetic background including aging, flexible and convenient operation, low cost of cultivation, and other characteristics, *C. elegans* plays an important role in studies on antioxidant activities, thermal stress, and anti-aging activities of natural plant polysaccharides. Lee *et al.*[10] found that a brazilin from *Caesalpinia sappan* significantly extends the lifespan and improves the resistance of *C. elegans* by increasing the expression of stress proteins. Icariin II can also extend the lifespan of *C. elegans*, increase the expression of daf-16 in wild nematode, and make it more resistant to heat and oxidative stress.[11] Zhang *et al.*[12] researched the antioxidant and neuroprotective effects and mechanisms of *Dictyophora indusiata* polysaccharides by *C. elegans*. Xiang *et al.*[13] used nematodes to study the effects of *Epimedium* polysaccharides on the neurotoxicity induced by poly-Q by reducing oxidative stress. Therefore, a large number of experiments have proved that *C. elegans* can be used as an antioxidant and anti-aging drug activity evaluation model organism. Based on the previous experimental researches of our research group, we took 5.0 mg/ml PRR and PRRP as the research object and nematode as the biological model to study the effects of PRR and PRRP.

MATERIALS AND METHODS

Preparation of polysaccharides from *Rehmannia glutinosa*

RR and RRP will be cut into 0.5 cm^2 small pieces, and 10 times more acetone was added to purge fat-soluble components. We added 10 times more water to pigment and decoct it on a radiant cooker for 3 times, each time for 1 h. Then, we merged the decoctions and concentrated the filtrate by reduced pressure distillation at 70°C to an appropriate amount. Next, we added 95% ethanol until the content of it in filtrate as 80%, placing it for 12 h under 4°C. Then, we filtered and froze it to dry. We dissolved it in distilled water and deproteinized by Sevage (chloroform: n-butanol = 4:1) and repeated more than 10 times until no protein absorption was detected by UV spectrum analysis. Finally, the PRG were obtained by freeze-drying.

Determination of purity of polysaccharides

The purity of polysaccharides was determined by phenol-sulfuric acid method. We prepared a series of glucose standard reference solutions and measured their absorbance at the wavelength of 491 nm. The absorbance was used as the ordinate. The glucose mass concentration was used as the abscissa. We can draw the standard curve of absorbance and concentration and get the standard curve equation. The absorbance of the prepared polysaccharide solution was measured under the same conditions. By calculation, we can get the purity of the polysaccharides.

In vitro antioxidant assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) method is used to determine the antioxidant activity of polysaccharides *in vitro*;[14] we prepared DPPH original solution, a series concentration of polysaccharides sample solution and Vc solution as a positive control. 517 nm was the measured wavelength. Methanol was the reference to adjust to zero. First, accurately absorbing 2 mL DPPH solution and 2 mL deionized water are mixed uniformly, and then, we measured the absorbance by UV-spectrophotometer, denoted as A_0. Finally, Vc sample solution and polysaccharides solution of different concentrations were mixed evenly with 2 mL DPPH solution, and absorbance was measured after 40 min. Measure three times and take the average. Clearance rate (SR) % = $(A_0 - A_{sample})/A_0$. The linear curves of Vc and PRG concentrations and clearance rates were, respectively, performed. The EC_{50} of Vc and polysaccharides was calculated and compared.

Worm strain maintenance

The strains used in this study were wild-type N2 (obtained from the Chinese Academy of Sciences). Nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C as described.[15] The nematodes were transferred to a fresh NGM plate every 2 days.

Synchronization of nematodes

Under aseptic conditions, 70–80 adult nematodes in the spawning period were selected on a new NGM plate coated with *E. coli* OP50, and all the adults were removed after laying eggs for 3 h. "These spawn plates were put at 20°C germ-free biochemical incubator for 2 days. Then we got homochronous nematodes in the L4 period." Meanwhile, We have decided not to cite any literature here.

Exposure experiments

The polysaccharide was added to the *E. coli* OP50 fluid at a concentration of 5mg/ml. Add the fluid to the surface of NGM. Nematodes were inoculated the synchronization process in the L4 period and then cultured under 20°C for 24 h. In the control group, nematodes were cultured on the NGM that *E. coli* OP50 containing Dimethyl sulfoxide was added.

Paraquat resistance assay

The paraquat assay was performed as described previously.[16] Paraquat is a kind of herbicide with internal adsorption, which can lead to oxidative damage in nematodes, and is used to study the antioxidant effect of polysaccharides. The L4 nematodes were treated with the polysaccharide for 24 hours before exposing to 70 mM paraquat. We put these plates at

20°C incubator, every 12 h to observe and record the survival nematodes. The live nematodes were transferred to new NGM plates coated with *E. coli* containing 70 mM paraquat every 24 h until all nematodes died. There were 30 nematodes in each NGM plates.

Thermal stress assay

Reference to the methods of nematodes of thermal stress experiment,[17] nematodes were exposed to the polysaccharides, 0.04 mg/ml of aspirin as the positive drug for 24 h, and transferred to normal NGM plates. Each plate had 30 nematodes and put them in an oven at 36°C medium, every 2 h to observe and record the survival condition of nematodes, until all nematodes were killed.

Lifespan assay

Nematodes lifespan experiment with reference to the literature,[18] the L4 nematodes were respectively transferred to the NGM plates with or without polysaccharides. There were 30 nematodes in each plate, per 48 h to observe and record the survival nematodes. The survival nematodes were transferred to the new NGM with or without polysaccharides until all nematodes died.

Determination of body length of nematode

The L4 nematodes were treated with the polysaccharide for 24 hours. Subsequently, *C. elegans* were exposed to thermal stress at 50°C for 5 min. Nematodes were killed. We observed and photographed by a Somatic microscope with CDD and used Image J software to measure worm body length. Thirty nematodes were measured in each group.

Fertility assay

The nematodes exposed to PRG for 24 h and the blank group were transferred to new NGM for spawning and permitted to lay eggs for 5 days. There is one nematode on per plate, 12 nematodes per group. We transfered them from a plate to a new every 24 h. Eggs spawned by a single worm were incubated at 20°C for 72 h. Then, the numbers of offspring were counted.

Locomotion behavior

According to the measurement method reported in the literature,[19] 30 nematode worms exposed to PRG for 24 h were placed on the NGM without the addition of *E. coli* OP50 and the number of body bending times within 20 s was recorded.

Intracellular reactive oxygen species content

As mentioned in the literature,[20] the L4 nematodes were treated with the polysaccharide for 24 hours before exposing to 70 mM paraquat. Then, worms were washed for 3 times using M9 to remove bacteria. Subsequently, worms were incubated in M9 containing 250 μM H_2DCF-DA for 2 h. Fluorescence was measured at excitation/emission wavelengths of 485 and 520 nm by a fluorescence microscope (ECLIPSE, TSR, Nikon). The fluorescence intensity was quantified by Image J software (National Institutes of Health, Bethesda, MD, USA). Six worms per group were used in each experiment.

Criteria for nematodes death

No swimming and swallowing movements and no reaction aftertouch were the criteria.

Statistical analyses

All the tests were carried out in parallel three times and the average value of the three times was taken for the result analysis. SAS 8.2 statistical software SAS 8.2 (SAA institute, Gary, NC,USA) was used to analyze the difference between the groups with *t*-test, and GraphPad Prism 5 (Prism, GraphPad Software, San Diego, CA) was used for the survival curves and survival analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Purity of polysaccharides

The standard curve regression equation of glucose concentration and absorbance was obtained through experimental data as $y = 1.6616x + 0.229$, $r = 0.999$. The purity of PRR is 71.2%. The purity of PRRP is 66.7%.

1,1-Diphenyl-2-picrylhydrazyl radical scavenging effect

PRG have some antioxidant activity *in vitro*. The curves of concentration and clearance rates of Vc, PRR, and PRRP are, respectively, shown in Figures 1-3. Using clearance standard curve equation, we obtained half clearance (EC_{50}) of Vc, PRR, and PRRP, which the values were 0.0021, 0.1845, and 0.1291, respectively. Namely, compared with VC, PRR and PRRP had weaker antioxidant activity *in vitro*. It showed that PRR and PRRP have certain antioxidant capacity *in vitro* and PRRP were stronger than PRR. The antioxidant capacity of PRR and PRRP *in vitro* had been verified, and their antioxidant capacity *in vivo* was worth further investigation.

Effect of polysaccharides on stress response

Survival rates of nematodes under paraquat action are shown in Figure 4. Compared with the blank group, nematodes exposed to polysaccharides had a higher survival rate of after 36 h and a longer maximum survival time of 36–48 h. Compared with the PRR group, the nematodes exposed to PRR had a higher survival rate and the maximum survival time was extended for 12 h. The above results indicated that PRG have certain antioxidant effect *in vivo* and the antioxidant effect of PRRP *in vivo* was stronger than PRR at the same concentration, which also showed the possibility of the anti-aging effect of PRG.

As shown in Figure 5, compared with the blank control group, the PRR, PRRP, and aspirin groups largely increased the nematode survival rates and the survival rates: PRRP group > PRR group > aspirin group > blank control group. The results indicated that the thermal stress resistance of PRRP was stronger than PRR and aspirin at a certain concentration, which further proved that PRG can resist the aging of nematodes.

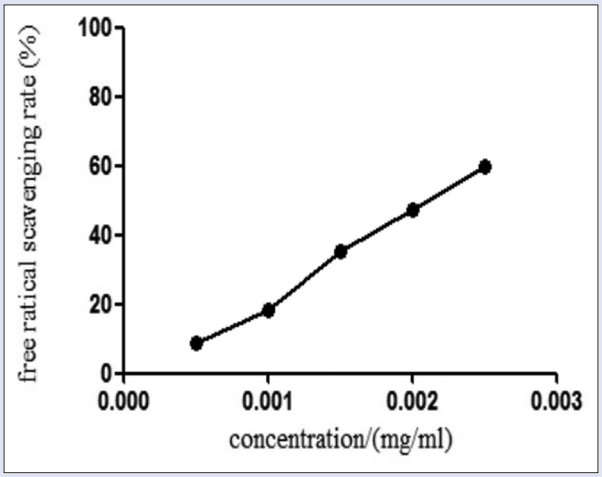

Figure 1: Free radical scavenging curve at different concentrations of Vc

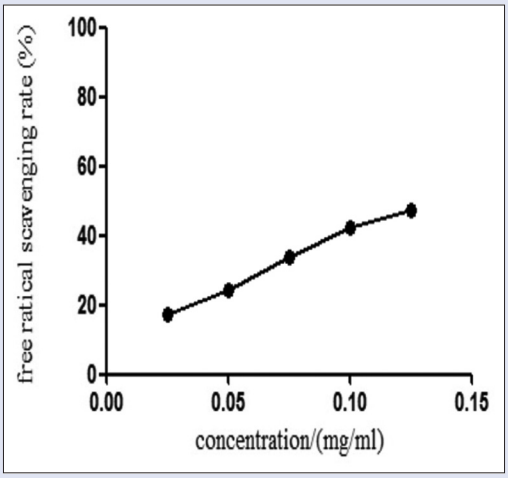

Figure 2: Free radical scavenging curve at different concentrations of the polysaccharides from Rehmanniae radix preparata

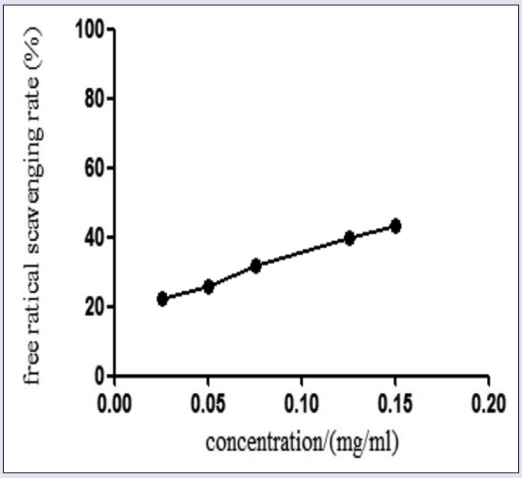

Figure 3: Free radical scavenging curve at different concentrations of the polysaccharides from Rehmanniae radix

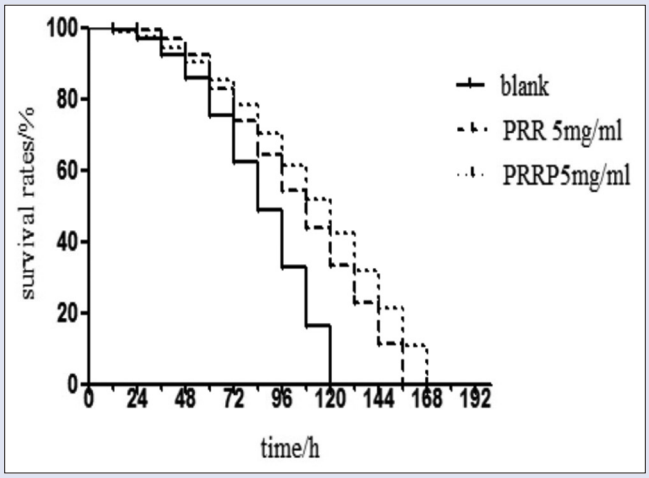

Figure 4: Curve of survival rate of nematodes under paraquat oxidation. It can be seen from the graph that PRR and PRRP can increase the survival rate of nematodes and prolong the maximum survival time of nematode, indicating that they had antioxidant effects *in vivo*. PRRP: Polysaccharides from Rehmanniae radix preparata; PRR: Polysaccharides from Rehmanniae radix

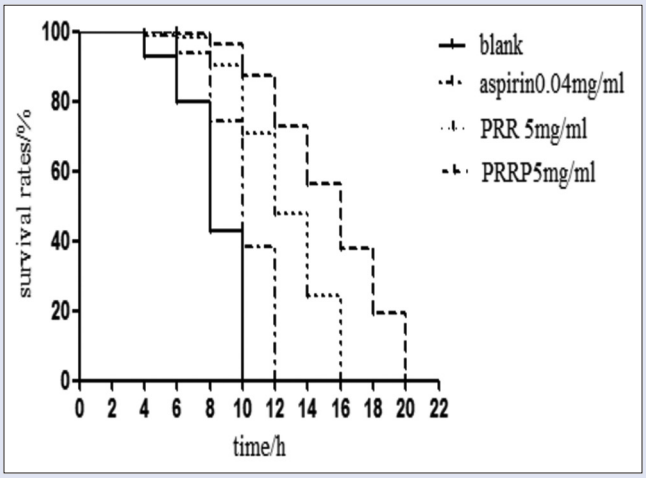

Figure 5: Curve of survival rate of nematodes under thermal stress. Compared with the blank group and aspirin group, PRR and PRRP can significantly increase the survival rate of nematodes and prolong the maximum survival time of nematodes, which indicates that they had the effect of thermal stress resistance. PRRP: Polysaccharides from Rehmanniae radix preparata; PRR: Polysaccharides from Rehmanniae radix

Lifespan-extending activity of polysaccharides from *Rehmannia glutinosa*

As shown in Figure 6, compared with the blank control group, the survival rates and maximum lifespan of the nematodes exposed to PRRP were increased. The survival rate of nematodes was reduced and the maximum life span was shortened by 2 days under the exposure of PRR. This indicated that the PRRP have remarkable anti-aging effect on nematodes. The causes of the difference in the lifespan of nematodes induced by the PRR in this concentration should be further studied.

Effects of polysaccharides from *Rehmannia glutinosa* on the growth and reproduction

As shown in Figure 7, compared with the blank control group, the body lengths of nematodes exposed to PRRP were significantly greater than the blank group, while the body lengths of nematodes exposed to PRR after treatment were not significantly different from that of the blank one. The experimental results showed that the PRR can promote the growth and development of nematodes and make them grow longer. However, there was no significant difference between the blank group and the PRR group.

Through Figure 8, compared with the blank group, after treatment of PRG, there was no significant difference of the nematodes' reproduction rate, showing that PRR and PRRP have no remarkable impact on nematode reproduction rate.

Effect of polysaccharides from *Rehmannia glutinosa* on movement behavior of nematodes

According to Figure 9, we knew that PRG can promote the movement of nematodes by comparing the blank control group with the experimental group. To some extent, PRG was not toxic to the nervous system of nematode worms.

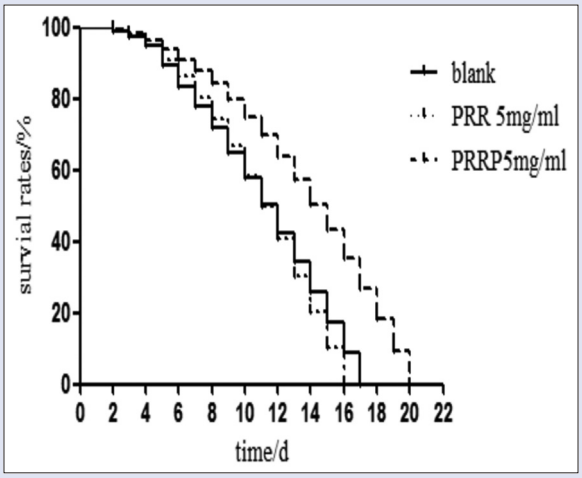

Figure 6: The survival curves of the lifespan assay of nematodes. The results of PRR and PRRP on life span. Compared with the blank group, PRRP significantly extended the lifespan of nematode worms, but PRR did not. PRRP: Polysaccharides from Rehmanniae radix preparata; PRR: Polysaccharides from Rehmanniae radix

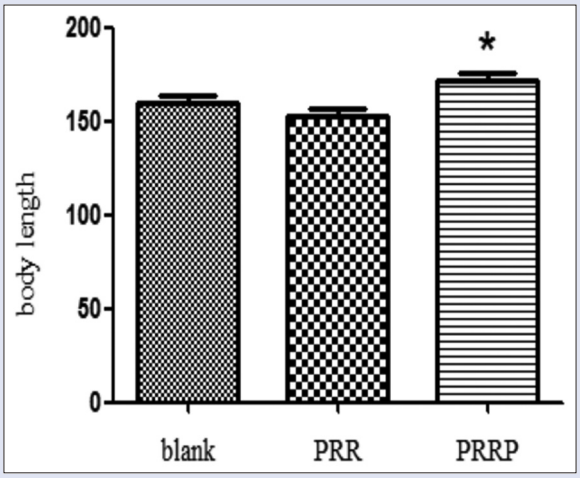

Figure 7: *$P < 0.05$. The body length of *Caenorhabditis elegans*. As shown, compared with the blank group, polysaccharides from Rehmanniae radix preparata can increase the body length of nematode worms, which indicates that polysaccharides from Rehmanniae radix preparata can promote the growth and development of nematodes

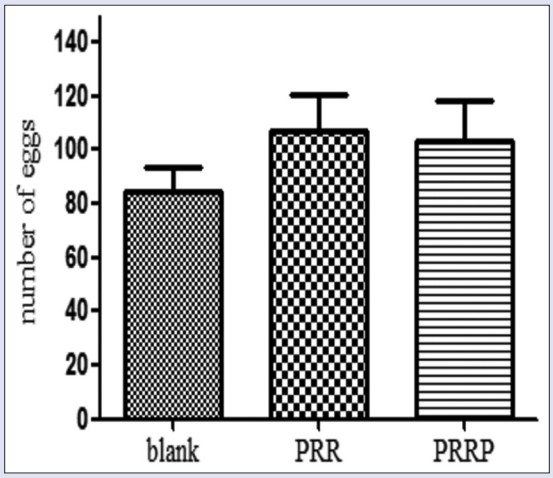

Figure 8: The number of eggs. Compared with the blank group, there was no significant difference in the number of nematode eggs, indicating that polysaccharides from *Rehmannia glutinosa* had no effect on the reproductive ability of nematodes. PRRP: Polysaccharides from Rehmanniae radix preparata; PRR: Polysaccharides from Rehmanniae radix

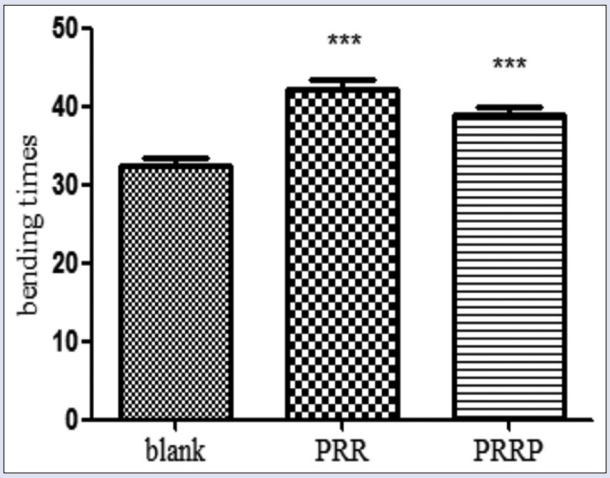

Figure 9: ***$P < 0.001$. Effect of polysaccharides from *Rehmannia glutinosa* on body bending frequency of nematodes. As shown in the figure, compared with the blank group, PRR and PRRP can increase the body bending frequency of nematodes, which indicates that they did not inhibit the motor ability of nematodes. PRRP: Polysaccharides from Rehmanniae radix preparata; PRR: Polysaccharides from Rehmanniae radix

Polysaccharides from *Rehmannia glutinosa* affects reactive oxygen species formation in *Caenorhabditis elegans*

The free radical aging hypothesis holds that aging is caused by the accumulation of molecular damage caused by ROS, a by-product of normal metabolism. To explore the possible mechanism of action of PRG on promoting oxidative stress resistance and prolonging life of nematodes, we used 2,7-dichlorodihydrofluorescein diacetate as an indicator to directly measure intracellular ROS formation after pretreatment of PRG. When membrane-permeable H_2DCF-DA diffuses into cells, it is deacetylated and retained in the cell. The nonfluorescent H_2DCF-DA can be oxidized by intracellular ROS and then becomes the highly fluorescent 2',7'-dichlorofluorescein. As shown in Figure 10, PRG could reduce the content of ROS in wild-type worms due to oxidation paraquat condition *in vivo*. These results suggested that PRG may reduce excessive intracellular ROS induced by the external environment to reduce oxidative damage, which may be the reason and mechanism of extended lifespan, in a good agreement with the increase survival rate of worms under oxidative stress condition and extended lifespan under the action of PRRP.

DISCUSSION

It has come to our attention that a lot of plant polysaccharides are able to reduce the antioxidant action of free radicals. Antioxidant activity and anti-aging potential of the natural plant polysaccharides *in vivo* were studied in mice with oxidative injury model. In recent years, studies on polysaccharides with nematode have become more and more popular.

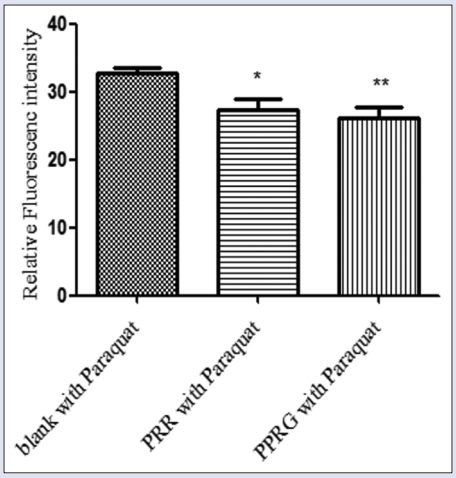

Figure 10: *$P < 0.05$, **$P < 0.01$. Effect of polysaccharides from *Rehmannia glutinosa* on reactive oxygen species formation in *Caenorhabditis elegans*. It can be seen from the graph that the fluorescence intensity of nematodes decreased after the treatment of PRR and PRRP. This shows that they can reduce the content of reactive oxygen species under oxidative stress to reduce oxidative damage and prolong life. PRRP: Polysaccharides from Rehmanniae radix preparata; PRR: Polysaccharides from Rehmanniae radix

According to the current researches on the anti-aging mechanism of nematodes, mitochondrial pathways related to ROS,[21,22] heat restriction,[23] insulin signaling pathways,[24] and steroid hormone signaling pathways are involved.[25] It is believed that excessive oxidative stress and free radicals are responsible for human aging and most diseases.[26] First, we demonstrated that PRRP and PRR have a certain ability to scavenge DPPH free radicals *in vitro*. Then, we found that they can significantly enhance survival rate of nematodes in the oxidative and thermal environments. Moreover, they not only greatly improved oxidative stress ability but also enhanced thermal stress ability. Thermal stress and oxidative stress are often used as external indicators to evaluate the effect of life extension. We used the lifespan experiment to get the fact that PRRP can extend the life of nematodes but PRR cannot. This suggests that the mechanism of prolonging lifespan of PRRP may be related to improve the stress capacity of the external environment. A defect or loss of reproductive ability can lead to increasing life expectancy, a "trade-off" between reproduction and longevity.[27] We have proved that PRRP can prolong life without affecting the reproductive ability of nematodes through reproduction experiment, which indicated that the mechanism of PRRP to prolong life is not "weight the advantages and disadvantages." Similarly, PRR had no remarkable impact on nematode reproduction. We evaluated the growth and development of nematodes by measuring their body length. We found that not only PRR but also PRRP can promote the growth and development of nematode worms. Movement is the index reflecting the fundamental function of the nervous system.[28] By measuring the motor ability of nematodes, PRRP and PRR showed no effect on the motion and neurotoxicity of the nematodes. Through ROS formation experiments, we found that PRRP and PRR can scavenge excessive ROS radicals *in vivo*. It demonstrates that the mechanism of PRRP anti-aging may be to extend life by effectively scavenging excessive radicals and inhibiting oxidative damage.

In summary, compared to PRR, PRRP not only displayed powerful anti-aging ability *in vivo* but also could alleviate stress damage and increase the survival rate of wild-type *C. elegans* under thermal and oxidative stress. PRG could be further studied as promising natural antioxidant.

CONCLUSION

Through the above studies, we found that the PRRP have the effect of extending the life span of nematodes without affecting the ability to reproduction and locomotion. PRG could improve the resistance to heat stress and oxidative stress of nematodes; in addition, PRRP could promote the growth and development of nematodes. Through the experiment on the formation of ROS, we further verified that the mechanism of PRRP to prolong the lifespan of nematodes is to improve the ability of oxidative stress and reduce oxidative damage by abating the content of ROS.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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