Food & Medicine Homology

ISSN 3006-6867 (print); 3006-4252 (online)
https://doi.org/10.26599/FMH.2025.9420037

Research Article

The anti-aging effect of a nutritional supplement based on Lycopene and grape extract

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Received: 19 June 2024 / **Revised:** 19 July 2024 / **Accepted:** 19 July 2024

Abstract: The demand for healthcare products is growing worldwide beyond the safety and convenience. This study examined the anti-aging effects of a nutritional supplement (lycopene/grape extract = 1.5:1), which is a healthcare product. The mean lifespan, athletic and reproductive abilities were examined in *Caenorhabditis elegans* (*C. elegans*). Additionally, the levels of lipofuscin, reactive oxygen species (ROS), and several critical genes related to lifespan regulation in *C. elegans* were determined. According to the results, the nutritional supplement effectively extended the mean lifespan and improved the abilities of athletic and reproductive of *C. elegans* dose-dependently. Meanwhile, the levels of lipofuscin and ROS decreased significantly in the three dosage groups ($P < 0.05$). Furthermore, the nutritional supplement upregulated the mRNA expression of SOD3, GST4 ($P < 0.01$), and HSP16.2 ($P < 0.05$) genes. Our findings showed the nutritional supplement could prolong the lifespan of *C. elegans* through reducing the levels of lipofuscin and ROS, and enhance the resistance against oxidative stress.

Keywords: nutritional supplement; *Caenorhabditis elegans*; antioxidants; anti-aging; reactive oxygen species

Citation: Xie Y., Lu Z. J., Du L. B., et al. The anti-aging effect of a nutritional supplement based on Lycopene and grape extract. *Food & Medicine Homology*, 2025, 2: 9420037. https://doi.org/10.26599/FMH.2025.9420037

1 Introduction

Aging is a natural, time-dependent degenerative process of physiological functions that results in a decline in overall function of the human body. This decline is the primary risk factor for major human pathologies, including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases[1]. Therefore, reducing many negative effects of aging populations and extending healthy life span has been an important goal of aging and anti-aging research. Genomic instability, epigenetic changes, dysregulation of nutrient senescence, mitochondrial dysfunction, cellular senescence, and altered intercellular communication are considered to be the main molecular and cellular features of senescence[1]. According to these characteristics and the pathogenesis of aging, several well-known aging theories have been proposed, including the evolutionary theory, free radical theory, antagonistic pleiotropy theory, and energy maintenance theory[2-5]. However, the free radical theory is the most widely recognized, suggesting a critical role of overproduced free radicals during the aging process. The extreme of free radicals, particularly the reactive oxygen species (ROS), results in oxidative stress[6], cell damage, lipid peroxidation, lipofuscin production[7], further aggravating aging. To examine the effect of oxidative stress, Sohal *et al.*[8] studied the distinct characteristics of flies and observed that longer-lived flies had a lower level of H_2O_2, limited oxidative damage to DNA and protein, while higher activities of antioxidant enzymes including superoxide dismutase (SOD) and catalase. A study conducted by Mossad *et al.*[9] revealed that the gut microbiota of mice plays a significant role in driving the age-related elevation of ROS levels and impairing metabolic function in microglia derived from aged mice. These studies concluded that enhanced antioxidant defense mechanisms indeed play an important role in extending longevity.

Meanwhile, a series of well-established antioxidants with proven bioactivities, including curcumin, lycopene, astaxanthin, resveratrol[10-13], have been used to counter the overproduction of free radicals. For instance, a study conducted by Wang *et al.*[14] demonstrated the efficacy of vitamin C and vitamin E supplementation mitigate oxidative stress-induced embryotoxicity and enhance the rate of blastocyst development. Zhu *et al.*[15] reported that *Ginkgo biloba* extract and Aspirin synergistically suppress oxidative stress through attenuating activated platelet-induced ROS production and LOX-1 expression in human coronary artery endothelial cells. Additionally, lycopene (a carotenoid found in tomatoes) and grape extract (rich in procyanidin) have also gained much attention due to their protective roles in neurodegenerative diseases[12, 16]. Meanwhile, they exhibit high antioxidant effects and could be used as food additive in the field of food processing[12]. Therefore, nutritional supplements with lycopene and grape extract are being explored

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as a healthcare product. Previous studies have provided compelling evidence for the notable efficacy and safety of nutritional supplements containing lycopene and grape extract in preventing and treating various diseases (Chrono and metabolic syndrome)[17-19]. However, it would be necessary to evaluate their protective effect on the extension of lifespan.

In the past three decades, *Caenorhabditis elegans* (*C. elegans*) have been accepted as a model organism to study the molecular mechanism of aging and screening of novel anti-aging drugs[7,20]. The theory of "aging is controlled by genes" and the discovery of famous longevity genes (*age-1*, *daf-2*) were both studied in *C. elegans*[21-23]. Moreover, *C. elegans* is regarded as a perfect model to study aging due to its ease of culturing and short life cycle. Also, it is easy to observe the stages of life by detecting relevant biological markers, because they are transparent animals. With these features, *C. elegans* undeniably is the best model organism to study the diverse biological problems, including growth, neurobiology, tumorigenesis, siRNA, and aging[24-28].

Hence, *C. elegans* were selected as a suitable model in this study. The aim was to investigate the anti-aging and anti-oxidative effects of nutritional supplement against oxidative stress. Paraquat (PQ) was widely used because it could produce a variety of ROS, mainly including hydrogen peroxide and hydroxyl radicals, further causing oxidative damage. Therefore, in this study, the anti-aging and anti-oxidative effects both determined in *C. elegans* induced by PQ for the first time. Moreover, the relevant mechanisms were clarified by measuring the changes of several genes in *C. elegans*.

2 Materials and methods

2.1 Life span assay of *C. elegans*

The life span assay was performed as described previously with a few modifications[7,29]. Wild type N2 *C. elegans* were cultured[30] and divided into five groups, namely N2, PQ, PQ + 0.2 mg/mL, PQ + 0.5 mg/mL, PQ + 1 mg/mL, each consisting of approximately 120 nematodes. Synchronized *C. elegans* were obtained using an egg-laying assay in line with previous description[31]. Then they were placed onto new nematode growth medium (NGM) culture plates and fed with *Escherichia coli* OP50 (*e. coli* OP50) (China Center for Type Culture Collection (CCTCC), China), the day was denoted as day 0. The nematodes in the control group (N2) grew normally and naturally while the model group (PQ) was treated with PQ (No.36541, Sigma Aldrich, USA) on day 2. Before selecting the synchronized nematodes, the experimental groups (PQ + 0.2 mg/mL, PQ + 0.5 mg/mL, PQ + 1.0 mg/mL) were treated with 100 μL (0.2, 0.5, or 1.0 mg/mL) nutritional supplement solutions (Beijing Dawn Aerospace Bio-Tech Co. Ltd, China) for 30 min, and then treated with PQ until the nematodes reached the L4 larval stage. Accordingly, the OP50/NGM plates were replaced. From then on, the number of surviving nematodes was recorded every two days until the last nematode died. In addition, the data related to abnormally dead nematodes were excluded. The Origin 9.0 software was used to perform statistical analysis.

2.2 Analysis of *C. elegans* athletic ability

The experimental groups remained the same as section 2.1. Briefly, synchronous L4 larval stage nematodes were transferred to OP50/NGM plates, treated as 2.3, and transferred to the fresh plates every 24 h. As an index of athletic activity, the sinusoidal movements (for a period of 30 s) of *C. elegans* were recorded for the corresponding groups using a stereomicroscope. Data were analyzed by the Origin 9.0 software.

2.3 Analysis of *C. elegans* reproductive ability

Synchronized nematodes, obtained from the egg stage, were transferred to OP50/NGM plates and treated as 2.1. These were transferred to fresh OP50/NGM plates once the nematodes developed to the L4 larval stage. Once the nematodes started laying eggs, the number of eggs was recorded every day. During the spawning period, the total number of eggs laid was used as a measure of fertility ability. Results were analyzed using the Origin 9.0 software.

2.4 Measurement of lipofuscin level in *C. elegans*

The wild type nematodes were cultured according to 2.1. After 10 days, in each group, the nematodes were washed with M9 buffer, transferred to tubes, anesthetized with 0.03 mol/L NaN_3 solution, and then fixed on 2% agarose slides. A laser scanning confocal microscope was used to detect the autofluorescence of lipofuscin (Olympus Fluoview FV1200, Japan). Besides, the fluorescence intensity, corresponding to each group, were analyzed using the Image J software.

2.5 Determination of reactive oxygen species (ROS) level in *C. elegans*

ROS levels were estimated with the 2', 7'-dichlorodihydro-fluorescin diacetate (DCFH-DA) fluorescent probe (Suzhou Comin Biotechnology Co. Ltd, China). Since intracellular ROS can oxidize DCFH-DA into 2'-7'dichlorofluorescein (DCF), the fluorescence intensity of DCF can be utilized as an indirect measure of the level of ROS using a fluorescence microplate reader[32,33]. When the wild type nematodes were synchronized, the L4 larval stage nematodes were transferred onto the new OP50/NGM plates. Like earlier, after 10-day culture, the nematodes were washed with M9 buffer, transferred to tubes, and then subject to incubation with 10 μmol/L DCFH-DA for 30 min. Post-incubation, extra DCFH-DA was removed by washing thrice with M9 buffer. Animals were anesthetized with 0.03 mol/L NaN_3 solution and fixed on 2% agarose slides. The fluorescence intensity was observed using a laser scanning confocal microscope (Olympus Fluoview FV1200, Japan). The data were explored by the Image J software.

2.6 RT-qPCR analysis of gene expression in *C. elegans*

Similar to section 2.3, after 12 days of culture, the nematodes belonging to each group were washed with M9 buffer, transferred to tubes, centrifugated at 12,000 r/min for 30 min. Corresponding to each group, the supernatants were recovered, total RNA was extracted with the use of the Trizol reagent (Invitrogen, Thermo Fisher, China), and the sample concentration was estimated. The total RNA was reverse-transcribed to cDNA by adopting the reverse transcription kit, as per the manufacturer's instructions[34]. The mRNA expression were identified by real-time PCR (RT-PCR) detection system using the SYBR Green qPCR Master Mix (Suzhou Comin Biotechnology Co. Ltd, China)[35]. The relative gene expression of *SOD3*, *GST4*, *SKN-1*, *DAF-16*, and *HSP16.2* were estimated with the comparative $2^{-\Delta\Delta Ct}$ method. Data were analyzed by the Origin 9.0 software.

The corresponding 5'-3' sequences of the primers used in the study are as follows (**Table.1**).

Table 1 Primer sequences

Primer	Upstream primers (5'-3')	Downstream primers (5'-3')
SOD-3	*CACACTCTCCCAGATCTCCC*	*AATTTCAGCGCTGGTTGGAC*
GST-4	*GCTGAGCCAATCCGTATCAT*	*GGCCTTCAGCTTTGACCATTC*
SKN-1	*AGTGTCGGCGTTCCAGATTTC*	*GTCGACGAATCTTGCGAATCA*
DAF-16	*TTTCCGTCCCCGAACTCAA*	*ATTCGCCAACCCATGATGG*
HSP-16.2	*TCCATCTGAGTCTTCTGAGCTTGTTA*	*TGGTTTAAACTGTGAGACGTTGA*

2.7 Statistical analysis

All experiments included three or more statistical analyses using Graphpad prism 8 without specific instructions. All data are shown to be mean ± SD, and statistical analysis was conducted by Student's t-test (unpaired, two-tailed) for significance analysis. Log-rank method was used to compare the differences among all groups. $P < 0.05$ is thought to be of statistical significance and $P < 0.01$ is thought to be of remarkable difference.

3 Results and discussion

3.1 Nutritional supplement extended the lifespan *of C. elegans*

After treating with PQ ($P < 0.05$), compared to the control group, the average and the maximum lifespan of nematodes were 9.88 days and 18.00 days respectively. However, upon treatment with 0.2, 0.5, or 1.0 mg/mL nutritional supplement, the average lifespan of nematodes extended to 12.00 days, 12.30 days, and 12.35 days respectively ($P < 0.05$). A respective increase by 21.50%, 24.50%, and 25.00%, exhibiting the concentration-dependent effect of the nutritional supplement (Fig.1). These results suggest that distinct concentrations of the nutritional supplement effectively delayed the aging process and prolonged the lifespan of *C. elegans*.

3.2 Nutritional supplement improved the athletic ability of *C. elegans*

As displayed in Fig.2, the number of sinusoidal movements (per 30 s) of nematodes decreased to 6.80 after PQ treatment ($P < 0.01$) compared to the control group. However, upon treatment with 0.20, 0.50, or 1.00 mg/mL nutritional supplement, the number of sinusoidal movements increased to 8.00, 9.10, and 10.40, respectively. The findings suggested that compared with the PQ - group, the athletic ability of *C. elegans* in the nutritional supplement group (PQ + 0.5 mg/mL and PQ + 1.0 mg/mL) elevated by 33.80%, and 52.90%, respectively ($P < 0.01$).

3.3 Nutritional supplement enhanced the reproduction ability of *C. elegans*

In general, the reproductive ability of *C. elegans* are relevant with egg-laying capacity. We observed that PQ treatment significantly decreased the egg-laying capacity of the nematodes ($P < 0.05$) (Fig.3). While after 0.2, 0.5, or 1.0 mg/mL nutritional supplement, compared to PQ treated nematodes, the egg-laying ability of nematodes increased by 27.80%, 40.10%, and 42.00%, respectively ($P < 0.05$). These data indicated that the nutritional supplement significantly restored the reduced reproduction ability of PQ-treated nematodes.

3.4 Nutritional supplement reduced lipofuscin levels in *C. elegans*

During the L1 to L4 larval stages, with the growing nematodes, the accumulation of lipofuscin also increased gradually. Once nematodes entered the adult stage, a sharp increase in lipofuscin level was observed. Using the laser scanning confocal microscope, the blue autofluorescence of lipofuscin in the nematodes was observed, as presented in Fig.4A. The fluorescence intensity of

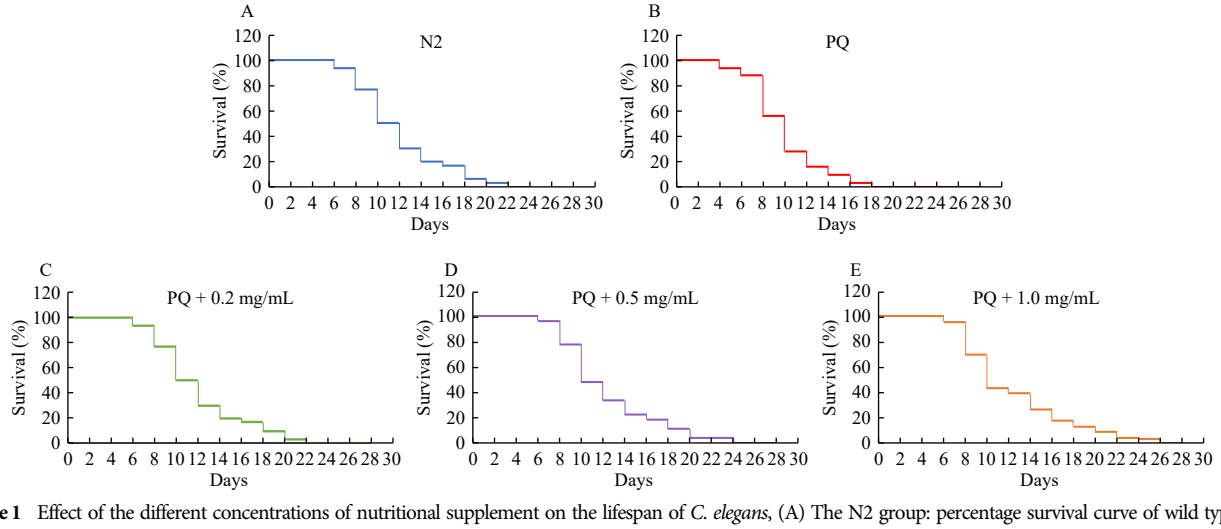

Figure 1 Effect of the different concentrations of nutritional supplement on the lifespan of *C. elegans*, (A) The N2 group: percentage survival curve of wild type N2 nematodes in control group without additional treatment, (B) The PQ group: percentage survival curve of N2 nematodes treated with PQ, (C) The PQ + 0.2 mg/mL group: percentage survival curve of nematodes, treated with PQ and 0.2 mg/mL nutritional supplement, (D) The PQ + 0.5 mg/mL group: percentage survival curve of nematodes, treated with PQ and 0.5 mg/mL nutritional supplement, (E) The PQ + 1.0 mg/mL group: percentage survival curve of nematodes, treated with PQ and 1.0 mg/mL nutritional supplement.

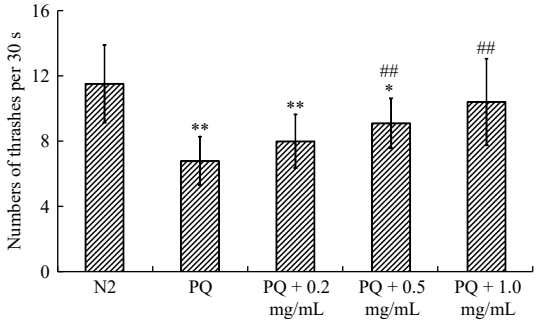

Figure 2 The athletic ability of *C. elegans*. treated with different concentrations of nutritional supplement. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the N2 group, $^{\#\#}P < 0.01$ compared with the PQ group.

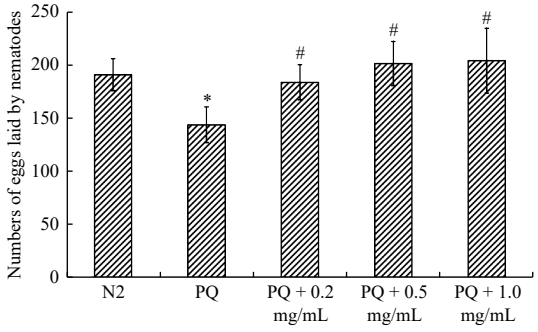

Figure 3 The nutritional supplement treatment improved the reproductive ability of PQ treated *C. elegans*. $^{*}P < 0.05$, compared with the N2 group, $^{\#}P < 0.05$ compared with the PQ group.

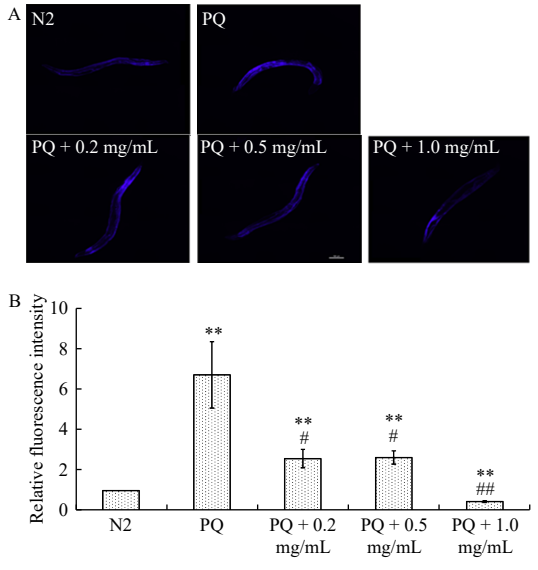

Figure 4 The accumulation of lipofuscin in *C. elegans*. treated with different concentrations of nutritional supplement. (A) The lipofuscin related fluorescence intensity of each treatment group, (B) The quantitative analysis of the relative fluorescence intensities of each treatment group. $^{**}P < 0.01$, compared with the N2 group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the PQ group.

lipofuscin in the PQ group was higher (~6.00 times) than the N2 group($P < 0.01$). Upon treatment with different concentrations of the nutritional supplement, the lipofuscin related fluorescence decreased significantly ($P < 0.01$), compared to the PQ group. Particularly, in the high-dose group (1.0 mg/mL), the fluorescence intensity of lipofuscin was even lower than the N2 group ($P < 0.01$). The statistical analysis of the relative fluorescence intensities was shown in Fig.4B. These results indicated that PQ treatment led to the accumulation of lipofuscin in the nematodes, which upon treatment with the 0.2, 0.5, or 1.0 mg/mL nutritional supplement was reduced remarkably.

3.5 The nutritional supplement effectively decreased the ROS levels in *C. elegans*

Studies have reported an essential role of oxidative stress in aging. It was demonstrated that oxygen-derived free radicals were produced in the mitochondrial and peroxisome. In *C. elegans*, an accumulation of damaged macromolecules and membranes further increased the production of ROS and accelerated the aging process [36-37].

In our study, we examined the anti-aging effect of a nutritional supplement in a PQ-induced oxidative damage model. PQ is an environmental pollutant that causes strong toxicity in humans and animals and has been widely used in establishing the oxidative stress damage model[38]. Based on Fig.5A, PQ treatment significantly increased the levels of ROS in the nematodes ($P < 0.01$). The intensity increased approximately 3.00 times than N2 group ($P < 0.01$). However, compared to the PQ group, treatment with 0.2, 0.5, or 1.0 mg/mL nutritional supplement, the levels of ROS reduced dose-dependently ($P < 0.05$ or $P < 0.01$). The statistical analysis of the average fluorescence intensity of ROS was shown in Fig.5B.

3.6 The nutritional supplement upregulated the mRNA expression of SOD3, GST4, and HSP16.2 in *C. elegans*

From the aforementioned findings, the nutritional supplement exhibited strong anti-aging and anti-oxidative ability. However, the relevant mechanisms were not clear. Furthermore, the mRNA expression of *SOD3*, *GST4*, *SKN-1*, *DAF-16*, and *HSP16.2* were quantified using the RT-PCR method. We observed that PQ treatment downregulated *SOD3*, *GST4*, *SKN-1*, *DAF-16*, and *HSP16.2* (Fig.6). However, treatment with either 0.5 or 1.0 mg/mL nutritional supplement significantly upregulated the expression of

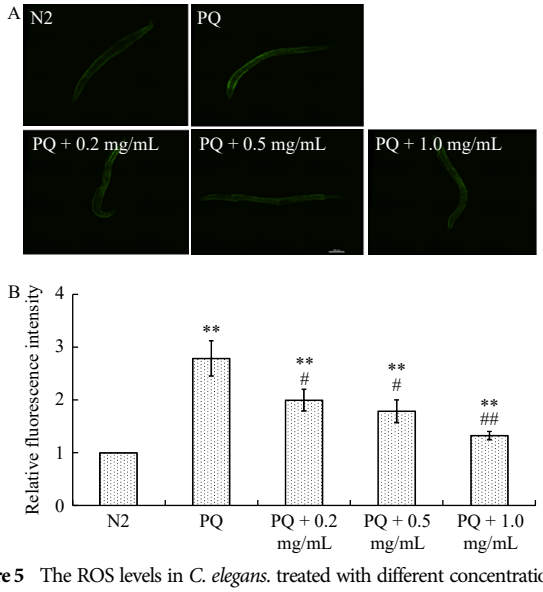

Figure 5 The ROS levels in *C. elegans*. treated with different concentrations of nutritional supplement. (A) The related fluorescence images of the different treatment groups, (B) The statistical analysis of the relative fluorescence intensities of the different treatment groups. $^{**}P < 0.01$, compared with the N2 group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the PQ group.

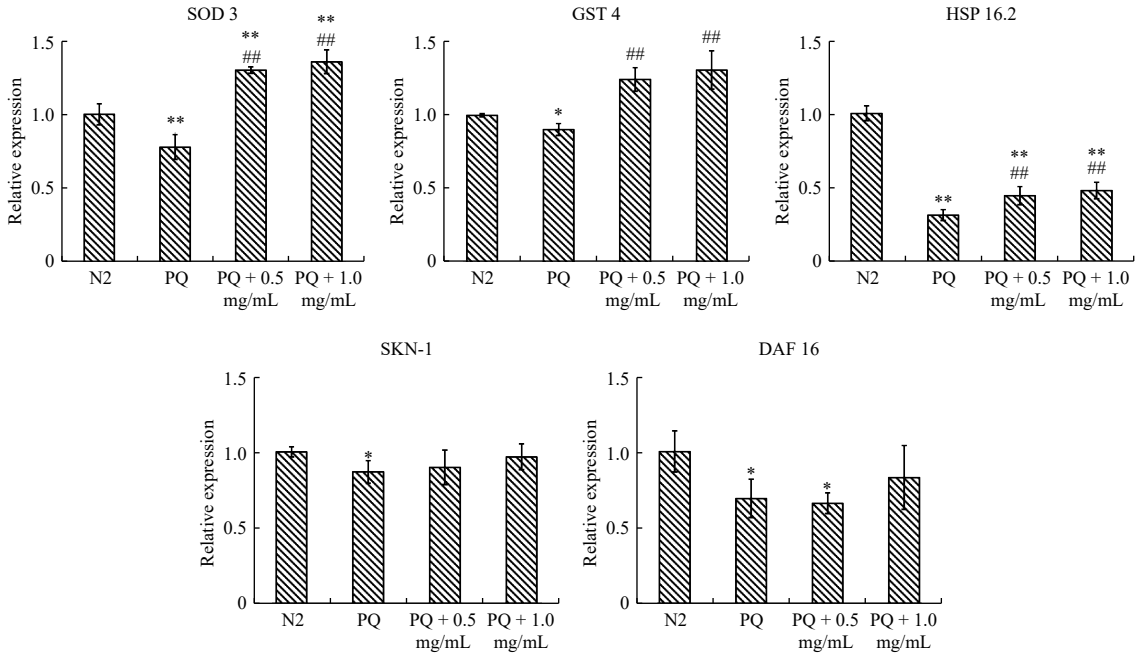

Figure 6 The expression of *SOD3*, *GST4*, *SKN-1*, *DAF16*, and *HSP16.2* estimated by qT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the N2 group, $^{\#}P < 0.05$, $^{\#\#}P < 0.05$ compared with the PQ group.

SOD3, *GST4* ($P < 0.01$). This increase was higher than the N2 group. Also, compared to the PQ group, the expression of *HSP16.2* was upregulated ($P < 0.05$), after nutritional supplement. The expression of *SKN-1*, *DAF-16* did not show a significant difference between PQ and nutritional supplement groups. Concisely, these results indicate that under oxidative stress the extension of nematodes life-span in the nutritional supplement group is possibly due to the upregulation of *SOD3*, *GST4*, and *HSP16.2*.

4 Conclusion

Aging is an inevitable and irreversible process[39]. With the rapidly increasing global average median age, more effective strategies are needed to conquer problems related to old age. Studies suggested that dietary intervention could be an effective way to improve health and enhance longevity[40].

To conclude, in this study, for the first time, the anti-aging effects of the nutritional supplement were examined *in vivo*. The nutritional supplement could increase the lifespan of nematodes against the oxidative stress, improve their athletic and reproductive abilities. The possible mechanisms of anti-aging effect could have two aspects. On the one hand, the nutritional supplement effectively inhibited the oxidative stress-induced production of lipofuscin and ROS. On the other hand, the nutritional supplement upregulated the mRNA expression of critical genes including *SOD3*, *GST4*, and *HSP16.2*. Overall, this study suggests that the nutritional supplement protected *C. elegans* against aging mainly by improving the antioxidative defense systems. Moreover, the findings provide a deep insight into the properties of the nutritional supplement for promoting life and health.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 31571020, 21375133), Henan Science and Technology Office Key Research Project (No. 132102310260), and Beijing Nova Program Interdisciplinary Cooperation Project (No. Z191100001119002).

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