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Antiaging Effects of Astaxanthin-Rich Alga *Haematococcus pluvialis* on Fruit Flies under Oxidative Stress

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ABSTRACT: The microalga *Haematococcus pluvialis* (HP) is the best natural producer of astaxanthin (AX), which is a potent antioxidant with broad health benefits. The present study investigated the antiaging potential of HP biomass using the fruit fly *Drosophila melanogaster* as the animal model. The results showed that in wild-type flies the treatment of HP induced the early mortality at a concentration of 20 mg/mL, which was associated with the decreased enzymatic activities of CuZn-superoxide dismutase (SOD1) and Mn-superoxide dismutase (SOD2) as well as the down-regulation of SOD1, SOD2, and catalase (CAT) at the transcriptional level. In SOD^{n108} mutant flies, the supplementation of HP (10 or 20 mg/mL) significantly extended their lifespan and ameliorated the age-related decline in locomotor function. Further studies suggested that HP may play a role as a complement to the defective endogenous antioxidant system to exert such lifespan elongation effects. These results, taken together, strongly support the antiaging properties of HP and its therapeutic rather than preventive potential against aging-related diseases.

KEYWORDS: *aging, microalgae, Haematococcus pluvialis, antioxidant, astaxanthin, Drosophila melanogaster*

INTRODUCTION

Aging is a progressive impairment of function causing decreased defensive capacity against environmental challenges as well as an increased risk for many diseases, such as cancer, diabetes, and neuro-degeneration.[1] As proposed by the free-radical theory, the development of aging and age-related diseases is largely attributed to the cumulative damage caused by the generation of reactive oxygen species (ROS) in cells.[2] As the degree of oxidative stress is determined by the balance between ROS production and antioxidant defense, antioxidants are regarded as promising agents to protect against aging and age-related diseases.

Astaxanthin (AX), a keto-carotenoid, is known as one of the most powerful antioxidants. It is capable of suppressing free-radical production as well as ROS-induced cellular toxicity.[3] The antioxidant activity of AX is as much as 10 times higher than that of other carotenoids such as zeaxanthin, lutein, canthaxanthin, and β-carotene, and 100 times higher than α-tocopherol. Thus, AX is described as a super vitamin E.[4] The commercial production of AX comes from both natural and synthetic sources. However, only natural AX has been generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA). Besides, natural AX outperforms synthetic AX in antioxidant capabilities.[5] Some previous studies have suggested the antiaging potential of AX.[6,7]

The best natural producer of AX is a single-celled microalga, *Haematococcus pluvialis* (HP). This green alga has long been highlighted for its strong antioxidative capability, which might be attributed to the high content of AX.[8] Although a number of microalgae have started to gain attention from both academic and industrial circles for use as potential antiaging cosmetics or healthcare products,[9] the research on HP is still limited. Given the potent antioxidant activity of AX, it is logical and meaningful to propose the benefits of AX-rich HP. To test this hypothesis, the antiaging effects of HP were evaluated in the present study for the first time. The fruit fly *Drosophila melanogaster* was used as the animal model.

MATERIALS AND METHODS

HP Biomass and Analysis of AX. The red HP biomass powder was provided by Guangdong Runke Biological Engineering Co., Ltd. To confirm its AX level, the algal biomass (20 mg) was extracted with acetone at room temperature. The extraction was repeated 2 to 3 times until the algal cells showed no color. The extracts were then centrifuged at 4500*g* for 10 min, and the supernatant was collected.

Astaxanthin as well as other major caronteoids within the HP extracts were analyzed using an HPLC system consisting of a Waters 2695 separations module and a Waters 2996 photodiode array detector, as described previously.[10,11] After the gas-dried samples were dissolved in 1 mL of acetone and filtered, 20 μL of the samples was separated using a Waters Spherisorb 5 μm ODS2 4.6 × 250 mm^2 analytical column. Pigments were eluted at a flow rate of 1.2 mL/min

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with a linear gradient from 100% solvent C (84% acetonitrile, 2% methanol, and 14% 0.1 M Tris-HCl, pH 8.0) to 100% solvent D (68% methanol and 32% ethyl acetate) over a 15 min period followed by 10 min of 100% solvent D. The peaks were compared at 450 nm. Carotenoids were identified by comparing the retention times and absorbance spectra of standard compounds purchased from Sigma (St. Louis, MO, U.S.A.). The calibration curves of the standards were prepared for the quantification of the carotenoids in the samples.

Fly Strains and Diet. Fly strains used in this study were wild-type *D. melanogaster* Oregon-R-C (OR) and a SOD^{n108}/TM3 (SOD^{n108}) mutant with one pair of single SOD genes knocked out on chromosome 3L, causing decreased enzymatic activities of SOD1 and SOD2 (Bloomington Drosophila Stock Centre, Department of Biology, Indiana University, Bloomington, IN, U.S.A.).

A basal diet was cooked according to the standard formulation described by Peng et al.[12] The basal diet was made up of corn flour (105 g/L), yeast (21 g/L), glucose (105 g/L), and bacto agar (13 g/L). Ethyl-4-hydroxybenzoate (0.4%) was used to prevent the growth of fungi. For the experimental groups, the diet was cooked and poured into each vial (5 mL). For the stocks and mating vials, 15 mL of basal medium was poured into each vial.

Effects of HP on the Longevity of Fruit Flies. Male flies (3 days old) freshly developed from eggs were collected and divided into five groups. Each group had 200 flies rearing in 10 vials (100 flies in 5 vials for the SOD^{n108} mutant). The control group was raised on the basal diet, whereas the rest of the groups were fed with HP biomass with concentrations of 0.1, 1, 5, and 20 mg/mL respectively (1, 5, 10, and 20 for the SOD^{n108} mutant). Dead flies were counted every 3 to 4 days, and the remaining living flies were transferred to fresh medium with the same composition. The feeding lasted until all flies died.

Climbing Assay. The locomotor function of fruit flies was examined using a climbing assay as previously mentioned with a minor amendment.[13] In brief, 10 male flies were placed in a glass tube and given 20 s to climb. At the end of each trial, the number of flies that reached the height of 10 cm or above was recorded.

Chronic Paraquat Challenge. To examine the resistance of the flies against paraquat-induced chronic oxidative stress, 400 newly born male OR flies were divided into two groups randomly and fed with the basal diet or the experimental diet containing 20 mg/mL of HP biomass. The flies were put under starvation for 2 h in vials containing filter paper soaked with distilled water every 3 days and transferred into new vials containing a filter paper saturated with 600 μL of 20 mM paraquat in a 6% glucose solution. After 24 h of culture under paraquat-induced oxidative stress, the flies were moved to new vials with water-soaked filter paper for 2 h before they were transferred back into vials containing freshly prepared diets. The experiment lasted for 69 days (until all flies died).

Evaluation of SOD1, SOD2, and CAT mRNA Expression. Quantitative real-time (RT) PCR was used to evaluate the expression of the SOD1, SOD2, and CAT genes. Total RNA was extracted from the fruit flies using the commercial extraction agent TRIzol (Invitrogen, Carslbad, CA, U.S.A.). Fruit flies (n = 15) were homogenized in 800 μL of TRIzol solution and centrifuged at 12 000*g* at 4 °C for 10 min. The supernatant was transferred to a tube containing 160 μL of chloroform. The mixed solution was subjected to centrifugation at 12 000*g* at 4 °C for 15 min. The upper layer was transferred to a new tube and mixed with 400 μL of isopropanol. After 10 min of incubation at room temperature, the mixture was centrifuged at 12 000*g* at 4 °C for 10 min, and the pellet was washed with 1 mL of 75% ethanol followed by centrifugation. Diethylpyrocarbonate (DEPC)-treated water (25 μL) was used to resuspend the RNA pellet. The purity and concentration of the obtained RNA was determined by measuring its absorbance at 260 and 280 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). The SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carslbad, CA, U.S.A.) was used to construct cDNA. Two micrograms of RNA was used for each reaction. cDNA was synthesized in the Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.) and stored at −20 °C.

Real-time PCR amplification was carried out on Applied Biosystems Step One Plus Real-Time PCR System (Applied Biosystems). The PCR reaction system, primers, and gene-specific detectors were provided by Taqman Gene Expression Assays (Applied Biosystems). The three target genes tested in the present study were SOD1 (NCBI Reference Sequence NM_057387.3), SOD2 (NCBI Reference Sequence NM_057577.2), and CAT (NCBI Reference Sequence NM_080483.2). The relative levels of the amplified mRNA were evaluated according to the $2^{-\Delta\Delta CT}$ method using the Rp49 gene (NCBI Reference Sequence NM_079843.2) for normalization. The levels of gene expression of all experimental groups were shown as a ratio against the day 0 control group.

Statistical Analysis. All experiments were determined in triplicate and repeated two times to ensure reproducibility. The results were expressed as the mean ± SD. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, U.S.A.). The paired-samples *t* test and Kaplan−Meier test were applied. Statistical significance was achieved when $p < 0.05$.

RESULTS

Analysis of Astaxanthin. HPLC data showed the amount of AX in HP extracts was 16.3 mg/g, which is consistent with previous findings.[5,8] The level of AX was much higher than other major carotenoids present in the extracts (i.e., lutein and canthaxanthin) (Figure 1a, Table 1). Because the long

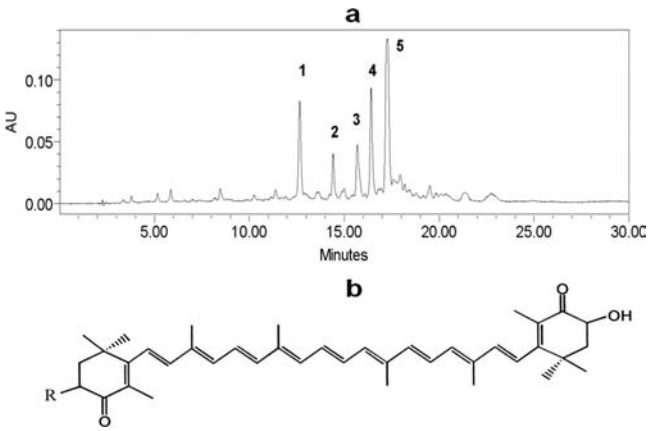

Figure 1. (a) HPLC analysis of the carotenoids profile in the HP extract. The peaks are 1, lutein; 2, canthaxanthin; 3−5, astaxanthin monoesters. (b) Chemical structure of AX monoester from HP in the form of 3*S*, 3′*S*; the hydroxyl group is substituted by a fatty acyl group (R), which could be C16:0, C18:1, C18:2, or C18:3.[23]

Table 1. Content of the Major Carotenoid Components in HP Extracts

carotenoids	mg/g of the cell dry weight
lutein	3.09
canthaxanthin	3.51
astaxanthin monoesters	16.3

conjugated-double-bond structure is susceptible to oxidation and not very stable, AX in HP needs to be esterified with fatty acids to stabilize the molecules and maintain its high antioxidant ability.[14] This is why AX in the algal extracts was found predominantly in the form of monoesters. The chemical structure of the AX esters was shown in Figure 1b. After administration to humans or animals, it is believed that the esters will be activated to the free form by esterase enzymes. Thus, AX monoesters and AX free possess the same activity when used in the diet.[4]

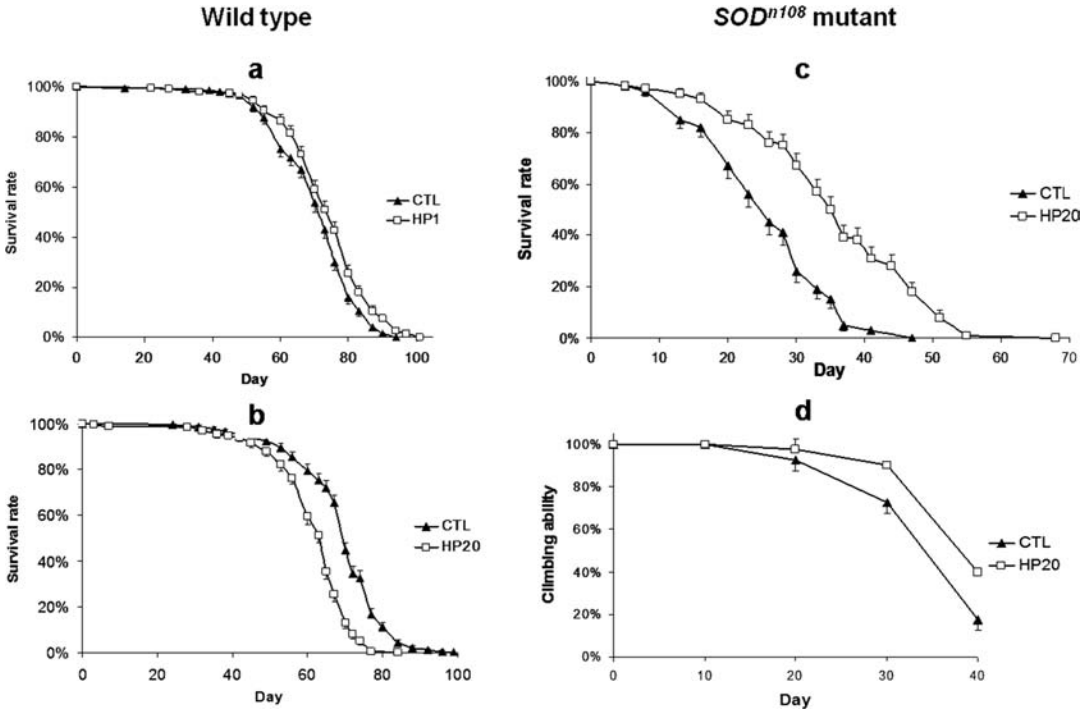

Figure 2. (a) Life-span curve of wild-type OR flies fed with diets containing 0 (CTL) or 1 mg/mL (HP1) of HP. (b) Life-span curve of wild-type OR flies fed with diets containing 0 (CTL) or 20 mg/mL (HP20) of HP. (c) Life-span curve of *SOD*^{n108} mutant flies fed with diets containing 0 (CTL) or 20 mg/mL (HP20) of HP. (d) Effect of HP on the climbing ability of the CTL and HP20 groups of *SOD*^{n108} mutant flies.

Effects of HP on the Longevity of Fruit Flies. Four different concentrations of HP biomass (0.1, 1, 5, and 20 mg/mL) were studied for their life-span-extending effects on wild-type OR flies. At the concentration of 1 mg/mL, HP slightly elongated the life span of OR flies compared with the control (CTL) group (Figure 2a). On the contrary, 20 mg/mL of HP seemed to accelerate the death of the flies (Figure 2b). At other concentrations (0.1 and 5 mg/mL), no significant effects on the life span of OR flies were observed (data not shown).

The effects of the HP biomass on the life span of *SOD*^{n108} mutant flies were subsequently studied. The results showed that the supplementation of algal cells at high concentrations (10 and 20 mg/mL) significantly extended the life span of *SOD*^{n108} mutant flies ($p < 0.05$) (Figure 2c, Table 2). Low concentrations of HP (1 and 5 mg/mL) had no significant life-span-extending effect on *SOD*^{n108} mutant flies (Table 2). The results of the climbing assay showed that the age-dependent declining trend of locomotor function in *SOD*^{n108} flies was effectively rescued by the intake of HP (Figure 2d).

Table 2. Lifespan of *SOD*^{n108} Mutant Flies Fed with the Control Diet and Experimental Diets Containing 1, 5, 10, and 20 mg/mL of HP

group	maximum lifespan (days)	50% survival (days)	mean lifespan (days)[a]
control	47	26 ± 1	26 ± 1[a]
1 mg/mL HP	44	23 ± 1	24 ± 1[a]
5 mg/mL HP	55	26 ± 1	26 ± 1[a]
10 mg/mL HP	64	30 ± 1	30 ± 1[b]
20 mg/mL HP	68	35 ± 1	36 ± 1[c]

[a]Means with different letters differ significantly at $p < 0.05$.

Effects of HP Against Chronic Paraquat Stress in OR Flies. The effects of HP against chronic oxidative stress induced by paraquat were subsequently studied using OR fruit flies. With the supplementation of 20 mg/mL HP biomass in diets, the paraquat-induced mortality was significantly ameliorated ($p < 0.05$) (Figure 3).

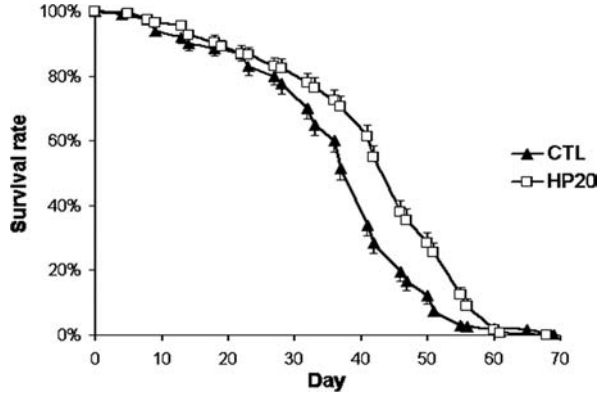

Figure 3. Effect of chronic paraquat-induced oxidative stress on the survival time of OR flies fed with the diets containing 0 (CTL) or 20 mg/mL (HP20) of HP.

Effects of HP on Endogenous Antioxidant System. The mRNA levels of genes involved in endogenous antioxidant system were studied in OR flies to elucidate the functional mechanism of HP. RT-PCR results showed that the mRNA levels of SOD1, SOD2, and CAT were down-regulated by the supplementation of 20 mg/mL of HP biomass on the aging stage after day 45 (Figure 4).

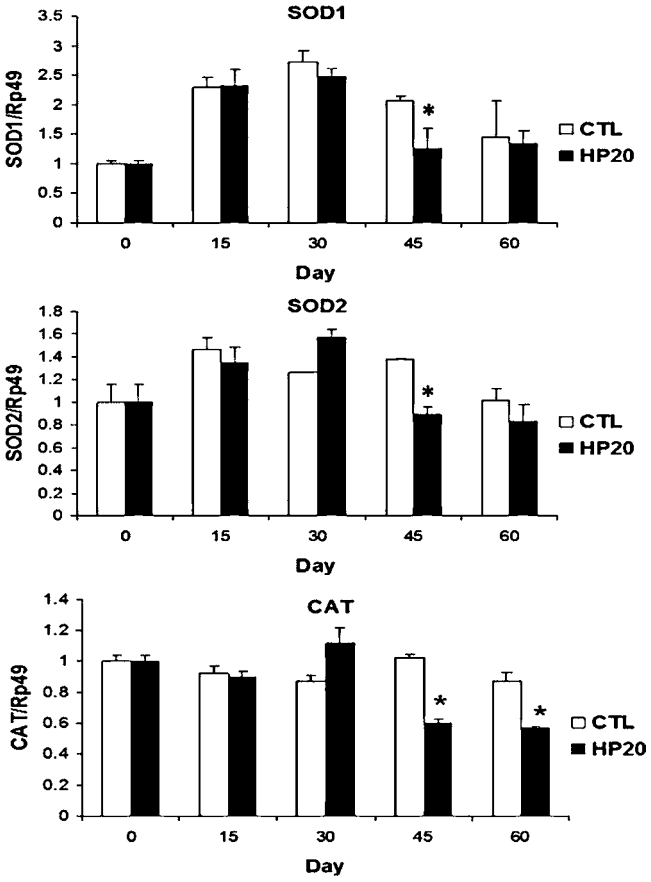

Figure 4. Effects of HP supplementation (20 mg/mL in diet) on the mRNA levels of SOD1, SOD2, and CAT measured in wild-type fruit flies. The flies were incubated at 25 °C, collected at day 0, 15, 30, 45, and 60 respectively, and stored at −80 °C until RNA extraction and analysis were performed. The data are expressed as the mean ± SD and * denotes $p < 0.05$ compared with the value of the corresponding CTL group.

DISCUSSION

Research on the antiaging potential of antioxidants from natural resources have gained particular attention because they have less side effects and are safer than chemically synthesized antioxidants. A wide range of natural products with antioxidant capability have been evaluated.[12,13] The microalga HP has long been highlighted for its strong antioxidative activity, which is related to the high level of AX within it. In the present study the antiaging effect of the AX-rich extracts from microalga HP was investigated for the first time. The animal model used in this work, the fruit fly *D. melanogaster*, has been regarded as an ideal research model of aging since 1915 because of its sophisticated genetics, high fecundity, small genome size, and short generation time.[15,16] Moreover, *D. melanogaster* has evolved a lipid metabolism and transport system that are analogous to mammalian hepatocytes.[17]

As shown in Figure 2a, at a low concentration (1 mg/mL) the supplementation of HP slightly extended the life span of wild-type flies. When the concentration increased to 20 mg/mL, however, the survival rate of the wild-type flies decreased (Figure 2b). This might be related to the unique roles of ROS: although excessive production of ROS may cause cellular damage, a certain amount of ROS is also needed, which serves as specific signaling molecules that reflect physiological changes and trigger cellular-signaling pathways that enhance metabolic health.[18] This biphasic response to a potentially detrimental compound is referred to as hormesis.[19] According to hormesis theory, although antioxidants can protect against the damage induced by high level of ROS generation, the long-term intake of large quantities of dietary antioxidants in healthy organisms may interrupt the endogenous balance of ROS or even alleviate the defending capabilities of endogenous antioxidant enzymes. The phenomenon in Figure 2b may be explained by this theory: as the endogenous antioxidant in wild-type flies were capable of maintaining the balanced ROS level as well as the normal metabolic functions, the intake of excessive strong antioxidant from HP could break such balance, which eventually caused early mortality. In the SOD^{n108} mutant flies, because the endogenous antioxidant enzymes were defective and could not provide enough defense against the ROS generated, the supplementation of a high dose of HP helped to eliminate the excessive ROS and resulted in an extended life span (Figure 2c).

In the chronic paraquat assay, the level of oxidative stress in fruit flies continuously treated with paraquat (every 3 to 4 days) remained at a relatively high level, and under this condition the supplementation of HP helped scavenge excessive free radicals and maintain the normal metabolic functions of the fruit flies (Figure 3). Because long-term exposure to paraquat has been revealed as an environmental factor inducing neurodegenerative syndrome,[20] the life-span-extending effects of HP on OR flies exposed to chronic paraquat-induced oxidative stress also indicated the protective role of HP against neurodegenerative diseases.

Some antioxidants, including AX, may interact with intracellular enzymes to exert antioxidant actions, as evidenced by a report that a few similar antioxidants are recycled by endogenous enzymes or other shuttle molecules.[21] To elucidate the impact of HP extracts on the endogenous stress-defending system, the mRNA expression of several important endogenous antioxidative enzymes was studied. SODs and CAT serve in tandem as the first line of defense against superoxide in the endogenous antioxidant-defense system. Defects in these enzymes were reported to be linked with neurodegeneration and shortened life span.[22] The results showed that the mRNA levels of SOD1, SOD2, and CAT were all decreased in the group of flies fed with 20 mg/mL of HP for 45 days (Figure 4). These data suggested that the transcription of endogenous antioxidant enzymes was reduced by the long-term intake of HP at a high dose, which was also in accordance with the hormesis theory and therefore proved the bifacial effects of HP on fruit flies.

The antiaging potential of HP biomass containing high AX levels was investigated. We found that at high concentrations, the supplementation of HP extended the lifespan of SOD^{n108} mutant fruit flies by protecting against oxidative stress. In contrast, a long-term intake of HP led to the early death of healthy fruit flies, which may result from the inactivation of the endogenous antioxidant-defense system and decreased stress-tolerance capabilities. These results strongly suggested the therapeutic rather than preventive potential of HP against aging-related diseases, such as Alzheimer's disease. With the scientific evidence provided by this work, a new direction for the research of HP may be explored in future. Further studies are needed to elucidate the contribution of AX.

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Author Contributions

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AX, astaxanthin; CAT, catalase; HP, *Haematococcus pluvialis*; OR, Oregon-R-C; ROS, reactive oxygen species; SOD1, CuZn-superoxide dismutase; SOD2, Mn-superoxide dismutase

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