Original Article

Anti-hypoxia effects of walnut oligopeptides (*Juglans regia* L.) in mice

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Abstract: Objective: To investigate the anti-hypoxia effects of walnut oligopeptides (WOPs) in mice. Methods: Randomly divide the mice into 4 experimental sets. Then randomly divide each set of mice into 5 groups, including one vehicle control group, one whey protein group (220 mg/kg), and three WOPs intervention groups (110 mg/kg, 220 mg/kg, 440 mg/kg). Test substances were administered orally to mice via the drinking water for 30 days. Results: WOPs significantly extended the normobaric hypoxia survival time, sodium nitrite toxicosis survival time, and acute cerebral ischemia survival time. Notably, WOPs increased red blood cell (RBC), hemoglobin (Hb) and hematocrit (Hct) levels, decreased malonaldehyde (MDA) content and lactate content in brain, enhanced brain lactate dehydrogenase (LDH) activity, and promoted the expression levels of hypoxia-inducible factor 1alpha (HIF1α) mRNA and vascular endothelial growth factor (VEGF) mRNA. Conclusion: WOPs have anti-hypoxia effects, and the mechanism may involve the following aspects: the first is to improve the blood's oxygen carrying capacity and oxygen utilization rate, the second is to minimize the lesion of lipid peroxidation, the third is to increase the brain's ability to buffer against lactic acidosis of mice, and the fourth is to promote angiogenesis and regulate hypoxia response.

Keywords: Walnut, oligopeptide, anti-hypoxia, mice

Introduction

Hypoxia is defined as a pathological process in which tissue cell metabolism and even morphological structure change abnormally because of lack of oxygen or obstacles to use the oxygen. The symptoms generally include rapid heartbeat, dry mouth, nausea, vomiting, diarrhea, palpitations, shortness of breath, and even malaise, coma, shock, etc. [1]. Hypoxia not only damages the physiological functions of various systems such as nerves, digestion, respiration, urination and endocrine, but also affects the metabolism of carbohydrates, proteins, lipids, water and electrolytes, and ultimately endangers the health and life. Thus, it is necessary to find safe and effective methods for the prevention of hypoxia. Currently, the normal vasodilating agents, acetazolamide and nifedipine are used to reduce the incidence and severity of hypoxia [2, 3]. However, multiple adverse effects, such as headache and cardiopalms, have been observed in clinical practice [4].

In recent years, nutrition intervention has received increasing attention, and a large number of studies have found the safety and effectiveness of natural food ingredients in anti-hypoxia [5-7]. To date, researchers have isolated various bioactive peptides from plants, animals and microorganisms. These peptides have been confirmed to have diverse biological activities, such as antimicrobial, antioxidant, anti-hypoxia, cholesterol-lowering, cyto-or immunomodulatory activities [8-12]. Bioactive peptides are considered to have great potential in medical and health field. It was estimated that in 2015, the share of peptide therapy in the global market was 17.5 billion US dollars, and this share is expected to reach 47 billion US dollars by 2025 [13]. As of February 2016, over 60 peptide-related drugs have been approved by the U.S. Food and Drug Administration (FDA) [14], and more than 400 of them are in preclinical or clinical trials [13].

Walnut (*Juglans regia* L.) is a deciduous tree of the Juglans family and one of the oldest culti-
Anti-hypoxia effects of walnut oligopeptides

Walnut, a nutritive nut varieties [15]. Its fruits contain numerous functional components, such as unsaturated fatty acids, proteins and peptides, polyphenols, dietary fibers and flavones, which makes them to have extensive biological activities including improving memory [16, 17], antioxidation [18, 19], hypolipidemic capacity [20], antihypertension [21], anti-tumor potency [19] and so on. What's more, protein hydrolysates of the walnut fruits also have a variety of activities including antioxidant, anti-cancer [22]. However, oligopeptides, an important biologically active ingredient in walnuts, have the characteristics of small molecular weight, easy absorption and high bioavailability, but are rarely reported about their effects on anti-hypoxia. We hypothesize that walnut oligopeptides (WOPs) have anti-hypoxia effects. Therefore, this study aimed to investigate WOPs anti-hypoxia effects in mice.

Materials and methods

Extraction of WOPs

WOPs were derived from the walnut fruits by enzymatic hydrolysis. In brief, wash the walnuts, chop them, homogenize them in distilled water, adjust the pH to 8.0, and then, the most critical step is to, treat them with compound protease at 40°C for 3 hours. Next, nanofiltration, freeze concentration, decolorization, purification and spray drying were performed to obtain WOPs powder. A Phenomenex C18 column (10 mm×250 mm) was used to purify the sample. WOPs content was 87.87%, and the proportion of free amino acids was 2.99%, besides, the relative molecular weight were between 180 Da and 1000 Da.

Chemicals and reagents

Malondialdehyde (MDA) and lactate detection kits were purchased from Beyotime Biotechnology (Shanghai, China). Lactate dehydrogenase (LDH) assay kit was obtained from Yingke Xinchuang Technology Co., Ltd. (Macao, China). Medical soda lime and sodium nitrite were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Animals

Adult male BALB/C mice, weighting 18-22 g, were purchased from the Laboratory Animal Science Department of Peking University Health Science Center. The temperature maintain at 25°C ± 1°C, the relative humidity was 55% ± 5%. A 12-hour: 12-hour light-dark cycle was performed. Animals could freely enjoy an AIN-93G diet and clean water. Feed the mice adaptively without intervention for 7 days. Mice were treated in compliance with the Principle of Laboratory Animal Care (National Institutes of Health publication NO.85-23, revised 1985).

Groups and treatment

Randomly divide the mice into 4 experimental sets (n=60), namely experimental set 1, 2, 3 and 4. Then randomly divide each set of mice into 5 groups (n=12), including one vehicle control group, one whey protein group (220 mg/kg), and three WOPs intervention groups (110 mg/kg, 220 mg/kg, 440 mg/kg, namely WOPs-LG, WOPs-MG, and WOPs-HG, respectively). The vehicle control group was given vehicle by gavage, and the whey protein group and 3 WOPs groups were administered whey protein and corresponding doses of WOPs respectively. Intervene once a day for a total of 30 days. The weight was recorded weekly.

Normobaric hypoxia assessment

Effects of WOPs on the normobaric hypoxia survival time of mice: 60 minutes after the final dose, each mouse in experimental set 1 was placed into a 250 ml sealed container containing 5 g of medical soda lime. The duration of survival in oxygen deprivation was recorded.

Effects of WOPs on MDA content, lactate levels, and LDH activity: After the death of the mouse, its brain was immediately separated and homogenized with physiological saline to a 10% solution at 4°C. Detect MDA content, lactate levels, and LDH activity in brain with corresponding kits.

Effects of WOPs on HIF-1α and VEGF mRNA levels: After the death of the mouse, its brain was immediately separated and total RNA was extracted from brain tissue using Ribospin (GeneAll, Inc., Seoul, Korea). The ABI 7300 real-time PCR detection system was used for real-time reverse transcription-PCR to detect target genes’ RNA expression. Use the M-MLV kit (Invitrogen) to determine reverse transcription-polymerase chain reaction (RT-PCR) analysis of target mRNA levels. The special primers were
Anti-hypoxia effects of walnut oligopeptides

as follows: HIF-1α, forward 5'-TCACCACAGGACAGTACAG GATGC-3' and reverse 5'-CCAGCAAAGTTAAAGCAT CAGGTTCC-3'; VEGF, forward 5'-ACG AAGTGGTGAAGTTCATGGATG-3' and reverse 5'-TTC TGTATCATCTTGCTGGTGA-3'; GAPDH, forward 5'-GCCAAAGGGTCATCATCCTC-3' and reverse 5'-GGCAGGGATGAGGCTT-3'. After normalizing the target mRNA value to the GAPDH mRNA level, the target mRNA value was measured by comparison with the control sample, then the comparison period threshold ($\Delta \Delta C_t$) method is used for calculation.

Sodium nitrite toxicosis assessment

60 minutes after the final dose, each mouse in experimental set 2 was injected intraperitoneally with 240 mg/kg sodium nitrite (0.1 ml/10 g), and the survival time was recorded.

Acute cerebral ischemia assessment

60 minutes after the final dose, each mouse in experimental set 3 was killed immediately by decapitation, and the time between decapitation and final breathing was recorded.

Whole blood analysis of mice

60 minutes after the final dose, a blood sample was obtained in EDTA-containing tubes from the eyeballs of each mouse in experimental
Anti-hypoxia effects of walnut oligopeptides

Results

Effects of WOPs on the weight

No significant difference in weight was found among all groups ($P>0.05$) (Figure 1).

Effects of WOPs on the normobaric hypoxia survival time

No significant difference in the normobaric hypoxia survival time was found between the vehicle control group and the whey protein group ($P>0.05$). Normobaric hypoxia survival time in WOPs-LG, WOPs-MG and WOPs-HG was 14.25%, 27.07% and 36.29% higher than the vehicle control group, respectively ($P<0.01$). The normobaric hypoxia survival time of WOPs-MG and WOPs-HG was significantly extended when compared with the whey protein group ($P<0.01$) (Figure 2).

Effects of WOPs on the sodium nitrite toxicosis survival time

No significant difference in the sodium nitrite toxicosis survival time was found between the vehicle control group and the whey protein group ($P>0.05$). Sodium nitrite toxicosis survival time in WOPs-LG, WOPs-MG and WOPs-HG was 11.88%, 18.75% and 32.81% higher than the vehicle control group, respectively ($P<0.05$ for WOPs-LG, $P<0.01$ for WOPs-MG and WOPs-HG). The sodium nitrite toxicosis survival time of WOPs-MG and WOPs-HG was significantly extended when compared to the whey protein group ($P<0.01$) (Figure 3).

Effects of WOPs on the acute cerebral ischemia survival time

No significant difference in acute cerebral ischemia survival time was found between the vehicle control group and the whey protein group ($P>0.05$). Acute cerebral ischemia survival time set 4. Use Sysmex XT-2000i blood analyzer (Roche Diagnostics) to analyze red blood cell (RBC), hemoglobin (Hb) and hematocrit (Hct) within 3 hours of blood collection.

Statistical analysis

Use SPSS software version 20.0 for statistical analysis. The homogeneity of the variances was checked. Then perform one-way analysis of variance test as well as LSD method. We considered $P$ less than 0.05 as significant difference.

Figure 2. Effects of WOPs on the normobaric hypoxia survival time. **$P<0.01$ versus vehicle control group; *$P<0.01$ versus whey protein group. WOPs, small molecule oligopeptides isolated from walnut; WOPs-LG, 110 mg/kg WOPs group; WOPs-MG, 220 mg/kg WOPs group; WOPs-HG, 440 mg/kg WOPs group.

Figure 3. Effects of WOPs on the sodium nitrite toxicosis survival time. *$P<0.05$, **$P<0.01$ versus vehicle control group; *$P<0.01$ versus whey protein group. WOPs, small molecule oligopeptides isolated from walnut; WOPs-LG, 110 mg/kg WOPs group; WOPs-MG, 220 mg/kg WOPs group; WOPs-HG, 440 mg/kg WOPs group.
Anti-hypoxia effects of walnut oligopeptides

in WOPs-LG, WOPs-MG and WOPs-HG was 7.89%, 12.63% and 17.11% longer than the vehicle control group, respectively \( (P<0.05 \text{ for WOPs-LG, } P<0.01 \text{ for WOPs-MG and WOPs-HG}) \). The acute cerebral ischemia survival time in WOPs-HG was significantly extended when compared to whey protein group \( (P<0.01) \) (Figure 4).

**Effects of WOPs on RBC, Hb and Hct**

No significant difference in RBC, Hb and Hct was found between the vehicle control group and the whey protein group \( (P>0.05) \). In comparison with vehicle control group, RBC in WOPs-MG and WOPs-HG was significantly enhanced \( (P<0.05 \text{ for WOPs-MG, } P<0.01 \text{ for WOPs-HG}) \), Hct in WOPs-LG, WOPs-MG and WOPs-HG was significantly increased \( (P<0.05 \text{ for WOPs-LG and WOPs-MG, } P<0.01 \text{ for WOPs-HG}) \), besides, Hb in WOPs-LG, WOPs-MG and WOPs-HG was significantly increased \( (P<0.05 \text{ for WOPs-LG, } P<0.01 \text{ for WOPs-MG and WOPs-HG}) \). Moreover, compared with whey protein group, RBC in WOPs-HG was significantly enhanced \( (P<0.05) \), Hct and Hb of WOPs-MG and WOPs-HG were significantly increased \( (P<0.05 \text{ for WOPs-MG, } P<0.01 \text{ for WOPs-HG}) \) (Figure 5).

**Effects of WOPs on brain MDA content**

No significant difference in the brain MDA content was found between the vehicle control group and the whey protein group \( (P>0.05) \). In comparison with vehicle control group, brain MDA content in WOPs-LG, WOPs-MG and WOPs-HG was significantly decreased \( (P<0.01) \). The brain MDA content of WOPs-MG and WOPs-HG was lower than the whey protein group \( (P<0.05 \text{ for WOPs-MG, } P<0.01 \text{ for WOPs-HG}) \) (Figure 6).

**Effects of WOPs on brain lactate levels and LDH activity**

There was no significant difference in brain lactate levels and LDH activity between the vehicle control group and the whey protein group \( (P>0.05) \). In comparison with vehicle control group, brain lactate levels were significantly decreased and serum LDH activity was significantly enhanced in WOPs-LG, WOPs-MG and WOPs-HG \( (P<0.05 \text{ for WOPs-LG, } P<0.01 \text{ for WOPs-MG and WOPs-HG}) \). Compared with whey protein group, brain lactate levels were significantly decreased and brain LDH activity was higher in WOPs-MG and WOPs-HG \( (P<0.05 \text{ for WOPs-MG, } P<0.01 \text{ for WOPs-HG}) \) (Figure 7).

**Effects of WOPs on HIF-1α and VEGF mRNA levels in brain**

No significant difference in HIF-1α and VEGF mRNA levels was found between the vehicle control group and the whey protein group \( (P>0.05) \). HIF-1α and VEGF mRNA levels in WOPs-LG, WOPs-MG and WOPs-HG was higher than in the vehicle control group \( (P<0.05 \text{ for WOPs-LG and WOPs-MG, } P<0.01 \text{ for WOPs-HG}) \). In addition, compared with whey protein group, levels of HIF-1α and VEGF mRNA in WOPs-MG and WOPs-HG were significantly enhanced \( (P<0.05 \text{ for WOPs-MG, } P<0.01 \text{ for WOPs-HG}) \) (Figure 8).

**Discussion**

Hypoxia is a stressor to the body, disturbing normal metabolic processes, especially the antioxidant function, and even leading to death due to insufficient energy supply to main organs such as the heart and brain. We evaluated the
Anti-hypoxia effects of walnut oligopeptides

supplement, we used whey protein as a protein control. Under our experimental conditions, the effects of whey protein against hypoxia were not observed.

In the normobaric hypoxia assessment, insufficient oxygen supply severely reduced partial pressure of intracellular oxygen, leading to mitochondrial dysfunction and affecting energy metabolism. In the sodium nitrite toxicosis assessment, sodium nitrite converted bivalent hemoglobin into trivalent hemoglobin, then disrupted the oxygen-carrying capacity of hemoglobin and resulted in tissue hypoxia. In the acute cerebral ischemia assessment, the decapitation terminated blood supply to the brain, but the brain could still work for a short time, presenting with a regular mouth gasping. The gasp time could be used as a crucial indicator to evaluate the protective effect of the tested samples on cerebral ischemic anoxia. The above results indicated that WOPs intervention significantly extended the survival duration in the above three assessments. Hence, WOPs had the function of improving the anoxia tolerance.

Red blood cell is the largest number of blood corpuscle and acts as the major media for transporting oxygen in the blood [24]. The number of red blood cells directly reflects the blood's oxygen carrying capacity. Hct refers to the volume ratio of red blood cells to whole blood, indirectly indicating the number and size of red blood cells. Hb is easily bound with oxygen in areas with high oxygen content and is readily separated from oxygen in places with low oxygen content. This characteristic enables erythrocytes to function as oxygen carriers [25]. The results in

anti-hypoxia effects of WOPS for the first time in mice.

Whey protein is a protein extracted from milk that has high bioavailability and a variety of biological activities, including anti-oxidation, immunomodulation, anti-fatigue, anti-viral and anti-bacterial [23]. In order to rule out false positive results that may be caused by protein

Figure 5. Effects of WOPs on RBC, Hb and Hct. *P<0.05, **P<0.01 versus vehicle control group; *P<0.05, **P<0.01 versus whey protein group. WOPs, small molecule oligopeptides isolated from walnut; WOPs-LG, 110 mg/kg WOPs group; WOPs-MG, 220 mg/kg WOPs group; WOPs-HG, 440 mg/kg WOPs group.

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4586 Am J Transl Res 2021;13(5):4581-4590
the present study suggested that WOPs could enhance RBC, Hb and Hct levels, and thus improve the oxygen carrying capacity of blood and increase the oxygen utilization rate of mice.

During hypoxia, oxygen can not be completely hydroxynated by the function of mitochondrial cytochrome oxidase. Therefore, reduced equivalents accumulated in respiratory chain, leading to ROS formation due to the autooxidation of mitochondrial complexes [26]. When ROS production exceeds the capacity of cellular antioxidant systems, oxidative stress happens. Membrane lipids in brain contain abundant polyunsaturated fatty acids, which are crucial targets for free radical attack [27, 28]. Furthermore, the brain has lower levels of antioxidant enzymes than other organs [29, 30]. Above two factors make the brain very susceptible to lipid peroxidation, in the process of which a mixture of alkenes, epoxides, alkanes, alkenals, alkanals, and aldehydes including MDA yielded [31]. In general, the MDA content is used as a crucial indicator for lipid peroxidation [32-34]. In the present study, WOPs remarkably decreased the brain MDA content of mice, and therefore, minimized the lesion of lipid peroxidation.

In the process of hypoxia, energy produced by aerobic respiration is not enough to meet tissue’s needs. Under these circumstances, due to anaerobic respiration, excessive lactic acid will be produced, reduce the pH value, affect related enzymes’ activity, and causing intracellular acidosis [35]. Because LDH is a key enzyme in this process, changes in the quantity and activity of LDH directly affect the energy metabolism in vivo. The results of this
Anti-hypoxia effects of walnut oligopeptides

Study suggested that WOPs could reduce lactate levels and enhance LDH activity in brain, and consequently increase the brain’s ability to buffer against lactic acidosis of mice.

Blood vessels are an important source of oxygen for tissue cells. Vascular endothelial growth factor (VEGF) is an important regulator that can stimulate vascular endothelial proliferation and migration, change vascular permeability, and promote angiogenesis. It is an important marker of angiogenesis [36]. Hypoxia-inducible factor 1alpha (HIF-1α) is considered a key transcription factor involved in the hypoxia response [37, 38]. It can regulate the transcription of a variety of genes. The transcription product of HIF-1α can reduce the oxygen consumption of cells or increase the oxygen supply of hypoxic tissues, thereby alleviating the contradiction between oxygen supply and demand and maintaining the stability of the internal environment. HIF-1α is also the core regulator of angiogenesis under hypoxia, and plays a key role in angiogenesis process of hypoxic damaged tissues. Studies have shown [39] that in a normal aerobic environment, HIF-1α is at a low concentration due to increased degradation and transcriptional inhibition; when tissue cells are hypoxic, HIF-1α is rapidly activated and highly expressed by the stimulation of various hypoxia response genes. The results of this study show that WOPs can induce an increase in the expression levels of VEGF mRNA and HIF-1α mRNA in the process of brain hypoxic injury, suggesting that the body initiates its own protective mechanism under hypoxia stimulation, and the high mRNA expression of HIF-1α promotes it by up-regulating downstream VEGF expression. Angiogenesis induces adaptation of local tissues to hypoxia and prevents further deterioration of tissue damage.

The present study demonstrated the anti-hypoxia effects of WOPs in mice for the first time. The effects might work through the following ways: one is to improve the blood’s oxygen carrying capacity and oxygen utilization rate, the other is to reduce the lesion of lipid peroxidation, the third is to increase the brain’s ability to buffer against lactic acidosis of mice, and fourth is to promote angiogenesis and regulate hypoxia response. We look forward to more in-depth research on the mechanism.

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Disclosure of conflict of interest

None.

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