

Original Research Article

Anti-hypoxia effects of ginseng (*Panax Ginseng* C A Meyer) oligopeptides in mice

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Abstract

Purpose: To study the anti-hypoxia effects of ginseng oligopeptides (GOPs) in mice.

Methods: Mice were randomly assigned to six groups: vehicle control group, whey protein-fed group (0.30 g/kg body weight, BW), and four groups given GOP at doses of 0.075, 0.150, 0.300, 0.600 g/kg BW. All treatments were administered via gavage once a day for a total of 30 days.

Results: GOPs significantly extended survival times under normobaric hypoxia, sodium nitrite toxicosis and acute cerebral ischemia. Moreover, GOPs enhanced the levels of RBC, Hb and Hct; decreased brain malonaldehyde (MDA) and lactate contents, enhanced brain lactate dehydrogenase (LDH) activity, and upregulated the mRNA expression levels of hypoxia-inducible factor 1alpha (HIF1α) and vascular endothelial growth factor (VEGF).

Conclusion: GOPs exert anti-hypoxia effects via mechanisms which may involve improvement of oxygen-carrying capacity of blood and oxygen utilization, reduction of lipid peroxidation-associated lesions, enhancement of brain capacity to buffer against lactic acidosis, promotion of angiogenesis, and regulation of response to hypoxia.

Keywords: Ginseng; oligopeptide; anti-hypoxia; mice

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INTRODUCTION

Hypoxia is defined as a pathological situation in which tissue metabolism and morphological structures change abnormally due to oxygen deprivation or obstruction of oxygen utilization. Symptoms of hypoxia generally include rapid heartbeat, dry mouth, nausea, vomiting, diarrhea, palpitations, shortness of breath, and in worse cases, malaise, coma and shock [1]. Hypoxia damages the nerves, and impairs digestion, respiration, urination and endocrine function.

Moreover, it affects the metabolism of carbohydrates, proteins, lipids, water and electrolytes, and ultimately endangers human health. Thus, it is very necessary to develop safe and effective methods for the prevention of hypoxia. Currently, vasodilating agents such as acetazolamide and nifedipine are used to reduce the incidence and severity of hypoxia [2,3]. However, multiple adverse effects such as headache and cardiopalmus, have been observed in clinical practice [4].

In recent years, the use of nutrition intervention has received increasing attention, and a large number of studies have shown the safety and effectiveness of natural food components as anti-hypoxia agents [5-7]. To date, researchers have isolated various bioactive peptides from plants, animals and microorganisms, and confirmed that they have diverse biological activities such as antimicrobial, antioxidant, anti-hypoxia, cholesterol-lowering, as well as immunomodulatory properties [8-11].

Ginseng (*Panax ginseng* C. A. Mey.) is a traditional Chinese medicine which has been used for thousands of years in China [12]. Ginseng was approved as a new food resource by the Chinese Ministry of Health in 2012 [13]. The ginseng root contains numerous bioactive components, including ginsenoside, amino acids, peptides and polysaccharides, all of which account for its extensive pharmacological effects such as antioxidant [14-16], anti-hypoxic [17], immunoregulatory [18,19], hypolipidemic [20,21] and aphrodisiac potential [22-25]. Most studies have attributed the anti-hypoxia effects of ginseng to ginsenoside [26-29]. However, oligopeptides which are important bioactive components of walnuts, are characterized by small molecular weight, ease of absorption and high bioavailability, although not much is known about their anti-hypoxia effects. For the purpose of this research, it was hypothesized that GOPs have anti-hypoxia effects. Therefore, this study was designed to investigate the anti-hypoxic effect of GOPs in mice.

EXPERIMENTAL

Extraction of GOPs

Using enzymatic hydrolysis, GOPs were extracted from Jilin ginseng roots. The walnuts were washed, chopped into bits and homogenized in distilled water. The pH of the extract was adjusted to 8.0, prior to treatment with protease at 40°C for 3 hours. This was followed by nanofiltration, freeze-drying, decolorization, purification and spray drying, resulting in powdered GOPs. A Phenomenex C18 column (10 mm×250 mm) was used to purify the sample. The content of GOPs was 95.42 %, while the proportion of free amino acids was 3.94 %. The relative molecular weights of the GOPs ranged between 180 and 1000 Da.

Chemicals and reagents

Malondialdehyde (MDA) and lactate 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 bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Animals

Adult male BALB/C mice weighing 18-22 g, were purchased from the Laboratory Animal Science Department of Peking University Health Science Center. The mice were maintained in a laboratory environment at a temperature 25 ± 1 °C, relative humidity of 55 ± 5 %, and a 12-hour light:12-hour dark cycle. They were permitted *ad libitum* access to AIN-93G diet and clean water, and were fed adaptively without intervention for 7 days. The animals were handled in compliance with the Principle of Laboratory Animal Care [30]. The animal study was reviewed and approved by Peking University Institutional Animal Ethics Committee, Beijing, China (approval no. GDPPKU2019-10/134).

Mouse groups and treatment

The mice were randomly divided into 4 groups: A, B, C and D, each having 72 mice. Each group was randomly divided into 6 sub-groups (n = 12): vehicle control group (VCG-2), whey protein-treated sub-group (0.300 g/kg, WPG-2), and four sub-groups given GOPs at doses of 0.075, 0.150, 0.300 and 0.600 g/kg. These were tagged GOPs-7, GOPs-8, GOPs-9, and GOPs-10, respectively. Mice in WPG-2 and 3 GOPs sub-groups were administered whey protein and corresponding doses of GOPs (0.1ml/10g), while mice in VCG-group were given vehicle. All treatments were administered by oral gavage, once a day for a total of 30 days. The animals were weighed and their weights were recorded weekly.

Determination of the effect of GOPs on normobaric hypoxia

Normobaric hypoxia survival time

Sixty minutes after the last drug dose, each mouse in A sub-group was placed in a 250-mL sealed container containing 5 g of medical soda lime. The duration of mouse survival under oxygen deprivation was recorded.

MDA content, lactate levels, and LDH activity

When each mouse died, its brain was immediately dissected out, and a 10 % homogenate was prepared in physiological saline at 4 °C. The MDA content, lactate levels, and

LDH activity in the homogenate were measured using their corresponding kits.

HIF-1 α and VEGF mRNA levels

When each mouse died, its brain was immediately dissected out and total RNA was extracted using Ribospin (GeneAll, Inc., Seoul, Korea). The ABI 7300 real-time PCR detection system was applied for real-time reverse transcription-PCR to determine the RNA expression levels of the target genes. Analysis of mRNA levels of target mRNA levels was done using M-MLV kits (Invitrogen). The sequences of primers used were as follows.

HIF-1 α : forward: 5'-TCACCACAGGACAGTACAG GATGC-3', reverse: 5'-CCAGCAAAGTTAAAGCAT CAGGTTCC-3');
 VEGF: forward: 5'-ACG AAGTGGTGAAGTTCATGGATG-3', reverse: 5'-TTC TGTATCAGTCTTTCCTGGTGAG-3';
 GAPDH: forward: 5'-GCCAAAGGGTCATCATCTC-3', reverse 5'-GTAGAGGCAGGGATGATGTT-3'. The relative mRNA expression levels of target genes were calculated using the 2^{- $\Delta\Delta C_t$} method, with GAPDH as internal standard.

Determination of survival time under sodium nitrite toxicosis

Sixty (60) minutes after the last dose of treatment, each mouse in B sub-group was injected intraperitoneally with sodium nitrite (0.1 ml/10g) at a dose of 240 mg/kg, and its survival time was recorded.

Evaluation of survival time in acute cerebral ischemia

Sixty (60) minutes after the last drug dose, each mouse in C sub-group was sacrificed via decapitation, and the time interval between

decapitation and the last breath (death) was recorded.

Whole blood analysis

Sixty (60) minutes after the last drug dose, blood samples were obtained from the eyeballs of each mouse in D subgroup. The blood samples were collected in EDTA tubes. Sysmex XT-2000i Blood Analyzer (Roche Diagnostics) was used to measure the levels of RBC, Hb and Hct within 3 hours of blood collection.

Statistical analysis

Statistical analysis was done using SPSS software version 20.0. The homogeneity of variances was checked. One-way analysis of variance (ANOVA) and LSD methods were used for comparison of data. Values of $p < 0.05$ were considered indicative of significant differences.

RESULTS

Effect of GOPs on body weight of mice

There were no significant differences in weight among all the mice groups ($p > 0.05$). These results are shown in Table 1.

Effect of GOPs on survival time under normobaric hypoxia

The normobaric hypoxia survival times in WPG-2, GOPs-7, GOPs-8, GOPs-9 and GOPs-10 were 8.8, 24.5, 41.5, 47.3 and 53.8 %, respectively, longer than that in VCG-2 ($p < 0.05$). The GOPs dose-dependently prolonged survival time in mice exposed to hypoxia, when compared to WPG-2 ($p < 0.05$). These results are presented in Figure 1.

Table 1: Effect of GOPs on body weight

Weight (g)	VCG-2	WPG-2	GOPs-7	GOPs-8	GOPs-9	GOPs-10
A sub-group						
Initial weight	20.8 \pm 1.3	21.5 \pm 1.2	20.9 \pm 1.1	20.3 \pm 1.1	21.3 \pm 1.0	21.8 \pm 1.0
Terminal weight	25.6 \pm 1.6	26.0 \pm 1.6	25.5 \pm 1.7	25.5 \pm 1.5	25.4 \pm 1.8	25.1 \pm 1.6
B sub-group						
Initial weight	20.9 \pm 1.3	21.0 \pm 1.2	21.5 \pm 1.1	21.2 \pm 1.0	21.3 \pm 0.9	20.7 \pm 1.0
Terminal weight	25.3 \pm 1.5	26.1 \pm 1.5	25.4 \pm 1.5	25.6 \pm 1.6	25.5 \pm 1.9	25.9 \pm 1.4
C sub-group						
Initial weight	21.6 \pm 1.2	20.1 \pm 1.1	21.0 \pm 1.0	21.3 \pm 1.0	20.9 \pm 1.1	21.1 \pm 1.2
Terminal weight	25.6 \pm 1.7	26.0 \pm 1.6	25.4 \pm 1.6	25.4 \pm 1.5	25.7 \pm 1.5	25.7 \pm 1.25
D sub-group						
Initial weight	21.3 \pm 1.1	20.5 \pm 1.0	20.8 \pm 1.2	21.0 \pm 1.0	20.4 \pm 1.1	21.2 \pm 1.3
Terminal weight	25.4 \pm 1.6	25.3 \pm 1.4	25.7 \pm 1.4	25.5 \pm 1.3	25.4 \pm 1.7	25.0 \pm 1.5

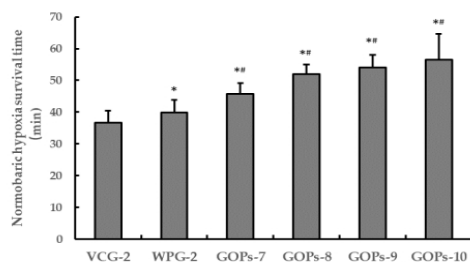


Figure 1: Effect of GOPs on normobaric hypoxia survival time of mice

Effect of GOPs on survival time under sodium nitrite toxicosis

As shown in Figure 2, there was no significant difference in the sodium nitrite toxicosis survival time between VCG-2 and WPG-2 ($p > 0.05$). However, the survival times in GOPs-8, GOPs-9, and GOPs-10 were 23.6, 41.4 and 45.9 %, respectively, longer than that in VCG-2 ($p < 0.05$). The sodium nitrite toxicosis survival times in GOPs-9 and GOPs-10 were significantly longer than that in WPG-2 ($p < 0.05$).

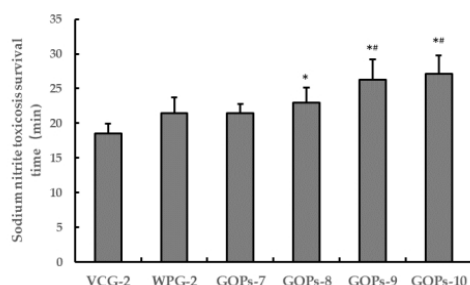


Figure 2: Effect of GOPs on survival time of mice under sodium nitrite toxicosis

Effect of GOPs on acute cerebral ischemia survival time of mice

Figure 3 shows that there was no significant difference in acute cerebral ischemia survival time of mice between VCG-2 and WPG-2 ($p > 0.05$). However, acute cerebral ischemia survival times in GOPs-7, GOPs-8, GOPs-9 and GOPs-10 were 4.5, 11.0, 11.0 and 9.0 %, respectively, longer than the corresponding survival time in

VCG-2 ($p < 0.05$). The acute cerebral ischemia survival times in GOPs-8, GOPs-9 and GOPs-10 were significantly extended, relative to the corresponding value in WPG-2 ($p < 0.05$).

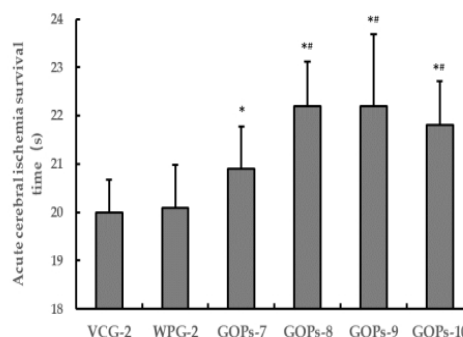


Figure 3: Effect of GOPs on acute cerebral ischemia survival time of mice

Effect of GOPs on RBC, Hb and Hct

As shown in Table 2, there were no significant differences in levels of RBC, Hb and Hct between VCG-2 and WPG-2 ($p > 0.05$). However, relative to VCG-2, levels of RBC and Hct in GOPs-8, GOPs-9 and GOPs-10 were significantly enhanced ($p < 0.01$). Moreover, Hb levels in GOPs-7, GOPs-8, GOPs-9, and GOPs-10 were significantly increased ($p < 0.05$). Compared with WPG-2, RBC levels in GOPs-8, GOPs-9 and GOPs-10 were significantly increased ($p < 0.05$). In GOPs-8, GOPs-9, and GOPs-10, Hct levels were markedly increased ($p < 0.05$). In addition, Hb levels were significantly increased in GOPs-8, GOPs-9 and GOPs-10 ($p < 0.05$).

Effect of GOPs on brain MDA content

No significant difference in brain MDA content was found between VCG-2 and WPG-2 ($p > 0.05$). However, compared with VCG-2, brain MDA levels in GOPs-7, GOPs-8, GOPs-9 and GOPs-10 were significantly decreased ($p < 0.01$).

Table 2: Effect of GOPs on levels of RBC, Hb and Hc in mice

Group	RBC ($10^{12}/L$)	Hct (%)	Hb (g/L)
VCG-2	7.59±0.66	39.41±3.88	120.86±11.27
WPG-2	8.05±0.80	41.01±4.09	127.14±11.57
GOPs-7	8.24±0.74	43.04±4.21	138.58±12.99*
GOPs-8	8.93±0.73***	46.22±4.35***	143.01±13.64***
GOPs-9	8.96±0.71***	47.01±4.59***	149.90±14.89***
GOPs-10	9.10±0.89***	48.60±4.75***	153.39±15.26***

Brain MDA levels in GOPs-8, GOPs-9 and GOPs-10 were lower than that in WPG-2 ($p < 0.01$). These results are shown in Figure 4.

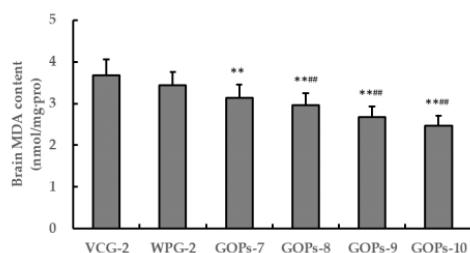


Figure 4: Effect of GOPs on brain MDA content. ** $P < 0.01$, versus vehicle control group; # $P < 0.01$, versus whey protein group. GOPs = ginseng oligopeptides; VCG-2 = vehicle control group; WPG-2 = 0.300 g/kg BW whey protein group; GOPs-7 = 0.075 g/kg BW GOPs group; GOPs-8 = 0.150 g/kg BW GOPs group; GOPs-9 = 0.300 g/kg BW GOPs group; GOPs-10 = 0.600 g/kg BW GOPs group

Effect of GOPs on brain lactate levels and LDH activity

There were no significant differences in brain lactate level and LDH activity between VCG-2 and WPG-2 ($p > 0.05$). Brain lactate levels in GOPs-7, GOPs-8, GOPs-9 and GOPs-10 were significantly increased, while their brain LDH activities were significantly decreased, when compared to VCG-2 ($p < 0.05$). Brain lactate levels were significantly decreased, while brain LDH activities were elevated in GOPs-8, GOPs-9 and GOPs-10, relative to WPG-2 ($p < 0.05$). These results are shown in Figure 5.

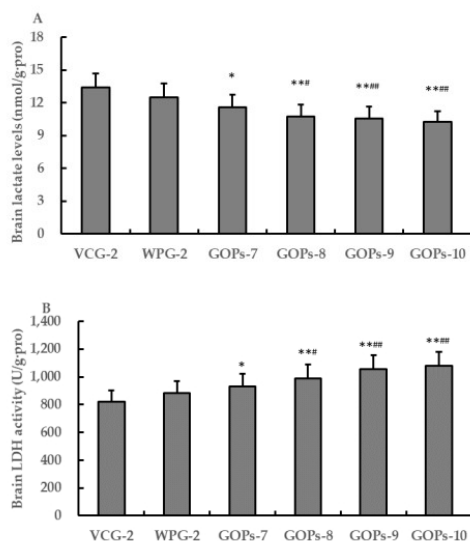


Figure 5: Effect of GOPs on brain lactate levels and LDH activities in mice

Effect of GOPs on mRNA expression levels of HIF-1 α and VEGF in mouse brain tissue

No significant differences in mRNA expression levels of HIF-1 α and VEGF were found between VCG-2 and WPG-2 ($p > 0.05$). However, mRNA levels of HIF-1 α and VEGF in GOPs-7, GOPs-8, GOPs-9 and GOPs-10 were higher than the corresponding levels in VCG-2 ($p < 0.05$). In addition, compared with WPG-2, the mRNA expression levels of HIF-1 α and VEGF in GOPs-8, GOPs-9 and GOPs-10 were significantly upregulated ($p < 0.05$). These results are presented in Figure 6.

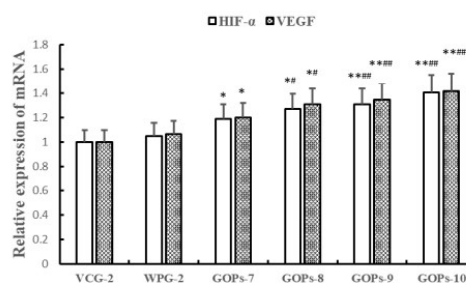


Figure 6: Effect of GOPs on brain mRNA levels of HIF-1 α and VEGF mRNA in mice

DISCUSSION

Hypoxia is a form of stress to the body. It impairs normal metabolic processes, especially antioxidant function, and it may even lead to death due to insufficient energy supply to major organs such as the heart and brain. In the present study, the anti-hypoxic effects of GOPs in mice were determined for the first time.

Whey protein is extracted from milk using advanced technology. It has high bioavailability, as well as various biological activities such as anti-oxidant, immunomodulative, anti-fatigue, anti-viral and anti-bacterial properties [13]. In order to rule out false positive results which may be caused by protein supplements, whey protein was used as a protein control. There were no effects of whey protein on hypoxia under the experimental conditions used in this study.

During normobaric hypoxia, insufficient oxygen supply severely reduces the partial pressure of intracellular oxygen, leading to mitochondrial dysfunction which impairs energy metabolism. In the sodium nitrite toxicosis studies, sodium nitrite converted bivalent hemoglobin into trivalent hemoglobin, thereby disrupting the oxygen-carrying capacity of hemoglobin, leading to tissue hypoxia. In the acute cerebral ischemia studies, decapitation terminated blood supply to the brain,

but the brain could still work for a short time, as manifested in regular gasping. The duration of gasp was used as a crucial indicator for evaluating the protective effect of the tested samples on cerebral ischemic anoxia.

results obtained indicate that GOPs significantly extended the duration of survival in the three assessments, while treatment with whey protein at a dose of 0.150 g/kg only extended the normobaric hypoxia survival time, but had no effect on the duration of survival in the sodium nitrite toxicosis and acute cerebral ischemia assessments. Besides, the normobaric hypoxia survival times in the four GOPs groups were longer than that of the whey protein group. Thus, GOPs improved anoxia tolerance, and the effect was better than that produced by whey protein.

Red blood cells are the largest number of blood corpuscles, and they are responsible for transporting oxygen in the blood [31]. The number of red blood cells reflects the oxygen carrying capacity of blood. Hematocrit (Hct) is defined as the volume ratio of red blood cells to whole blood, indirectly indicating the number and size of red blood cells. Hemoglobin (Hb) is the protein that carries oxygen in higher animals. It is easily combined with oxygen in areas with high oxygen contents, but it readily dissociates from oxygen in places with low oxygen levels. This characteristic enables erythrocytes to function as oxygen carriers [32]. The results of the present study suggest that GOPs enhanced levels of RBC, Hb and Hct, thereby improving the anoxia tolerance of mice.

During hypoxia, oxygen cannot be completely reduced to water by mitochondrial cytochrome oxidase. Therefore, reduced equivalents are accumulated in the respiratory chain, leading to ROS formation due to the autooxidation of mitochondrial complexes [33]. When ROS production exceeds the capacity of cellular antioxidant systems, oxidative stress occurs. Brain membrane lipids contain abundant polyunsaturated fatty acids which are crucial targets for free radical attack [34,35]. Furthermore, the brain has lower levels of antioxidant enzymes than other organs [36,37]. These two factors make the brain very susceptible to lipid peroxidation, a process which yields a mixture of alkenes, epoxy-fatty acids, alkanes, alkenals, alkanals, and aldehydes including MDA [38]. In general, MDA content is used as a crucial indicator of lipid peroxidation [39-41]. In the present study, GOPs markedly decreased mice brain MDA content, thereby minimizing lesions due to lipid peroxidation.

During hypoxia, energy produced by aerobic respiration is not enough to meet tissue needs. Under these circumstances, due to anaerobic respiration, excessive lactic acid is produced, resulting in lowered pH values which affect the activities of enzymes, and cause intracellular acidosis [42]. Since LDH is a key enzyme in this process, changes in the quantity and activity of LDH directly affect energy metabolism *in vivo*. The results of this study suggest that GOPs reduce brain lactate levels and enhance LDH activity, thereby enhancing the capacity of the brain to buffer against lactic acidosis in mice.

Blood vessels are important routes of oxygen supply to tissue cells. Vascular endothelial growth factor (VEGF) is an important regulator that stimulates vascular endothelial proliferation and migration, changes vascular permeability, and promotes angiogenesis. It is an important marker of angiogenesis [43]. Hypoxia-inducible factor 1 α (HIF-1 α) is considered a key transcription factor involved in hypoxic response [44,45]. It regulates the transcriptions of a variety of genes. The transcription product of HIF-1 α reduces the oxygen consumption of cells and increases oxygen supply to hypoxic tissues, thereby alleviating imbalance between oxygen supply and demand, while maintaining the stability of the internal environment. Moreover, HIF-1 α is the core regulator of angiogenesis under hypoxia, and it plays a key role in angiogenesis in hypoxic damaged tissues.

Studies have shown that in a normal aerobic environment, HIF-1 α is at a low concentration due to increased degradation and transcriptional inhibition, but when tissue cells are hypoxic, HIF-1 α is rapidly activated and highly expressed by the stimulation of various hypoxia response genes [46]. The results of this study show that GOPs induced increases in the mRNA expression levels of VEGF mRNA and HIF-1 α during brain hypoxic injury. This implies that the body initiates its own protective mechanism under hypoxic stimulation, and the high mRNA expression of HIF-1 α promotes the protective mechanism by up-regulating downstream expression of VEGF. Angiogenesis induces the adaptation of local tissues to hypoxia and prevents further deterioration of tissue.

CONCLUSION

The present study has demonstrated the anti-hypoxic effects of GOPs in mice for the first time. These effects are exerted via mechanisms involving improvement in oxygen carrying-capacity of blood and oxygen utilization, minimization of lipid peroxidation, increase in the

capacity of the brain to buffer against lactic acidosis, enhancement of angiogenesis, and regulation of hypoxic response. Further research is planned for more in-depth investigation of the mechanisms involved in the anti-hypoxic effect of GOPs.

DECLARATIONS

Acknowledgement

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Conflict of interest

No potential conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yong Li and Di Li conceived and designed the experiments; Di Li, Jinwei Ren, Jingqin Sun, Lan Wu and Peng Liu performed the experiments; Di Li and Jinwei Ren analyzed the data; Di Li wrote and revised the manuscript.

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