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Phenylethanoid glycosides of *Phlomis younghusbandii* Mukerjee ameliorate acute hypobaric hypoxia-induced brain impairment in rats

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ABSTRACT

High altitude cerebral edema (HACE), whose development process is associated with oxidative stress and inflammatory response, is a life-threatening condition caused by rapid ascent speed to high altitudes. Phenylethanoid glycosides (PhGCs) are primary active constituents isolated from *Phlomis younghusbandii* Mukerjee that reportedly exhibit potent anti-oxidant and anti-inflammatory activities. The present study aims to investigate the protective effect of phenylethanoid glycosides (PhGCs) from *P. younghusbandii* in acute hypobaric hypoxia (AHH) – stimulated HACE rats and its underlying mechanisms. The expression of pro-inflammatory cytokine levels (IL-1β, TNF-α, and IL-6) was detected by RT-PCR and ELISA at mRNA and protein levels in brain tissues. Western blotting was carried out to measure the major protein levels (IL-1β, TNF-α, and NF-κB) in brain tissues. The oxidative stress biomarkers (MDA, SOD, and GSH) were evaluated using kits. Results demonstrate that PhGCs significantly improved pathological changes in brain tissues, reduced the brain's water content, and attenuated the production and mRNA expression of pro-inflammatory cytokines. Furthermore, the increased oxidative stress and the decrease in anti-oxidant stress system under the AHH condition were also abrogated reversely through PhGCs treatment by elevating the levels of SOD and GSH and suppressing the accumulation of MDA. Simultaneously, there was also a significant reduction in NF-κB, IL-1β, and TNF-α protein expression levels in brain tissues, suggesting that blocking the NF-κB signaling pathway activation prevented the production of pro-inflammatory cytokines. Taken together, these findings indicate that PhGCs may afford a protectively intervene in HACE through the suppression of oxidative stress and inflammatory response via the inhibition of the NF-κB signaling pathway, indicating that PhGCs are promising agents for the treatment of acute HACE.

1. Introduction

Mountains cover one-fifth of the Earth's surface and are popular and challenging destinations for explorers (Patir et al., 2012). Approximately 140 million people in the world live at altitudes > 2500 m, with more than 8 million people living on the Qinghai-Tibet plateau of China at an average elevation of > 4000 m. People living at these altitudes require physiological adaptations to compensate for the lower partial pressure of oxygen (Moore et al., 2010). As to travelers, trekking expeditions, mountaineers, and military units who enter the high altitude environment too high or too quickly, especially for people with fatigue, infection, and psychological stress, hypobaric hypoxia is an important pathogenic factor for acute mountain sickness (AMS) (Pichler et al., 2017; Kushwah et al., 2016). Two independent or concomitant AMS fatal progressions may develop if ascensions of up to 3500 m are made: high altitude pulmonary edema (HAPE) and high altitude cerebral edema (HACE) (Li et al., 2017; Zhou et al., 2017). Clinically, AMS and HACE represent a continuum of an illness, because AMS usually precedes HACE, although not always. The two conditions may share common symptoms, such as a headache, loss of coordination, weakness, and decreasing levels of consciousness, including disorientation, loss of memory, hallucinations, and psychotic behavior (Kushwah et al., 2016; Davis and Hackett, 2017).

HACE has been recognized over the past two centuries, with

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altitude, speed, and mode of ascent known as the most significant determinants for its occurrence (Natah et al., 2009). Many mechanisms of HACE have been reported, with oxidative stress and inflammation debated as potentially the most crucial factors in its development (Kushwah et al., 2016). The reduced oxygen availability acts as an electron sink that is normally associated with hypoxia, which may result in the accumulation of reducing equivalents in the mitochondrial electron transport chain, further enhancing the production of reactive oxygen species (ROS), and leading to hypoxia-related oxidative stress (Schild et al., 2003). Previous findings also show cytokine activation and free radical-mediated damage to the blood-brain barrier (BBB) function and vascular injury under hypoxia as causative of fluid accumulation in the brain (Song et al., 2016). According to Gong et al., oxidative stress and inflammation play key roles in the development of HACE via the redox-sensitive transcription factor and nuclear factor kappa B (NF-κB) activation (Gong et al., 2018). Hensel et al., meanwhile, reported that blocking Rho-associated kinase, inhibiting NF-κB activation, and producing pro-inflammatory cytokines may reduce transvascular leakage in the brain of rats (Hensel et al., 2018).

NF-κB, a heterodimer that consists of two proteins; a p65 subunit and a p50 subunit, plays various transcriptional regulatory roles in inflammatory processes (Jeong et al., 2014; Sarada et al., 2008). This heterodimer is required for the maximal transcription of numerous cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), followed by the destruction of the microvascular basement membrane, which results in blood vessel hyperpermeability and vasogenic cerebral edema (Lou et al., 2015; Kalpana et al., 2008; Lafuente et al., 2016). It has been suggested that inhibitors of the NF-κB activation may be useful as anti-inflammatory agents (Niu et al., 2014). Therefore, eliminating oxidative stress or terminating persistent inflammation by an anti-oxidative and anti-inflammatory agent could be an important strategy to use as prophylaxis and in the treatment of HACE.

Phlomis younghusbandii Mukerjee, an essential Tibetan medicine called “Lu Mu Er”, is a perennial herb (Labiatae family) and only distributed in the eastern region of the Qinghai-Tibet Tibetan Plateau in China (Li et al., 2011a). The roots of *P. younghusbandii* has been traditionally used in Tibetan medicine as an important crude drug to treat anemopyretic cold, cough with profuse sputum, throat inflammation, skin infection, rheumatoid arthritis, pneumonia, and bronchitis (Zhao et al., 2009). In 2011, our lab isolated and identified three major phenylethanoid glycosides (PhGCs) from the roots of *P. younghusbandii*: acteoside, alyssonoside, and isoacteoside (Li et al., 2011b). Many studies have highlighted the fact that PhGCs possess a wide range of pharmacological activities; these include but not limited to antioxidant, anti-inflammatory, antiviral, antibacterial, antitumor, memory strengthening, and hepatoprotective effects (Wang et al., 2014). These properties of PhGCs enable them to inhibit malondialdehyde (MDA), the lipid peroxidation product and increase antioxidant levels, such as reduced glutathione hormone (GSH), myeloperoxidase (MPO), and superoxide dismutase (SOD) (Wang et al., 2015).

In the present study, we aimed to highlight the role of PhGCs in acute hypobaric hypoxia (AHH)-stimulated high altitude brain edema in rats. Additionally, we elucidated the potential mechanism involving oxidative stress and inflammation and evaluated the possible molecular mechanisms of the NF-κB signaling pathways.

2. Materials and methods

2.1. Reagents and antibodies

Phenylethanoid glycosides (PhGCs) (Purity > 98%) were obtained from our laboratory based on our previous study (Zhang, 2011). The MDA, SOD, and GSH kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). IL-1β, IL-6, and TNF-α ELISA kits were acquired from R&D Systems (Minneapolis, USA). TaKaRa BCA Protein Assay Kit, TaKaRa MiniBEST Universal RNA Extraction Kit, PrimeScriptTM RT Master Mix, and SYBR® Premix Ex TaqTM II were bought from Takara Biotechnology (Dalian, China). Primary antibodies against IL-1β, NF-κB, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA), while antibodies against TNF-α were obtained from Cell Signaling Technology (Beverly, USA). Secondary antibody, horseradish-peroxidase-conjugated goat anti-mouse IgG (H + L) was acquired from Beijing Zhongshan Jinqiao Biotechnology (Beijing, China). Other reagents were of commercially available analytical grade.

2.2. Animals

Wistar rats (male, 220–250 g) were purchased from the Laboratory Animal Center of Lanzhou General Hospital of PLA (Approval No. SCXX 2012-0020) and exposed to environmental and trainer-handling conditions for acclimation for seven days (Lanzhou, China). The animals were kept at an ambient temperature of 25 ± 1 °C and humidity of 55 ± 2%, with day and night cycles of 12 h each and given food and water ad libitum. 10% chloral hydrate was used to anesthetize the rats before the collection of tissues and blood for the experiment. All animal procedures and the experimental protocol were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Lanzhou General Hospital of PLA (LZ20120907-35). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Experimental design

All rats (n = 90) were randomly divided into 6 groups of 15 rats each: normoxia group, hypoxia group, PhGCs-L group, PhGCs-M group, PhGCs-H group, and dexamethasone group. The PhGCs-L, PhGCs-M, and PhGCs-H groups were given PhGCs 50, 100, and 200 mg/kg intragastrically (i.g.), respectively, the dexamethasone group was treated with 4 mg/kg dexamethasone daily for three days, and the normoxia and hypoxia groups received distilled water.

2.4. Establishment of a high altitude cerebral edema rat model

The HACE rat model was established according to the methods modified from a previous report (Yu et al., 2013). Briefly, on the fourth day of drug administration, the hypoxia, PhGCs-L, PhGCs-M, PhGCs-H, and dexamethasone groups were exposed to acute hypobaric hypoxia for three days in a specially designed animal decompression chamber with a chamber pressure of 267 mm Hg that reduced the barometric pressure (chamber pressure: 35.9 kPa; oxygen partial pressure: 7.5 kPa, and temperature: 25 °C) to simulate an altitude of 8000 m. The rats in the normoxia group were kept at normal atmospheric pressure with controlled temperature and humidity. The beginning rate of ascent to the desired altitude was 10 m/s according to the vacuum detection. To avoid secondary oxygen-enriched injuries in rats, the chamber was twigged to an altitude of 4000 m every day at 9:00 AM for 1 h to replenish food, water, and provide drug treatment. The rats had free access to food and water. After exposure to acute hypobaric hypoxia, the altitude of the chamber was brought down to 4000 m, the rats were anesthetized with 10% chloral hydrate (300 mg/kg), and blood samples were collected immediately from the retrobulbar venous plexus with capillary tubes, using 0.1 M EDTA as an anticoagulant. Brain tissues were removed after perfusion with 0.9% saline containing heparin, followed by fixation with an ice-cold 4% paraformaldehyde solution to collect blood frozen at −80 °C for further analysis.

2.5. Analysis of hematological parameters

Blood specimens (200 μl) were used for different hematological analyses (WBC: white blood corpuscles; RBC: red blood corpuscles;

HGB: hemoglobin; HCT: hematocrit; MCV: mean cell volume; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets; LYM%: lymphocytes; MON%: monocytes; and NEU%: neutrophilic granulocyte). The analysis was carried out with the Mindray Hematology Analyzer (BC-2300, Shenzhen, China).

2.6. Histopathological evaluations

For histological assessment, the brain specimens were fixed in 4% formalin, dehydrated with graded ethanol, embedded in paraffin, and cut into 5 μm thick sections. These sections were stained with hematoxylin and eosin (H&E) and examined using light microscopy. Pathological evaluation was conducted by experienced pathologists in a single-blind way. The results were scored in accordance with the reported method previously (Szapiel et al., 1979), and the score numbers (0–3) were respectively corresponded to the grades.

2.7. Determination of brain water content

To measure the brain water content (BWC) in the normoxia and hypoxia-exposed group rats, the left hemispheres were weighed with a precision electronic balance to obtain the wet weight. These left hemispheres were then dried at 55 °C for 72 h to achieve constant dry weights. The percentage of brain water content was calculated according to Elliot's formula: brain water content (%) = (wet weight − dry weight)/wet weight × 100% (Guo et al., 2013).

2.8. Biochemical analysis

Rat brains were homogenized at 4–8 °C in ice-cold 0.01 M phosphate-buffered saline (PBS, pH 7.4), centrifuged (4 °C, 12,000 rpm, 30 min), and the supernatant was collected for assays. The antioxidant status of the brain was evaluated using the activity of SOD and concentration of GSH. Lipid peroxidation was determined by the concentrations of MDA, which is its end product. The detailed analytical procedures were performed following the instructions provided with the test kits (Jiancheng Bioengineering Institute, Nanjing, China), and the results were expressed as nmol/mg protein, U/mg protein, and μmol/mg protein.

2.9. Enzyme-linked immunosorbent assay (ELISA)

To detect the anti-inflammatory effect of PhGCs, the brain tissues were weighed and homogenized (10% W/V) in a phosphate buffer solution. The homogenate was then centrifuged at 12,000 g for 30 min at 4 °C, and the supernatant was collected. The levels of IL-1β, TNF-α, and IL-6 in brain tissues were measured using the commercially available ELISA kits according to the manufacturer's instructions.

2.10. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the brain using the TaKaRa MiniBEST Universal RNA Extraction Kit according to the manufacturer's instructions. The concentration and purity of the total RNA of different samples were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Shanghai, China). The quantitative real-time polymerase chain reaction was run on the Applied Biosystems ViiA™ 7 DX detection instrument (Applied Biosystems, American) according to optimized PCR protocols. The PCR reaction system (20 μL in total) contained 10 μL SYBR Premix Ex TaqTM II (2X), 1 μL PCR Forward Primer (10 μM), 1 μL PCR Reverse Primer (10 μM), 0.4 μL ROX Reference dye (50X), 1 μL cDNA template, and 6.6 μL RNase Free dH_2O, with fluorescence monitored at each cycle. The cycling conditions were as follows: a preincubation step at 95 °C for 30 s, followed by 40 amplification cycles (95 °C for 5 s, 60 °C for 31 s), melting (95 °C for 10 s, 95 °C for 20 s, 97 °C for 1 s), and then cooling (56 °C for 31 s). Specific primers used for RT-PCR were: IL-1β (F: 5′-CCCTgAACTCAACTgTgAAATAgCA-3′; R: 5′-CCCAAgTCAAgggCTTggAA -3′); TNF-α (F: 5′-TCCCTTCCTTC CTTCCTTCC-3′; R: 5′-TAgCTggTAgTTTagC TCCgTTTTC-3′); IL-6 (F: 5′-ATTgTATgAACAgCgATgATgCAC-3′; R: 5′-CCAggT agAAACggAACT CCAgA-3); and GAPDH (F: 5′-ggCACAgTCAAggCTgAgAAT g-3′; R: 5′-AtggTggTgAAgAcgCCAgTA-3′). GAPDH served as a normalization control. The relative RNA expression of each gene was analyzed using the comparative Ct ($2^{-\Delta\Delta CT}$) method as previously reported (Livak and Schmitten, 2001).

2.11. Western blot analysis

Brains were lysed in RIPA lysis buffer and homogenized with an electric homogenizer. The protein concentration was determined using a bicinchoninic protein assay kit, separated with SDS-PAGE, and transferred to PVDF membranes. Next, the PVDF membranes were blocked with 5% BSA for 2 h and then incubated with anti-IL-1β (at a dilution ratio of 1:1000), anti-TNF-α (at a dilution ratio of 1:500), anti-NF-κB (at a dilution ratio of 1:500), and β-actin (at a dilution ratio of 1:1000), respectively, overnight at 4 °C, the latter serving as an internal control. The next day, after washing with TBST five times for 8 min, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies at 1:5000 dilution for 2 h and washed again. Immunoblots were detected with an ECL Plus chemiluminescence reagent kit (Bio-rad), and images of IL-1β, TNF-α, NF-κB, and β-actin were captured with a ChemiDoc-It2 610 chemiluminescence imaging system (UVP, American) for quantitative imaging of gels and blots. The densitometric values of the bands were calculated using the Image J software (National Institutes of Health, USA), and β-actin was used as the quality control.

2.12. Statistical analysis

All data from at least three experiments were analyzed by SPSS 16.0. All quantitative data were expressed as the mean ± standard deviations (S.D.). The statistical analysis was performed by a one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test (Gong et al., 2018). Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. PhGCs improved hematological parameters in AHH-stimulated HACE rats

As shown in Table 1, compared with the normoxia group, there were notable increases in the levels of WBC, RBC, HGB, HCT, MCV, PLT, LYM%, MON%, and NEU% in the hypoxia group. Compared with the hypoxia group, the administration of 50, 100, and 200 mg/kg body wt. of PhGCs (PhGCs-L, -M, and -H) and dexamethasone (4 mg/kg) led to a significant reduction in the levels of WBC, RBC, HGB, HCT, MCV, PLT, LYM%, MON%, and NEU% in a dose-dependent manners. However, the level of HGB remained high after the administration of PhGCs and dexamethasone.

3.2. PhGCs reduced brain water content in AHH-stimulated HACE rats

The degree of brain edema was determined by measuring brain water content as wet to dry weight ratio. To investigate the function of PhGCs in acute hypobaric hypoxia-caused brain edema, rats were exposed to acute hypobaric hypoxia. As shown in Fig. 1, exposure to acute hypobaric hypoxia significantly aggravated the brain water content relative to the normoxia group. Pre-conditioning with PhGCs (50, 100 and 200 mg/kg) and dexamethasone (4 mg/kg) dose-dependently ameliorated the adverse increase in brain water content levels.

Table 1
PhGCs (50, 100, and 200 mg/kg) improved hematological parameters in AHH-stimulated HACE rats.

Variable	Normoxia	Hypoxia	PhGCs-L (50 mg/kg)	PhGCs-M (100 mg/kg)	PhGCs-H (200 mg/kg)	Dexamethasone (4 mg/kg)
WBC	8.13 ± 1.19	15.38 ± 3.31**	11.59 ± 1.67#	11.24 ± 4.12#	8.79 ± 1.47##	8.17 ± 3.64##
RBC	8.57 ± 0.44	10.33 ± 0.44*	9.77 ± 0.69	9.67 ± 0.31	9.45 ± 0.37##	9.01 ± 0.67##
HGB	158.63 ± 12.01	191.63 ± 5.50*	195.70 ± 8.14	198.00 ± 5.37	196.75 ± 6.65	198.00 ± 7.16
HCT	47.79 ± 2.08	60.48 ± 1.40*	57.48 ± 3.79	55.80 ± 4.48#	54.63 ± 1.73#	53.71 ± 1.16#
MCV	55.81 ± 0.89	68.99 ± 0.84*	66.94 ± 0.91	59.01 ± 0.45	56.75 ± 1.28#	53.23 ± 1.89#
MCHC	317.38 ± 11.06	319.00 ± 14.96	323.70 ± 15.61	318.70 ± 7.30	328.88 ± 6.98	318.25 ± 5.06
PLT	508.13 ± 148.01	1001.30 ± 172.83**	949.86 ± 174.64#	883.13 ± 181.83#	763.80 ± 140.58#	703.88 ± 105.95#
LYM%	69.85 ± 5.21	80.15 ± 4.29*	79.34 ± 7.12	74.85 ± 5.13#	72.42 ± 4.72#	68.56 ± 5.32#
MON%	1.75 ± 0.89	6.59 ± 1.64**	5.67 ± 1.46	3.98 ± 1.87#	2.03 ± 0.97#	3.56 ± 0.81#
NEU%	27.65 ± 5.14	50.36 ± 6.68*	48.91 ± 7.96	33.79 ± 5.52#	28.45 ± 3.67#	30.13 ± 4.65#

The analysis was carried out with the Mindray Hematology Analyzer. Compared with normoxia group: $^{*}P < 0.05$, $^{**}P < 0.01$; Compared with hypoxia group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Hematological analysis. WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCV: mean cell volume; MCHC: mean corpuscular hemoglobin concentration; PLT: platelets; LYM%: lymphocytes; MON%: monocytes; NEU%: neutrophilic granulocyte.

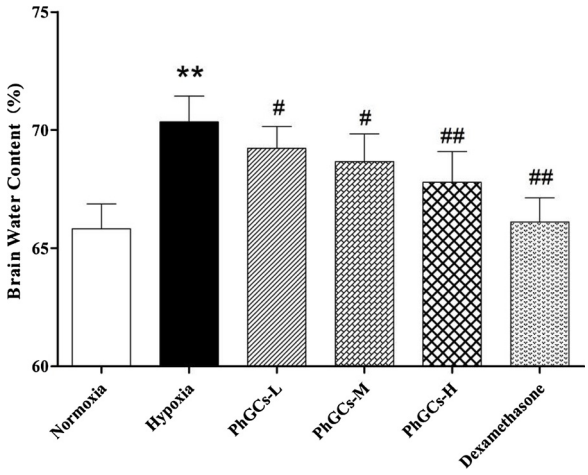

Fig. 1. PhGCs (50, 100, and 200 mg/kg) reduced the brain water content (BWC) in AHH-stimulated HACE rats. Rats were preconditioning with various doses of PhGCs or the same volume of distilled water, and exposure to AHH environment for three days. Then, the BWC was determined by wet/dry weight ratio. Data are expressed as the mean ± SD, $n = 10$ rats. Compared with normoxia group: $^{**}P < 0.01$; Compared with hypoxia group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

3.3. PhGCs shrunk brain tissue injuries in AHH-stimulated HACE rats

As shown in Fig. 2, brain sections from the normoxia group showed a normal arrangement, structure of cortical neurons and cerebral capillaries with a normal configuration (Fig. 2A). However, sections from the hypoxia group showed significant widening of the pericellular spaces, a dilatation of the cortical blood vessels, shrunken neurons with darkly stained pyknotic nuclei, and infiltration of inflammatory cells around the perivascular space (Fig. 2B), resulting in neuronal structural damage. Compared to the hypoxia group, after hypoxic preconditioning with PhGCs (50, 100 and 200 mg/kg) and dexamethasone (4 mg/kg), rats exposed to hypoxia showed only moderate widening or no widening of the pericellular space, while inflammatory cell infiltration lessened, showing a very mild cerebral edema (Fig. 2C–G), indicating that pre-administration of PhGCs has an antagonistic effect on pathological changes in the brain of rat caused by AHH exposure.

3.4. PhGCs attenuated oxidative stress in AHH-stimulated HACE rats

To determine oxidative stress, we examined the activity of SOD (Fig. 3A), the concentration of GSH (Fig. 3B), and the content of lipid peroxidation product MDA (Fig. 3C) in the brains of hypoxia-stimulated HACE rats pretreated with PhGCs. The activity of SOD and the concentration of GSH were revealed to be significantly reduced, while the content of MDA was shown to be significantly elevated in the hypoxia group compared to the same parameters in the normoxia group. Compared with the hypoxia group, the PhGCs (100 and 200 mg/kg) and dexamethasone (4 mg/kg) groups had significantly increased SOD activity and concentration of GSH, whereas, the MDA content in the PhGCs (100 and 200 mg/kg) groups was significantly reduced. There was, however, no significant difference in the activity of SOD and the concentrations of GSH and MDA between the PhGCs-L (50 mg/kg) and hypoxia groups.

3.5. PhGCs lessened the production of pro-inflammatory cytokines in AHH-stimulated HACE rats

Inflammatory response ranks as a critical cause of cerebral edema under various adverse conditions, including an acute hypobaric hypoxia-occurred high altitude environment (Himadri et al., 2010). To elucidate the mechanism underlying a PhGCs-mediated protective role in a hypobaric hypoxia-induced cerebral edema, we explored the effects of PhGCs on inflammatory responses. As shown in Fig. 4(A–C), hypoxia-stimulated cerebral edema led to significantly increased IL-1β, IL-6, and TNF-α levels in the hypoxia group when compared with the normoxia group. Pretreatment with PhGCs (50, 100 and 200 mg/kg) and dexamethasone (4 mg/kg), meanwhile, caused a decrease in IL-1β, IL-6, and TNF-α secretion in the PhGCs group (100 and 200 mg/kg) and dexamethasone (4 mg/kg) when compared with the hypoxia group.

3.6. PhGCs mitigated mRNAs' expression of pro-inflammatory cytokines in AHH-stimulated HACE rats

To further explore PhGCs' impact on mRNA expression levels of pro-inflammatory cytokines in hypoxia-stimulated HACE rats, RT-PCR analysis of IL-1β, TNF-α, and IL-6 in the brain was performed. As shown in Fig. 5, acute hypoxia-stimulated edema engineered significant increases in the IL-1β, TNF-α, and IL-6 mRNAs expression levels in the hypoxia group when compared with the normoxia group. Compared with the hypoxia group, pretreatment with PhGCs (100 and 200 mg/kg) and dexamethasone (4 mg/kg) led to significant suppression of the IL-1β, TNF-α, and IL-6 mRNAs expressions in a dose-dependent effect. These results suggest that PhGCs block the relative expression of pro-inflammatory cytokines in hypoxia-stimulated HACE rats. Subsequent studies focused on exploring the molecular mechanism underlying this anti-inflammatory activity of PhGCs are required.

3.7. PhGCs inhibited the activation of the NF-κB signaling pathway in AHH-stimulated HACE rats

The production of pro-inflammatory cytokines is closely related to the activation of the NF-κB signaling pathway and the translocation of NF-κB. Therefore, we further detected the protein expression of pro-

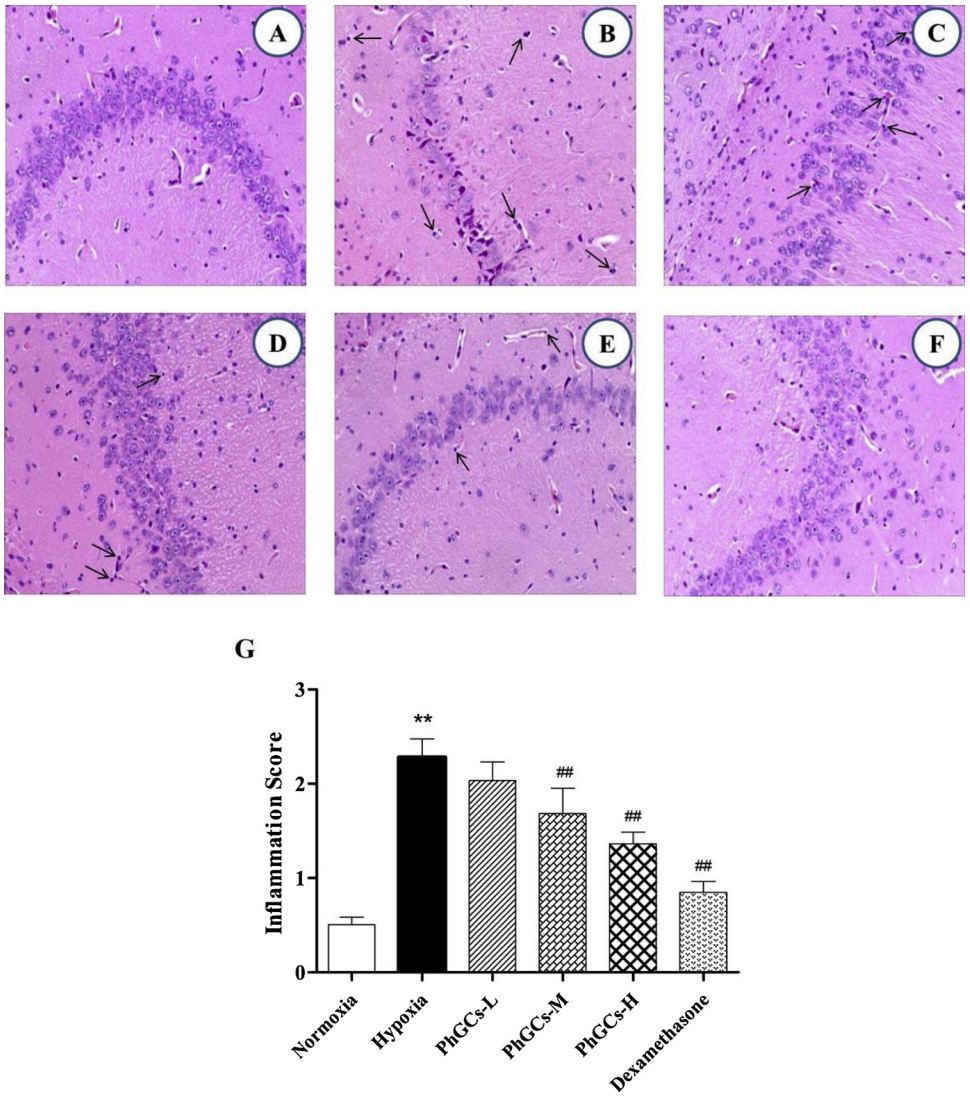

Fig. 2. PhGCs (50, 100, and 200 mg/kg) alleviated brain tissues impairment in AHH- stimulated HACE rats (HE staining, magnification 400×). The image represents H&E stained sections of brain tissues. (A): Normoxia group; (B): Hypoxia group; (C): PhGCs-L group; (D): PhGCs-M group; (E): PhGCs-H group; (F): Dexamethasone group; (G): Comparison of the inflammation score. Arrows show edema and infiltration of inflammatory cells in brain tissues, and the cell types are mainly lymphocytes and monocytes. Data are expressed as the mean ± SD, $n = 5$ rats. Compared with normoxia group: $^{**}P < 0.01$; Compared with hypoxia group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

inflammatory cytokines and NF-κB signaling pathways using the Western blotting analysis (Zheng et al., 2018). As shown in Fig. 6, our results indicated that acute hypoxia-stimulated edema enhanced the protein expression of IL-1β and TNF-α in hypoxia rats, compared to normoxia rats, while PhGCs (100 and 200 mg/kg) and dexamethasone (4 mg/kg) caused significant suppression of this phenomenon. PhGCs also led to a decrease in the translocation of the NF-κB protein. Taken together, these findings suggest that PhGCs have the potential to exert their anti-inflammatory activities through the inhibition of the NF-κB signaling pathway.

4. Discussion

HACE is a life-threatening disease that develops during rapid ascents to high altitudes, but its underlying mechanisms remain unclear. Growing evidence has implicated inflammation in the susceptibility to and development of brain edema (Schoch et al., 2002). High-altitude environments can cause the hypobaric hypoxia, which results in severe brain and lung functional disorders for humans or animals (Boos et al., 2012). Additionally, hypoxia and inflammation are closely associated and are important in a variety of pathological situations, with hypoxia eliciting tissue inflammation (Tu et al., 2016). PhGCs is reportedly a very potent anti-inflammatory and anti-oxidative agent with phytonutrient and protective properties (Li et al., 2011b). However, few studies have investigated the function of PhGCs in acute hypobaric hypoxia-induced inflammation in the central nervous system to date. Therefore, the aim of this study was to explore the potential anti-inflammatory and anti-oxidative effects and the underlying mechanisms of PhGCs on acute hypobaric hypoxia-stimulated HACE rats in the animal decompression chamber.

Recent studies have shown increases in hematological parameters, such as WBC, RBC, HGB, HCT, MCV, MCHC, PLT, LYM%, MON%, and NEU% in people who transferred to high altitude environments; these changes occur as compensatory responses to hypoxia (Bao et al., 2017). In our experiment, after exposure to a simulated altitude of 8000 m for three days, rats showed a significant elevation in certain blood parameters, including WBC, RBC, HGB, HCT, MCV, PLT, LYM%, MON%, and NEU%. However, the oral administration of PhGCs and dexamethasone instigated a significant reduction in most of the hematological changes in a dose-dependent manner. Adaptation to high altitude is related to complex hematological changes. A pervasive feature of adaptation to high altitude is hyperventilation, an extraordinary common phenomenon at high altitudes that may induce dehydration, partly because of the great insensible fluid loss resulting in a reduced plasma volume (Kaur et al., 2006). During the acute hypobaric hypoxia exposure, rats that drank less water and ate less food had decreased plasma volume and increased WBC, RBC, HCT, and MCV levels. People, who live on plains, often develop a rapid increase in erythrocyte levels when they transfer to high altitudes. This change is not caused by increased erythrocyte production, but by the decrease in plasma volume

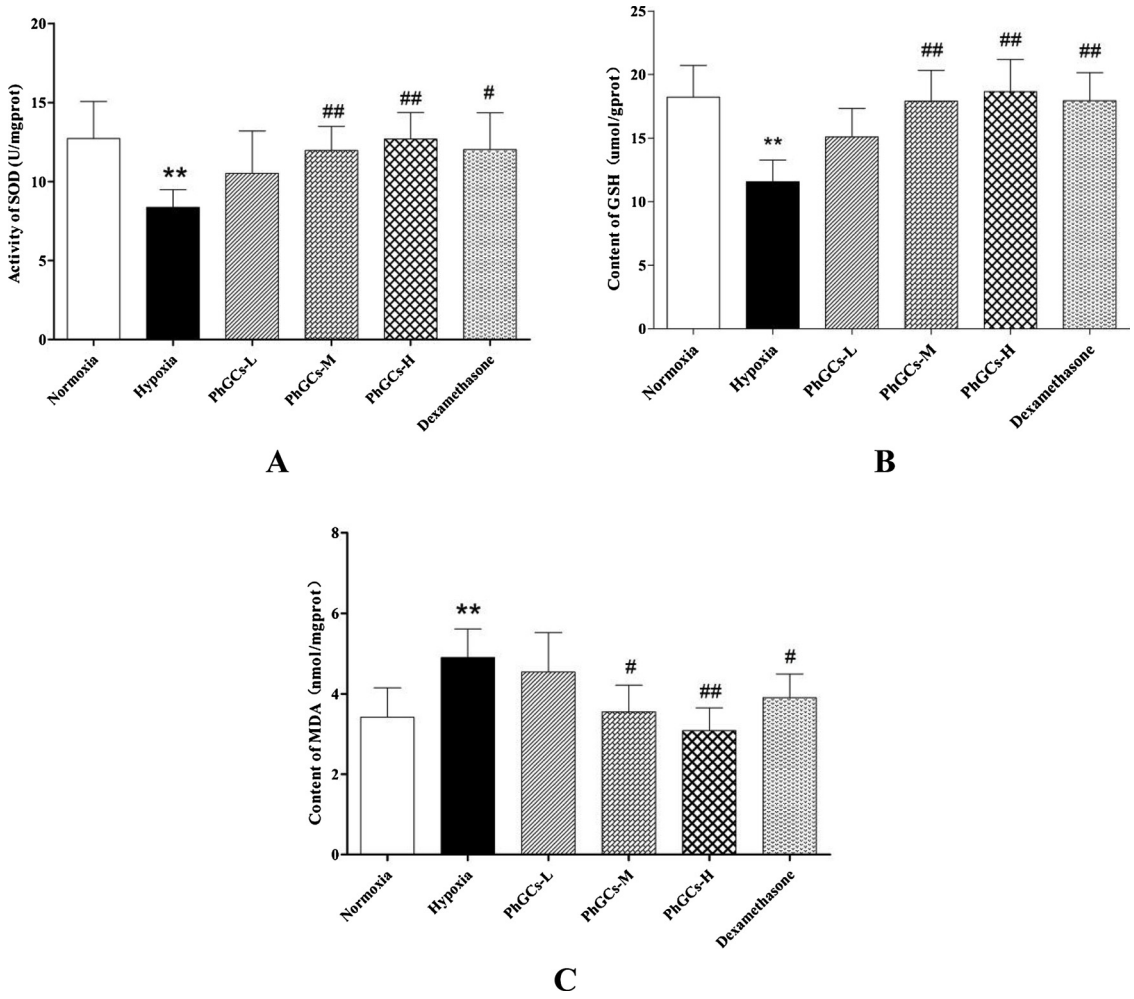

Fig. 3. PhGCs (50, 100, and 200 mg/kg) attenuated oxidative stress in AHH-stimulated HACE rats. Brain tissues were collected from various groups to prepare the 10% homogenate. The corresponding effects on anti-oxidant status in brain tissues from various groups were analyzed by detecting the concentration of SOD (A) and GSH (B). Levels of MDA were measured to evaluate lipid peroxidation (C). Data are expressed as the mean ± SD, $n = 10$ rats. Compared with normoxia group: $^{**}P < 0.01$; Compared with hypoxia group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

due to high altitude dehydration (Singh et al., 1990).

Brain water content was evaluated as an index of brain edema, a typical symptom of inflammation not only in local inflammation but also in systemic inflammation (Zhong et al., 2014). We found that pre- or after-treatment with PhGCs (50, 100, and 200 mg/kg) and dexamethasone (4 mg/kg) caused significant decreases in the acute hypobaric hypoxia-stimulated brain water content in a dose-dependent manner. These results suggest that PhGCs may have a protective effect on acute hypobaric hypoxia-stimulated HACE in rats.

Exposure to acute hypobaric hypoxia could cause an increase in reactive oxygen species and oxidative stress, accompanied by an enhanced-formation of free radicals and lipid peroxidation in the brain (Behn et al., 2007; Maiti et al., 2006). The brain is vulnerable to hypoxia stress and has a limited ability to withstand oxidative stress, due to the accumulation of endogenous ROS and the deficiency in anti-oxidant defense. Increasing evidence indicates that oxidative stress worsens HACE by destroying the structure of the BBB and inducing cell swelling (Himadri et al., 2010). We have shown in this study that the activity of SOD and concentration of GSH increased significantly, while the concentration of MDA decreased substantially in PhGCs (100 and 200 mg/kg) and dexamethasone (4 mg/kg) pretreatment groups in a dose-dependent manner. These findings suggest that PhGCs could shrink high altitude-induced brain injuries in rats by enhancing the activity of endogenous antioxidants and suppressing lipid peroxidation and oxidative stress. PhGCs could exert their protective effect through their anti-oxidant property.

Some studies have shown that altered NF-κB can mediate an inflammatory response and induce gene transcription and protein expression of pro-inflammatory and cell adhesion molecules (Sarada et al., 2008; Kalpana et al., 2008). It is well- known that inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are important biomarkers of brain injuries. The present study showed that acute hypobaric hypoxia caused a significant increase in the brain protein and mRNA expression levels of IL-1β, IL-6, and TNF-α, compared with the normoxia group. Pretreatment or after-treatment with PhGCs (100 and 200 mg/kg) and dexamethasone (4 mg/kg), on the other hand, triggered a markedly decreased production of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) at protein and mRNA levels after acute hypobaric hypoxia challenge, an outcome consistent with previous findings (Wang et al., 2014). These results imply that PhGCs exerted anti-inflammatory activities through the inhibition of pro-inflammatory cytokines.

Based on the findings above, we further explored the anti-inflammatory mechanism of PhGCs. There was plenty of evidence to show that NF-κB modulated the gene expression of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6 (Gu. et al., 2017; Fan et al., 2017). Paeoniflorin is a bioactive component extracted from *Paeonia lactiflora* and traditionally used to treat inflammatory disorders in China. Paeoniflorin reportedly ameliorates ulcerative colitis via the inhibition of the MAPK/NF-κB pathway in mice (Gu. et al., 2017). Research shows that quercetin-3-*O*-β-D-galactoside can inhibit lipopolysaccharide-

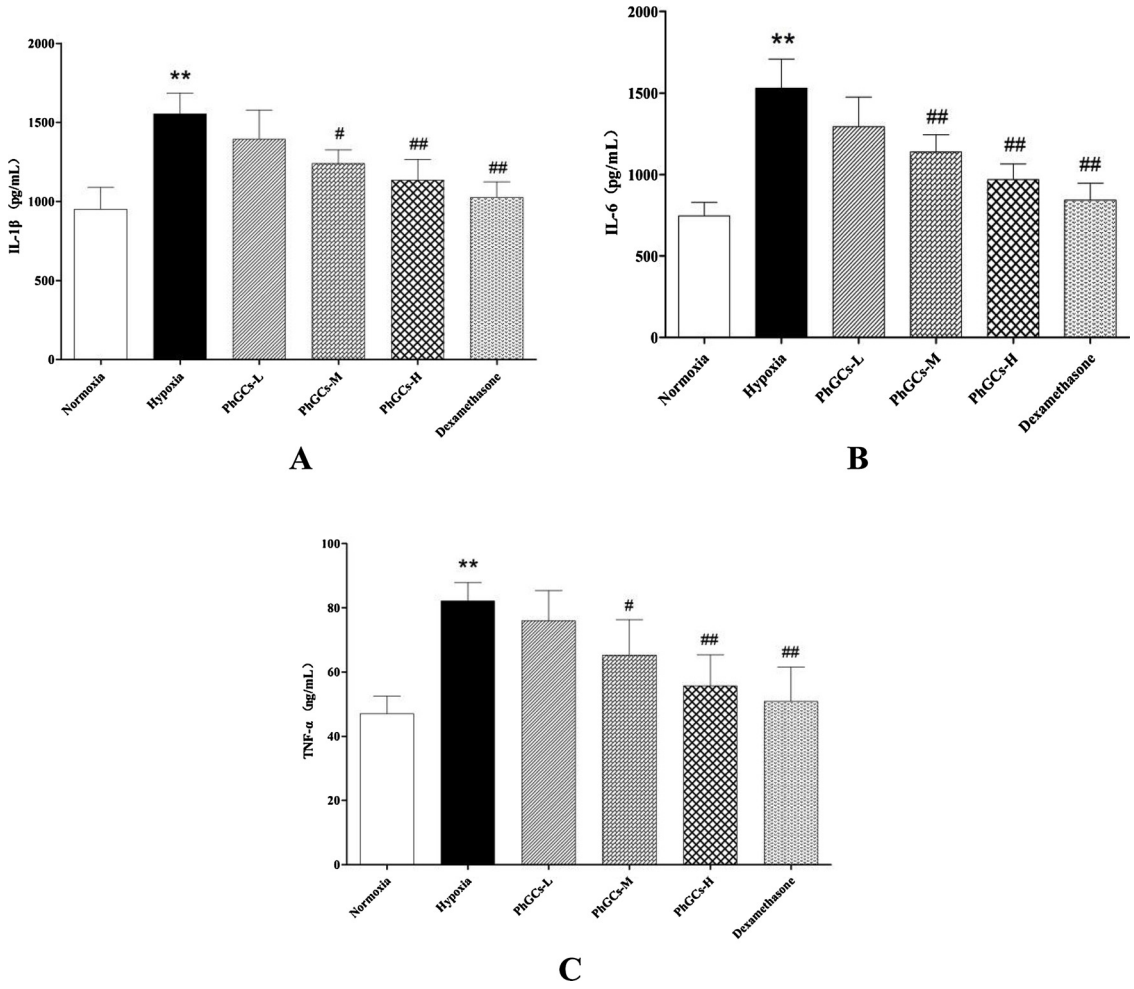

Fig. 4. PhGCs (50, 100, and 200 mg/kg) lessoned the production of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) in AHH-stimulated HACE rats. ELISA assay was then carried out to measure inflammatory cytokines IL-1β (A), IL-6 (B), and TNF-α (C). Data are expressed as the mean ± SD, $n = 10$ rats. Compared with normoxia group: $^{**}P < 0.01$; Compared with hypoxia group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

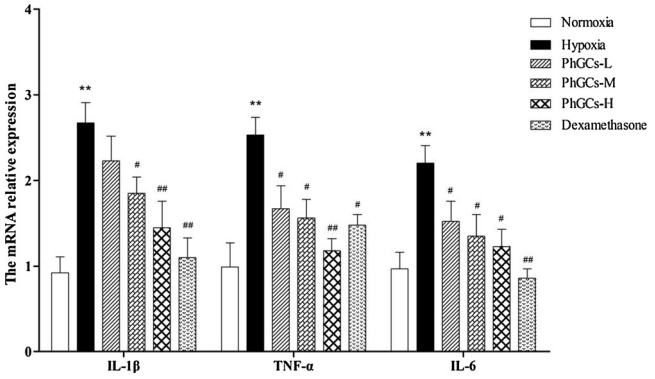

Fig. 5. PhGCs (50, 100, and 200 mg/kg) mitigated the mRNAs expressions of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) in AHH-stimulated HACE rats. After extraction from the brain tissues, equal volume of cDNA was used to perform the qRT-PCR assay to measure the relative transcripts of IL-1β, TNF-α, and IL-6. Data are expressed as the mean ± SD, $n = 10$ rats. Compared with normoxia group: $^{**}P < 0.01$; Compared with hypoxia group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

induced inflammatory responses in microglial cells via the NF-κB pathways (Fan et al., 2017). Similar to these findings, our studies establish that PhGCs triggered significant suppression of the relative expression of the NF-κB protein. Since NF-κB activation could highly induce pro-inflammatory factors by enhancing their transcription, and our study demonstrated that PhGCs could inhibit the production of pro-inflammatory mediators and NF-κB signaling pathways, we are inclined to suggest that the NF-κB signaling pathway might well be involved in the regulation of the production of pro-inflammatory mediators through PhGCs. Furthermore, PhGCs decreased the IL-1β and TNF-α protein expressions in acute hypoxia-stimulated HACE rats, indicating that PhGCs possess the ability to attenuate NF-κB activation and lead to the inhibition of pro-inflammatory cytokines' (IL-1β and TNF-α) production. Supposedly, the activation of the NF-κB pathway is critical to ameliorating acute hypobaric hypoxia-evoked brain edema by blocking oxidative stress and inflammatory response. However, in the present study, blocking the NF-κB pathway did not completely counteract the protective effects of PhGCs on acute hypobaric hypoxia-induced brain edema, oxidative stress, and inflammatory response. Whether other pathways are also involved in the above process or not, is lingering doubt. This question and others will be investigated and presented in our next report.

In summary, our results suggest that PhGCs have the ability to exert anti-inflammatory properties on acute hypobaric hypoxia-stimulated HACE in rats by inhibiting the expression of pro-inflammatory cytokines regulated by the NF-κB signaling pathways and rehabilitating the oxidative stress levels. PhGCs could, therefore, be a potential choice for the future treatment of HACE. Further studies are required to determine the detailed mechanisms for the protective effects of PhGCs on HACE.

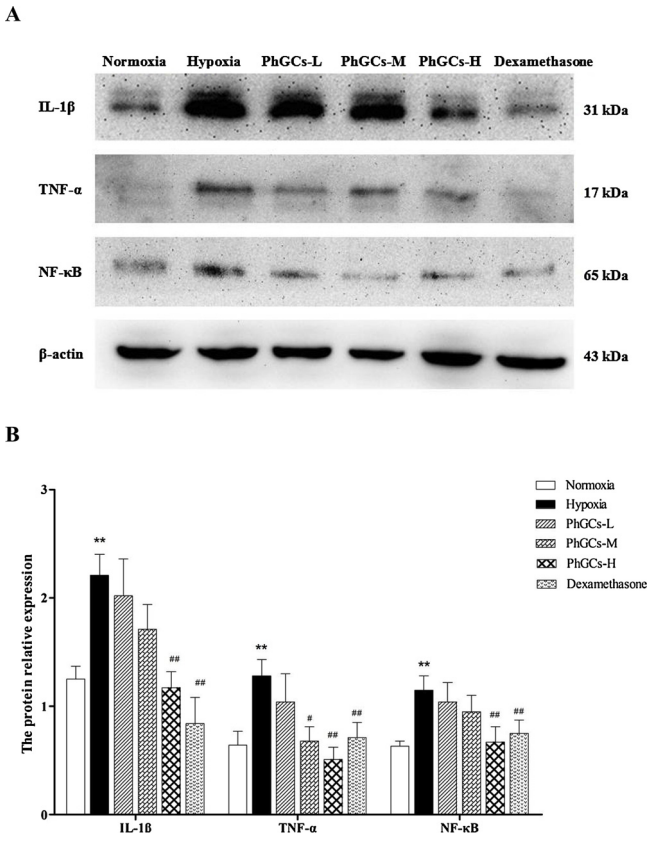

Fig. 6. PhGCs (50, 100, and 200 mg/kg) inhibited the activation of NF-κB signaling pathway in AHH-stimulated HACE rats. (A) Western blotting bands of IL-1β, TNF-α, NF-κB, and β-actin. (B) The corresponding quantification of the Western blot data by densitometric analysis and normalization to β-actin. Data are expressed as the mean ± SD, $n = 4$ rats. Compared with normoxia group: $^{**}P < 0.01$; Compared with hypoxia group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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