

Huangqi Baihe Granules Attenuate Hypobaric Hypoxia-Induced Brain Injury via HIF-1 α /p53/Caspase-3 Pathway

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Background: High altitude cerebral edema (HACE), a severe central nervous system dysfunction caused by acute plateau hypoxia, involves oxidative stress and apoptosis. Huangqi Baihe granules (HQBHG) show efficacy against these processes, but their mechanism remains unclear.

Purpose: This study evaluated Huangqi Baihe granules (HQBHG)'s efficacy in treating High altitude cerebral edema (HACE) and elucidated its mechanism.

Methods: UPLC-MS/MS characterized Huangqi Baihe granules (HQBHG)'s chemical composition. Seventy-two SD rats were divided into six groups: No-treatment Control (NC), Hypobaric Hypoxia Model (HHM), positive drug Dexamethasone (Dex, 5 mg/kg), and HQBHG low/medium/high-dose groups (1.105 g/kg d, 2.21 g/kg d, 4.42 g/kg d). Except NC, all underwent 72-hour 6000 m hypobaric hypoxia to establish High altitude cerebral edema (HACE). Brain barrier permeability (wet-dry ratio, Evans Blue staining), oxidative stress markers (Reactive oxygen species, Superoxide dismutase), and histopathology (HE/Nissl staining) were assessed. Network pharmacology (TCMSP, GenGards, OMIM, Drugbank) and transcriptomics identified Huangqi Baihe granules (HQBHG) targets and pathways. Apoptosis signaling (HIF-1 α /p53/Caspase-3) was validated via immunofluorescence, TUNEL, Transmission Electron Microscope, Western Blotting, and qRT-PCR.

Results: Hypobaric hypoxia caused brain injury and blood-brain barrier disruption. Network and transcriptome analyses linked Huangqi Baihe granules (HQBHG)'s effects to HIF-1 α /p53/Caspase-3 pathway, involving key genes. Huangqi Baihe granules (HQBHG) intervention attenuated brain injury, oxidative stress, and apoptosis, suppressing HIF-1 α /p53/Caspase-3 pathway activation.

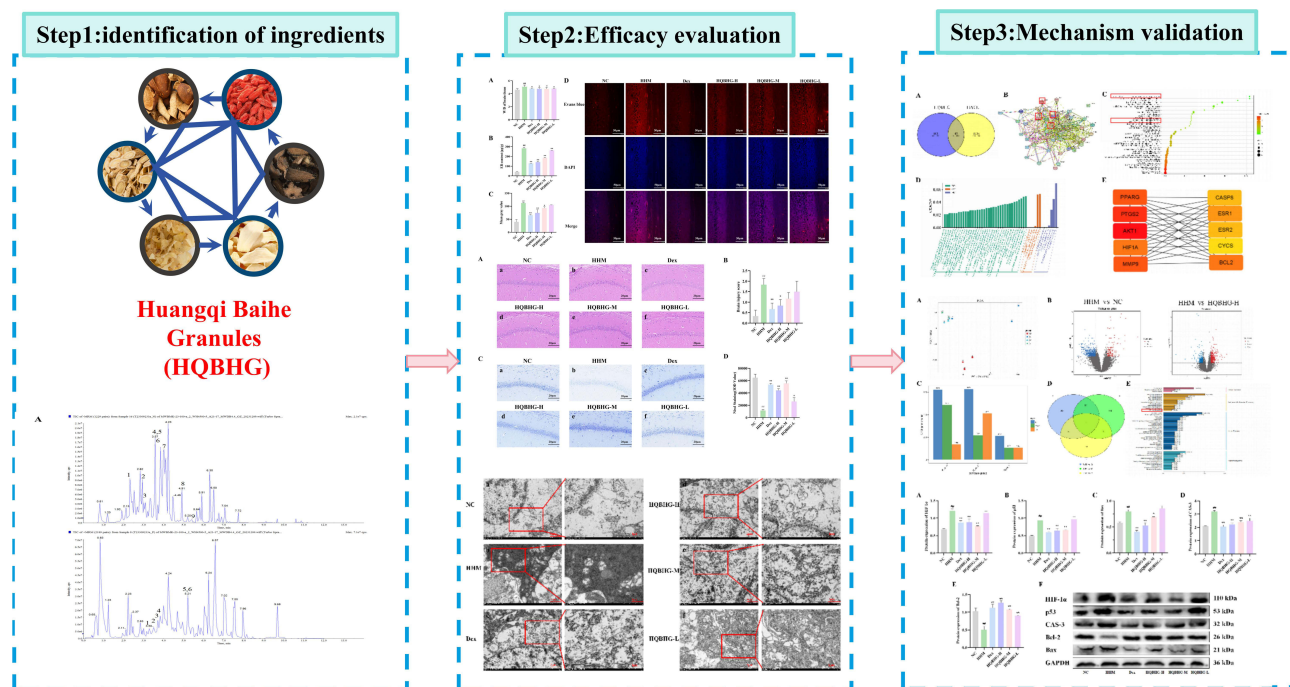
Conclusion: We demonstrated for the first time that Huangqi Baihe granules (HQBHG) may reduce brain tissue injury by regulating the HIF-1 α /p53/Caspase-3 signaling pathway, ameliorating blood-brain barrier disruption induced by low-pressure hypoxia, imbalance of oxidative stress in the brain tissues, and inhibiting apoptosis in the brain cells.

Keywords: Huangqi Baihe Granule, hypobaric hypoxia, high altitude cerebral edema, HIF-1 α /p53/caspase-3, apoptosis

Introduction

High altitude hypoxic cerebral edema (HACE) is a severe central nervous system dysfunction caused by acute hypoxia, which has been widely noted for its high lethality.¹ The clinical features of HACE include symptoms such as impaired consciousness, ataxia, and increased intracranial pressure, and in severe cases, it can progress to coma or even death within 24 hours.² Approximately 40 million highland travelers are at risk of HHBI each year, and more than 80 million permanent residents worldwide live in hypoxic environments at altitudes above 2500 meters, making the condition a major public health problem

Graphical Abstract



in the field of highland medicine.³ Because of the difficulty of early diagnosis and the short therapeutic window for HACE, the development of effective strategies to prevent HACE has become an urgent issue requiring immediate attention.

Although the underlying pathophysiological mechanisms of HACE have not been clearly elucidated, several evidences suggest that its pathogenesis focuses on blood-brain barrier damage,⁴ mitochondrial dysfunction,⁵ neuronal apoptosis,⁶ oxidative stress,⁷ and inflammatory response.⁸ When aerobic capacity is limited due to high altitude, organelles such as mitochondria become targets of hypoxia, leading to a dramatic increase in the production of oxygen free radicals. Hippocampal neuronal damage during oxidative stress is mainly due to the accumulation of ROS, apoptosis of brain cells, leading to cellular bioenergetic failure, inflammatory response, blood-brain barrier (BBB) damage triggered by disruption of cerebral vascular endothelial cell tight junctions, which induces vasogenic edema, resulting in damage to the morphology and structure of brain tissue. Meanwhile, hypoxia is a core feature of HACE, and abnormal activation of HIF-1 α promotes downstream expression of apoptotic genes, which are involved in the occurrence and development of HACE.

Currently, drug therapy is the primary approach for treating High-Altitude Cerebral Edema (HACE), with medications such as nifedipine, dexamethasone, and acetazolamide being commonly used. However, these treatments still lack ideal efficacy and may cause significant side effects. In Traditional Chinese Medicine (TCM), oxygen free radicals are categorized under “phlegm-dampness.” The thin air in high-altitude areas easily induces Qi deficiency, which impairs the propulsion force and leads to free radical accumulation. This results in phlegm-dampness and blood stasis, defining the pathogenesis of HACE as Qi deficiency, phlegm obstruction, and blood stasis. Huangqi Baihe Granule (HQBHG), derived from the folk remedy “Huangqi Baihe Porridge” and the Dunhuang medical formula “Huangqi Congrong Decoction”, alleviates HACE by tonifying Qi, nourishing Yin, drying dampness, and resolving phlegm. Previous studies demonstrated that HQBHG: Prevents high-altitude hypoxic lung injury by inhibiting the TLR4/NF- κ B p65/NLRP3 signaling pathway, exerting antioxidant and anti-inflammatory effects;⁹ Mitigates acute brain injury by regulating pyroptosis and suppressing inflammation through the HIF-1 α /NF- κ B/NLRP3 signaling pathway^{10,11}; Protects against oxidative stress damage in brain tissues of hypoxic mice;¹¹ Exerts protective effects on brain injury in normobaric hypoxia model mice via the HIF-1 α -VEGF signaling pathway¹². Given its promising pharmacological value in alleviating high-altitude

hypoxia and brain injury, this study aims to investigate whether HQBHG participates in preventing and treating HACE by modulating the HIF-1 α /p53/Caspase-3 signaling pathway.

Materials and Methods

Materials and Reagents

Dexamethasone Acetate Tablets were acquired from Changle Pharmaceutical Co., Ltd. (Henan, China). Evans Blue powder, gel preparation kit, 4% paraformaldehyde, SOD, MDA, and GSH kit were acquired from Solarbio, (Beijing, China). Tunel Reagent Kit were acquired from Servicebio (Wuhan, China). HIF-1 α , Bax, Bcl-2, HRP Goat Anti-Rabbit Antibody were acquired from Immunoway (USA), p53, Ngb, Caspase-3 were acquired from Beyotime, (Shanghai, China), GAPDH Antibody were acquired from GeneTex (USA), PVDF membrane were acquired from Millipore (USA). Reverse transcription and qPCR kits were acquired from YEASEN (Shanghai, China).

Preparation of HQBHG

The ingredients of the HQBHG compound are as follows: *Hedysarum multijugum Maxim* 9g, *Lilium brownii var. viridulum Baker* 9g, *Cistanche phelypaea (L.) Coult* 6g, *Polygonatum kingianum Collett & Hemsl* 9g, *Citrus reticulata Blanco* 3g, *Lycium chinense f. megistocarpa (Dunal) A. Terracc* 6g, 6 herbs. (All plant names from www.worldfloraonline.org), purchased from the Affiliated Hospital of Gansu University of Traditional Chinese Medicine (Gansu, China). After identification by the Chinese Medicine Pharmacology Laboratory of the School of Pharmacy, Gansu University of Traditional Chinese Medicine ([Table Supplement 1](#)), the decoction was boiled once, and the decoction was taken to be super-considered, concentrated under reduced pressure, dried under vacuum, crushed, and sieved, added with excipients, and granulated by dry method, and 0.585 g per gram of the original compound preparation was extracted. The prepared granules were stored at -80°C for spare use. The chemical composition of HQBHG was qualitatively analyzed by UPLC-MS/MS. The extraction rate of HQBHG was 0.585 and the final amount of the drug was $42\text{ g} \times 0.585 = 24.57\text{ g}$. Equivalent doses were converted according to the body mass of human and rat to give the amount of drug administered in the medium dose group: $24.57\text{ g}/70(\text{kg d}) \times 6.3 \approx 2.21\text{ g}/(\text{kg d})$. It was prepared in distilled water before gavage at 1.105 g/(kg d), 2.21 g/(kg d), 4.42 g/(kg d) for the low, medium, and high dose groups, respectively, and placed at 4°C for cold storage.

Identification of Active Ingredients of HQBHG

The bioactive components of HQBHG were analyzed using SHIMADZU Nexera X2 ultra-performance liquid chromatography and AB Sciex 6500 QTRAP Triple quadrupole linear ion trap mass spectrometer method.

Establishment of the HACE Rat Model and Group Administration of Drugs

72 SPF-grade male SD rats, weighing 110–120 g, age: 5–6 weeks, were purchased from SPF (Beijing) Biotechnology Co. Ltd (Beijing, China) under the license No. SCXK(Beijing)2019–0010. All experimental procedures were approved by the Ethics Committee of Gansu University of Traditional Chinese Medicine, China, Certificate of Ethical Review of Animal Testing No. SY2023-613. Rats were housed in the SPF-grade animal laboratory of the Animal Experiment Center of Gansu University of Traditional Chinese Medicine at a temperature ($22 \pm 2^{\circ}\text{C}$) and relative humidity ($55 \pm 10\%$), with 12/12h alternating light and dark environments for free movement, regular diet, and water intake.

After 7 d of acclimatization, the rats were divided into 6 groups of 12 rats each using the random number method: NC group: 0.9% saline (5 mL/kg) was given by gavage, 1 mL/d, for 14 d before entry; HHM group: 0.9% saline (5 mL/kg) was given by gavage, 1 mL/d, for 14 d before entry; Dex group: 0.9% saline (5 mL/kg) was given by gavage, 1 mL/d, for 14 d before entry; and 5 mg/kg HQBHG-L, HQBHG-M, and HQBHG-G groups: 1.105 g/kg, 2.21 g/kg, and 4.42 g/kg doses of HQBHG were given by gavage, 2 mL/d, and 14 d before entry, respectively.

At the end of drug administration, rats in all groups except the NC group were placed in a low-pressure hypoxic animal chamber at a simulated altitude of 6000 m (pressure $33.64\text{ kPa} \pm 0.5\text{ kPa}$; oxygen content $10.5\% \pm 0.5\%$) for 72 h of continuous low-pressure hypoxic exposure (ascending at a speed of 5 m/s to 3000 m for 10 min; ascending at the same speed to 4500 m for 10 min); The rats in the NC group were kept in a normobaric and normoxic environment. At the end

of the exposure, all rats were anesthetized with 3% sodium pentobarbital, blood was collected from the abdominal aorta, and brain tissues were extracted after decapitation.

Brain W/D Measurement

The brainstem and cerebellum of the brain tissue of each group of rats were separated, and the blood was removed leaving the left half of the brain, the wet weight value of the left half of the brain was weighed with an analytical balance and recorded, and then the left half of the brain was placed in a constant temperature oven at 80°C for 72 h continuously to make the weight of the brain tissue in a constant state (with an average error of less than 0.002 g), and the dry weight was recorded, and then the ratio was calculated based on the calculation formula ($W/D = \text{wet weight}/\text{dry weight}$).

Evans Blue Stain Evaluates BBB Changes

4 mg of EB dye was dissolved in saline, and the volume was fixed to 25 mL. It was diluted into 8 concentration gradients of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.5625 µg/mL and 0.78125 µg/mL. The above EB solutions were placed in a water bath at 60 °C for 24 h, and then the OD values at 620 nm were measured and the standard curves were plotted according to the data.

The rats were injected intraperitoneally with 2% EB solution (2 mL/kg) 24h before the experiment, and the exposed part of the skin of the rats turned blue, which indicated that the injection was successful. 100 mg of the left hemisphere of the rats was taken from the rats after 24 h, and the left hemisphere of the rats was cut and put into 3 mL of 2% formamide solution for 24 h of incubation at a constant temperature of 60 °C, and then centrifuged for 15 min at 5000 r/min and the supernatant was extracted. The OD value of the supernatant was measured at 620 nm. The EB content in the brain tissue of rats in each group was calculated according to the standard curve. The right half of the brain was frozen sectioned after fixation, then placed under a laser confocal microscope to observe the change in fluorescence intensity.

Hematoxylin and Eosin and Nissl Staining

Brain tissues were fixed in 4% neutral paraformaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin and eosin stain or 1% tar violet. Pathologic damage assessment was performed¹³ after observation under a light microscope.

Kit to Detect Oxidative Stress Indicators Such as SOD, MDA, and GSH

Preserved serum/plasma or brain tissue was pretreated and assayed according to the steps of each kit instruction.

Network Pharmacology

The TCMSP (<https://old.tcm-sp-e.com/tcm-sp.php>) database was used to retrieve the main chemical constituents of the herbs Astragalus (HQ) Lily (BH), Goji Berry (GQ), Cistanchia (RCR), Rhizoma Polygonati (HJ) and Pericarpium Citri Reticulate (CP) in the HQBHG and to screen for their corresponding target genes (oral bioavailability (OB) $\geq 30\%$ and druglikeness (DL) ≥ 0.18 , and blood-brain-barrier permeability $BBB > 0.3$). The Universal Protein Resource (UniProt) database (<https://www.uniprot.org/L>) was used to obtain UniProt IDs and gene names for targets and to screen for human-related genes. Three databases, GenCards (<https://www.genecards.org/>), OMIM (<https://omim.org/>), and DrugBank (<https://go.drugbank.com/>), were searched for “High Altitude Cerebral Edema” to identify candidate targets. The HACE targets were plotted against the drug action targets to obtain the intersecting targets and Venny plots were drawn. The intersecting targets were imported into the String database (<https://cn.string-db.org/>) for PPI protein network interactions analysis with “Homo sapiens” and a high confidence score (0.7), and then the results were visualized by Cytoscape 3.9.0. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the intersection targets were performed using the DAVID database (<http://david.abcc.ncifcrf.gov/>), and histograms of GO information and bubble plots of KEGG information were plotted on Wei Sheng Xin (www.bioinformatics.com.cn).

Transcriptomics Sequencing Analysis

For transcriptomics analysis, Fold Change ≥ 2 and FDR < 0.01 were used as screening criteria during differential expression gene detection. Total RNA from rat brain tissue was extracted by the Trizol method and tested for concentration,

purity, and integrity. Eukaryotic mRNA was enriched with magnetic beads with Oligo (dT); mRNA was randomly interrupted by adding Fragmentation Buffer; the first cDNA strand and the second strand were synthesized using mRNA as a template and cDNA purification was performed; purified double-stranded cDNA was then subjected to end repair, addition of A-tail, and ligation of sequencing junctions, and then fragment size selection was performed using AMPure XP beads for fragment size selection; finally, the cDNA library was enriched by PCR. QC of the library was performed by Q-PCR. After the library QC was qualified, PE150 mode sequencing was performed using the Illumina NovaSeq6000 sequencing platform. After the sequencing data were downloaded, the data were analyzed using the bioinformatics analysis process provided by the BMKCloud platform BMKCloud (www.biocloud.net).

Detection of Ngb Expression in Brain Tissue by Immunohistofluorescence

The prepared brain tissue sections were given Triton X-100 and stored at 37 °C for 20 min. 10% goat serum blocking solution was added and the primary antibody Ngb (1:200) was added dropwise. The samples were then stored in a refrigerator at 4 °C overnight, fluorescence-coupled secondary antibody was added in the dark, and protein expression in the cells was observed under a fluorescence microscope using an anti-fluorescence burst sealant containing DAPI.

TUNEL Staining for Apoptosis in Brain Tissues

The brain tissue sections were deparaffinized and stained according to the kit procedure. Stored in a moist dark box and observed under a laser confocal microscope.

Transmission Electron Microscopic Observation of Ultrastructural Changes in Brain Tissue

About 1 mm³ volume of fresh brain tissue was cut and immersed in 3% glutaraldehyde for 12 h. After the steps of PBS washing, osmium fixation, acetone dehydration, epoxy resin embedding, sectioning, and staining with uranyl diacetate and lead citrate, the sections were observed by transmission electron microscopy.

Detection of mRNA Expression of Related Genes in Brain Tissue by qRT-PCR

The total RNA of rat brain tissue was extracted according to the Trizol method, and the CT value was obtained by gDNA removal, reverse transcription, Real-Time PCR, etc. The relative expression of mRNA of the target gene was calculated according to the $2^{-\Delta\Delta Ct}$ method. PCR primers were provided by Beijing Qingke Biotechnology Co. The primer sequences are shown (Table 1).

Western Blot Detection of Protein Expression of HIF-1 α , p53, Caspase-3, Bax and Bcl-2

Proteins were extracted by adding 1 mL of lysate containing 1% PMSF per 100 mg of brain tissue and quantified by the BCA method. The proteins were separated by 10% SDS-PAGE and transferred to the PVDF membrane, 5% skimmed milk powder was enclosed for 2 h and then incubated with specific antibody conjugation overnight at 4 °C. The proteins were observed and the gray value of the protein bands was recorded using ECL ultrasensitive luminescent liquid placed in the dark box of the exposure machine for exposure.

Table 1 Primer Sequences for PCR

Genes	Forword Sequence (5'-3')	Reverse Sequence (3'-5')	bp
<i>HIF-1α</i>	CTGTGTCAGAGTCCTTGGAG	GTAGTAGTAGCAGTGGCTGTGG	154
<i>P53</i>	AGCGACTACAGTTAGGGGGT	ACAGTTATCCAGTCTTCAGGGG	89
<i>CAS-3</i>	GCTTGAACGCGAAGAAA	CCATTGCGAGCTGACATTCC	222
<i>Bax</i>	GTCTGCGGGGAGTCACG	TTCTTGGTGGATGCGTC	245
<i>Bcl-2</i>	ATGTGTGTGGAGAGCGTCAA	GATGCCGGTTCAGGTACTION	85
<i>Bad</i>	GGCTTGAGGAAGTCCGATCC	TCACTCGGCTCAACTIONCTGG	113
<i>GAPDH</i>	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT	92

Statistical Analysis

SPSS 26.0 was used for statistical analysis, GraphPad Prism 8.0 was used for graphing, and the results of the experiments were expressed as mean±standard deviation.

Multiple groups meeting the assumption of normality were analyzed using one-way ANOVA, followed by Tukey's HSD test for pairwise comparisons or Dunnett's test when comparing multiple groups to a control. Two-group comparisons were made using an independent sample *t*-test with results considered to be significantly different at $P < 0.05$.

Results

Active Ingredients of HQBHG

The negative and positive ion chromatograms of the 15 major chemical components of HQBHG are shown (Figure 1A). A total of 15 compounds were identified, including related terpenoids, phenolic acids, flavonoids, and alkaloids (Table 2). Among them, Chlorogenic acid methyl ester, Luteolin-7,3'-di-O-glucoside derived from luteolin, Astragaloside VI

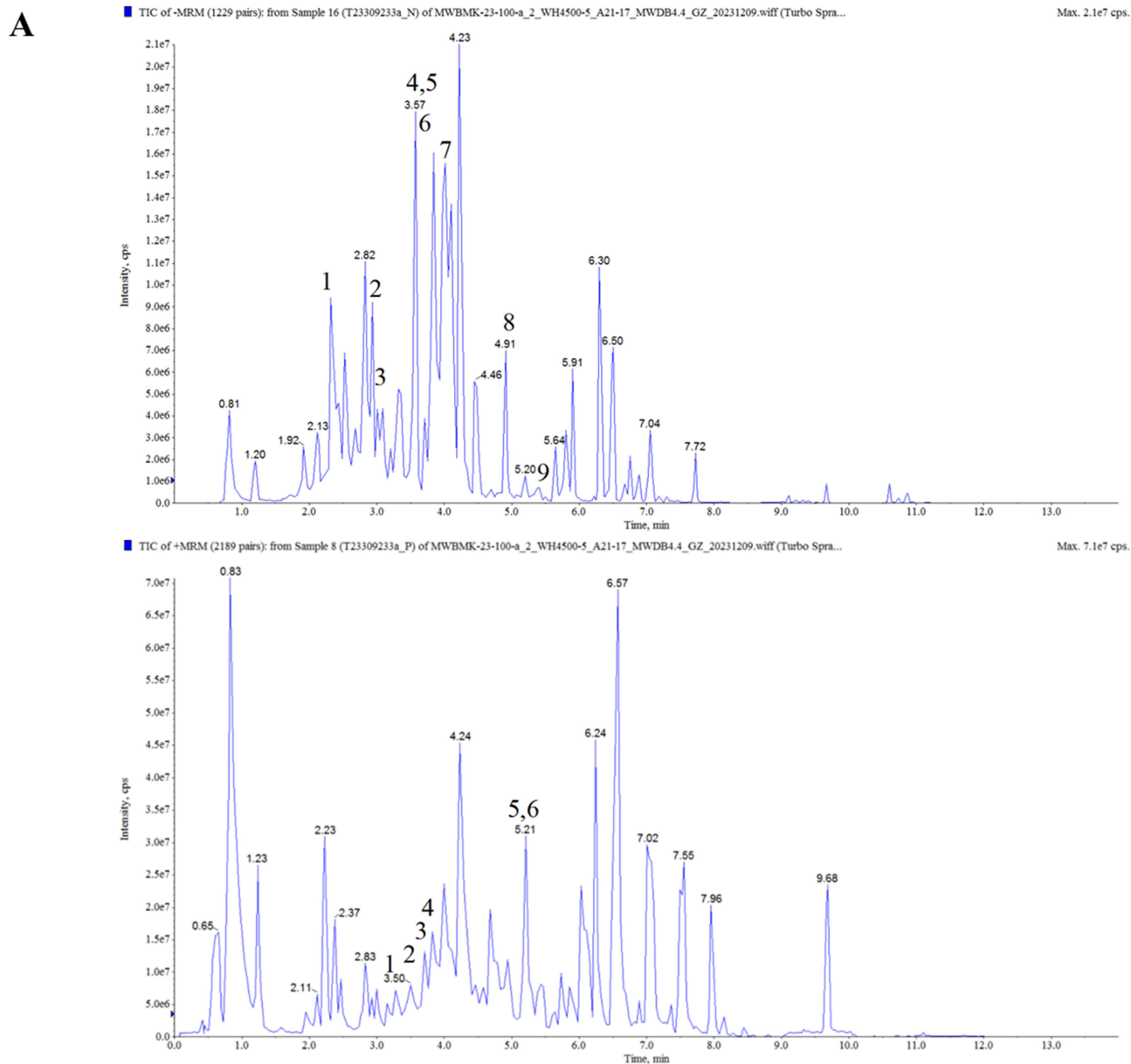


Figure 1 Functional ingredient analysis of HQBHG. (A) Total ion recent plots of HQBHG extract in negative and positive ion modes.

Table 2 The 15 Chemical Constituents of HQBHG

No.	Name	Formula	Measured value (Positive)	Theoretical value (Positive)	Measured value (Negative)	Theoretical value (Negative)	Remark
1	Bartsioside	C ₁₅ H ₂₂ O ₈	331.14	330.1315			[M+H] ⁺
2	5-Acetylsalicylic acid	C ₉ H ₈ O ₄	181.05	180.0423			[M+H] ⁺
3	Luteolin-7,3'-di-O-glucoside	C ₂₇ H ₃₀ O ₁₆	611.1607	610.1534			[M+H] ⁺
4	Quercetin-3-O-glucoside (Isoquercitrin)*	C ₂₁ H ₂₀ O ₁₂	465.1028	464.0955			[M+H] ⁺
5	Astragaloside VI	C ₄₇ H ₇₈ O ₁₉	947.521	946.5137			[M+H] ⁺
6	Pratensein-7-O-glucoside	C ₂₂ H ₂₂ O ₁₁	463.12	462.1162			[M+H] ⁺
1	6-DeoxyCatalpol	C ₁₅ H ₂₂ O ₉			345.12	346.1264	[M-H] ⁻
2	5-Hydroxyindole-3-acetic acid	C ₁₀ H ₉ NO ₃			190.051	191.0582	[M-H] ⁻
3	Coronarian	C ₁₂ H ₁₈ N ₂ O ₁₂			381.08	382.086	[M-H] ⁻
4	6-Hydroxyluteolin 5-glucoside	C ₂₁ H ₂₀ O ₁₂			463.09	464.0955	[M-H] ⁻
5	Plantamajoside	C ₂₉ H ₃₆ O ₁₆			639.193	640.2003	[M-H] ⁻
6	Chlorogenic acid methyl ester	C ₁₇ H ₂₀ O ₉			367.1	368.1107	[M-H] ⁻
7	Astraflavonoid C	C ₂₃ H ₂₈ O ₁₁			479.16	480.1637	[M-H] ⁻
8	Trifolirhizin (Maackiain-3-O-glucoside)	C ₂₂ H ₂₂ O ₁₀			445.11	446.1213	[M-H] ⁻
9	6'''-O-AcetylAmurensin	C ₂₈ H ₃₀ O ₁₂			557.16	558.1737	[M-H] ⁻

derived from *Astragalus membranaceus*, and 6-DeoxyCatalpol derived from *Cistanchis sinensis* have anti-inflammatory, antioxidant, and neuroprotective effects.

HQBHG Improves W/D and BBB in HACE Rats

W/D is the most intuitive index to observe and assess brain edema. As shown (Figure 2A), the wet-to-dry ratio of rat brain tissue in the HHM group was significantly increased compared with the NC group. The wet-to-dry ratio of the brain tissue of rats in the Dex group and the HQBHG-L, HQBHG-M, and HQBHG-G groups decreased significantly compared with the HHM group. The intervention of HQBHG successfully reduced brain edema in HACE rats.

The alteration of BBB is an important factor in the occurrence of HACE. Compared with the NC group, the brain tissue barrier permeability of rats in the HHM group was decreased, Evans Blue dye penetration was increased, and fluorescence expression was enhanced. Compared with the HHM group, rats in the Dex group and the HQBHG-L, HQBHG-M, and HQBHG-G groups showed increased brain tissue barrier permeability, decreased Evans Blue dye penetration, and weakened fluorescence expression (Figure 2B–D). HQBHG effectively improved BBB in HACE rats.

HQBHG Attenuates Histopathological Brain Damage in HACE Rats

HE and Nissl staining were used to observe the pathological morphology and neuronal cell survival in the brains of HACE rats and to evaluate the effect of HQBHG on brain tissue injury caused by hypoxic exposure. HE staining showed that the cells in the CA1 area of the hippocampus of the rats in the NC group were arranged neatly and densely, and the nuclei of the cells were dark-stained and full with clear boundaries; compared with that of the NC group, the cells in the CA1 area of the hippocampus of the rats in the HHM group were edematous, and the arrangement was irregular and the cytoplasm was sparse and light-stained. Compared with the NC group, the cells in the CA1 area of the hippocampus of the HHM group were edematous and irregularly arranged, and the cytoplasm of the cells was loose and lightly colored. Compared with the HHM group, the cone cells in the CA1 area of the hippocampus of rats in the Dex group and the HQBHG-L, HQBHG-M, and HQBHG-G groups were tightly arranged, with clearer borders, and the cellular edema was improved (Figure 3A and B). Nissl staining showed that the number of cells in the CA1 area of the hippocampus of the

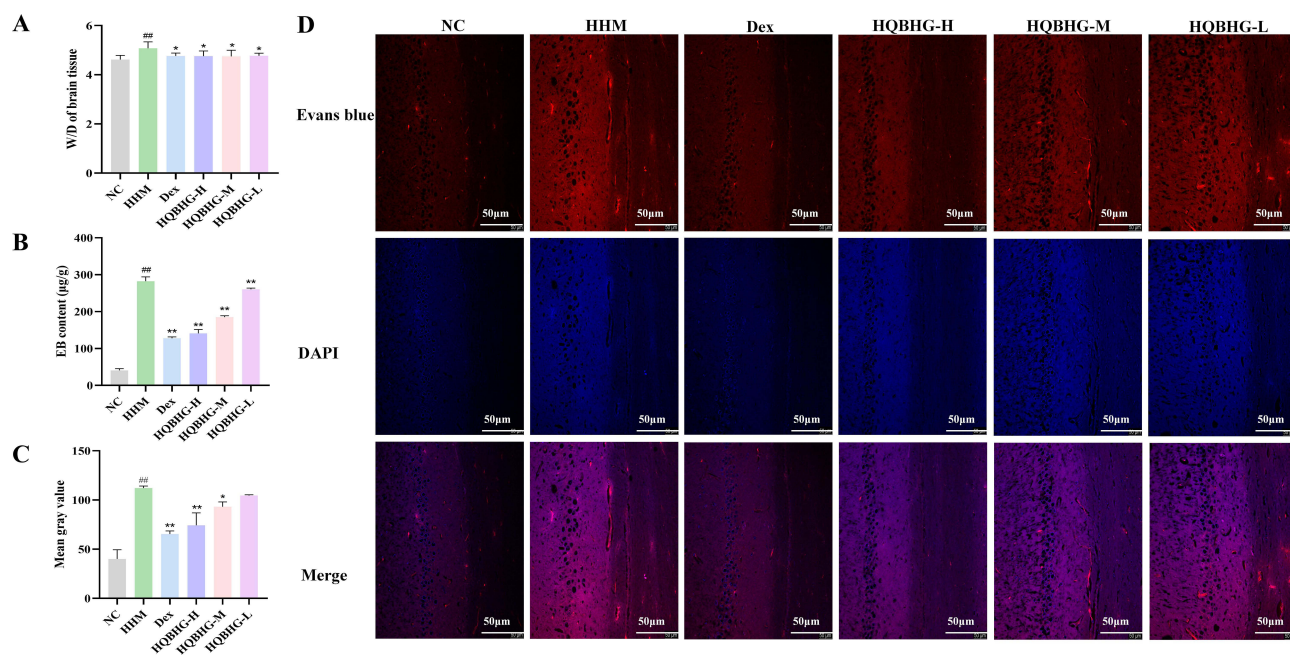


Figure 2 HQBHG ameliorates cerebral edema in plateau hypoxic rats. **(A)** Effect of HQBHG on brain tissue W/D ($n = 6$). **(B)** Effect of HQBHG on EB dye levels in brain tissue ($n = 6$). **(C and D)** Effect of HQBHG on fluorescent expression of EB dye in brain tissue ($n = 3$). (a) No-treatment Control group; (b) Hypobaric hypoxia model (HHM) group; (c) Dex (5 mg/kg) group; (d) HQBHG-H (4.42 g/kg) group; (e) HQBHG-M (2.21 g/kg) group; (f) HQBHG-L (1.105 g/kg) group. The mean standard deviation represents the data. ^{###} $P < 0.01$ compared to the control group. ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compared to the HHM group. (magnification 200 ×).

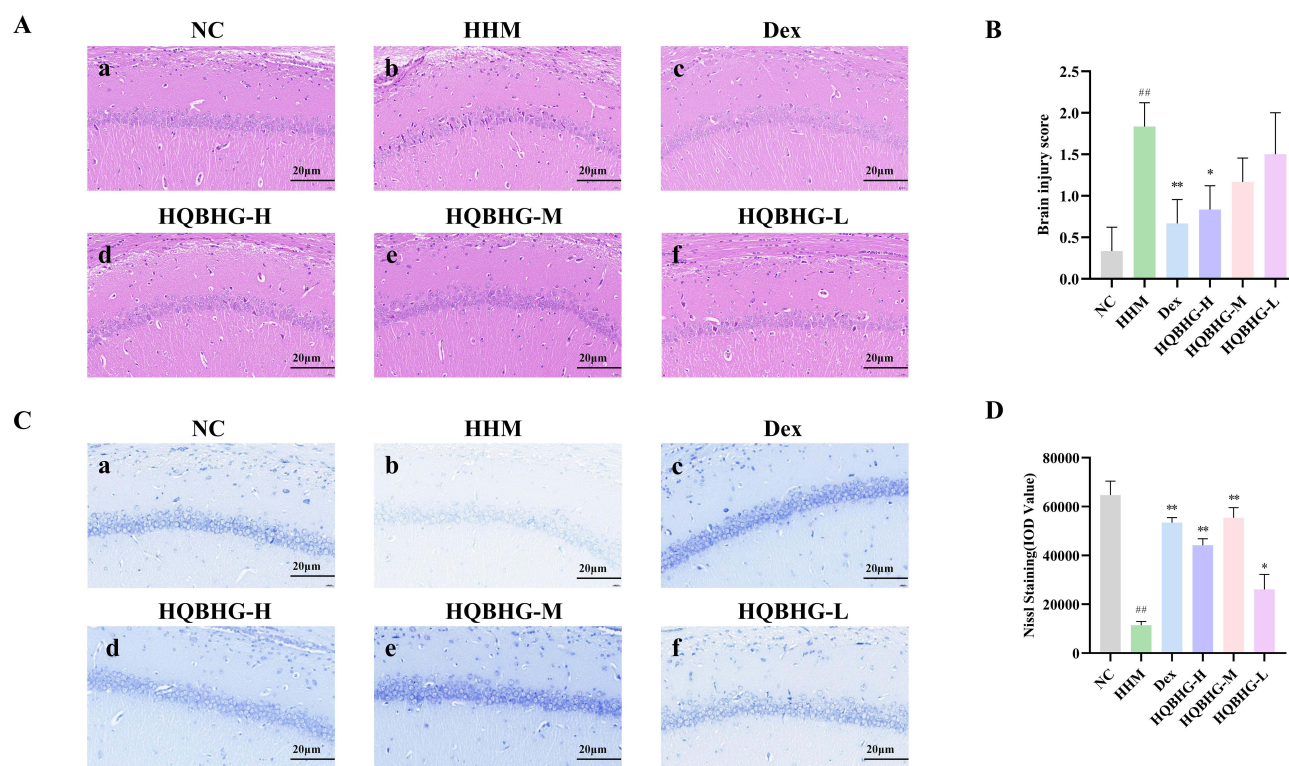


Figure 3 HQBHG ameliorates histopathologic brain damage in rats with HACE. (**A** and **B**) HE staining of the CA1 region of the cerebral hippocampus and brain injury pathology scores ($n = 3$). (**C** and **D**) Nissl staining and OD statistics in the CA1 region of the cerebral hippocampus ($n = 3$). (a) No-treatment Control group; (b) Hypobaric hypoxia model (HHM) group; (c) Dex (5 mg/kg) group; (d) HQBHG-H (4.42 g/kg) group; (e) HQBHG-M (2.21 g/kg) group; (f) HQBHG-L (1.105 g/kg) group. $###P < 0.01$ compared to the control group. $*P < 0.05$ and $**P < 0.01$ compared to the HHM group. (magnification 400 \times).

HHM group was significantly reduced, and the arrangement was disorganized with light staining of nissl vesicles, which indicated that the viability of the neurons was reduced significantly. The nissl vesicles in the CA1 area after HQBHG intervention were surrounded by increased, and neuronal cell viability was restored (Figure 3C and D). These results indicated that HQBHG could reduce the pathological damage of HACE by restoring neuronal viability and had a protective effect on the brain tissue of rats after HH exposure.

HQBHG Attenuates Oxidative Damage in HACE Rats

Since oxidative damage is an important hallmark of HACE, we investigated the effect of HQBHG on oxidative stress in HACE rats. The HHM group showed a decrease in the production of superoxide dismutase (SOD) and reduced glutathione (GSH), and an increase in the content of reactive oxygen species (ROS) and malondialdehyde (MDA) in the brain tissues of the rats after HH induction (Figure 4). The intervention of HQBHG effectively restored these oxidative products and antioxidant enzyme alterations in HACE rats, indicating that HQBHG has strong antioxidant effects.

HQBHG Reduces Neuronal Apoptosis in the CA1 Region of the Cerebral Hippocampus in HACE Rats

Various inflammatory or hypoxic injuries caused by low pressure and low oxygen are the most direct cause of neuronal cell apoptosis in the brain. In the CA1 area of the brain hippocampus, apoptosis of brain neuronal cells in the CA1 area of the hippocampus of rats in the HHM group was significantly increased compared with that in the NC group (Figure 5), and apoptosis of brain neuronal cells in the CA1 area of the hippocampus of rats treated with HQBHG was significantly reduced.

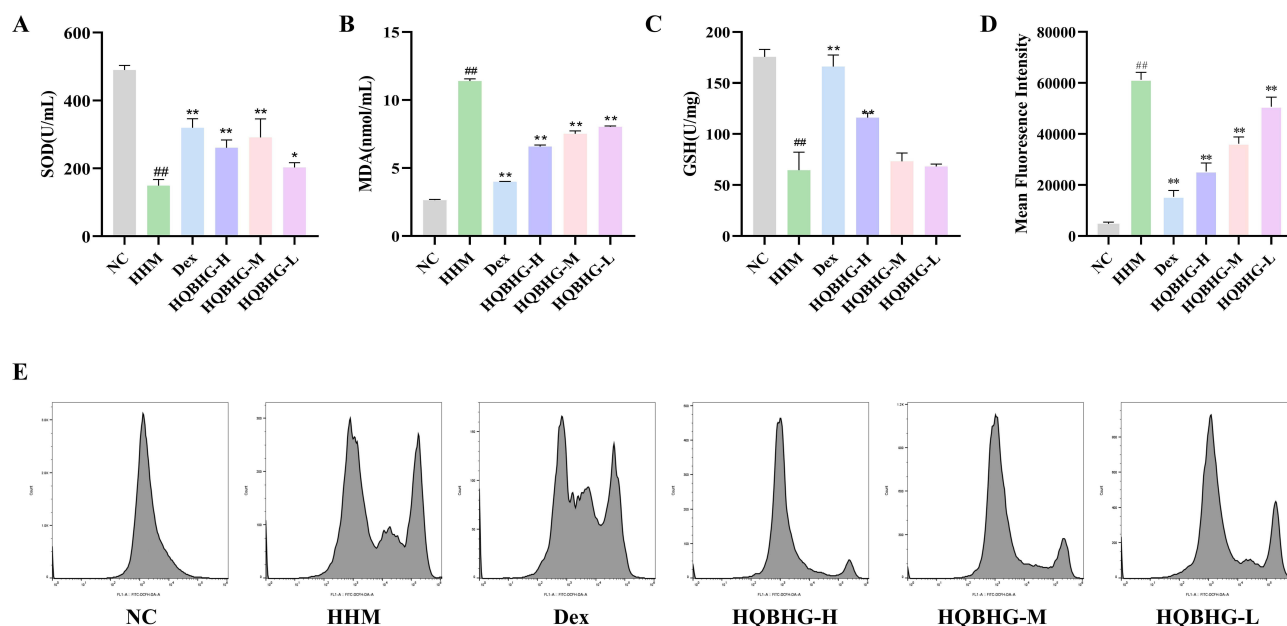


Figure 4 HQBHG improves oxidative stress in HACE rats. **(A)** Effect of HQBHG on SOD activity in serum ($n = 6$). **(B and C)** Effect of HQBHG on GSH and MDA contents in brain tissue ($n = 6$). **(D and E)** Effect of HQBHG on ROS contents in brain tissue ($n = 6$). (a) No-treatment Control group; (b) Hypobaric hypoxia model (HHM) group; (c) Dex (5 mg/kg) group; (d) HQBHG-H (4.42 g/kg) group; (e) HQBHG-M (2.21 g/kg) group; (f) HQBHG-L (1.105 g/kg) group. The mean standard deviation represents the data. $###P < 0.01$ compared to the control group. $*P < 0.05$ and $**P < 0.01$ compared to the HHM group.

HQBHG Improves Microstructure in Brain Tissue of HACE Rats

The accumulation of ROS caused by hypoxia mainly existed in the mitochondria, so the ultrastructural changes of neuronal cells in rat brain tissue were observed by transmission electron microscopy (Figure 6). The nuclear membranes of neuronal cells in the NC group were intact, the nuclei of the cells were normal in size, the morphology of the mitochondria was complete, and the cristae were clear and obvious, while the nuclear membranes of neuronal cells of the rat in the HHM group were crinkled, the nuclei of the cells were solidified and shrunken, the chromatin was accumulated, the mitochondria were edematous, and the cristae had broken off and disappeared. Compared with the HHM group, the nuclear consolidation and mitochondrial edema of rat neurons in the Dex group and the HQBHG-L, HQBHG-M, and HQBHG-G groups were improved to different degrees.

Network Pharmacology Target Prediction

Based on TCMSPP and databases, 163 HQBHG targets were identified, and 1,134 HACE-related disease targets were identified from three disease databases, with a total of 45 overlapping targets identified (Figure 7A).

Given the STRING database, PPI protein interaction analysis revealed that the core targets were HIF1A, TNF, AKT1, IL1B, PPARG, HOMX1, CYCS, CASP8, CXCL8, BCL2, and MMP9, respectively (Figure 7B).

KEGG predicted that the mechanism of HQBHG against HACE might be related to reactive oxygen species, herpes simplex virus type 1 infection, EBV infection, HTLV-1 infection, JAK-STAT signaling pathway, Alzheimer's disease, HIF-1 signaling pathway, and NF- κ B signaling pathway (Figure 7C).

The major BP (Biological Process) predicted by Gene Ontology is positive regulation of inflammatory response, response to ethanol, cellular response to insulin stimulus, etc., and the major MF (Molecular Function) was heme binding, protein homodimerization activity, etc. (Figure 7D).

The top 10 targets obtained by putting 45 overlapping targets through the Cytoscape MCC algorithm were AKT1, PTGS2, PPARG, MMP9, HIF1A, BCL2, ESR1, ESR2, CASP8, and CYCS (Figure 7E).

Combined with literature studies, we hypothesized that HQBHG may attenuate HACE by regulating reactive oxygen species, HIF-1 α , and apoptosis-related genes.

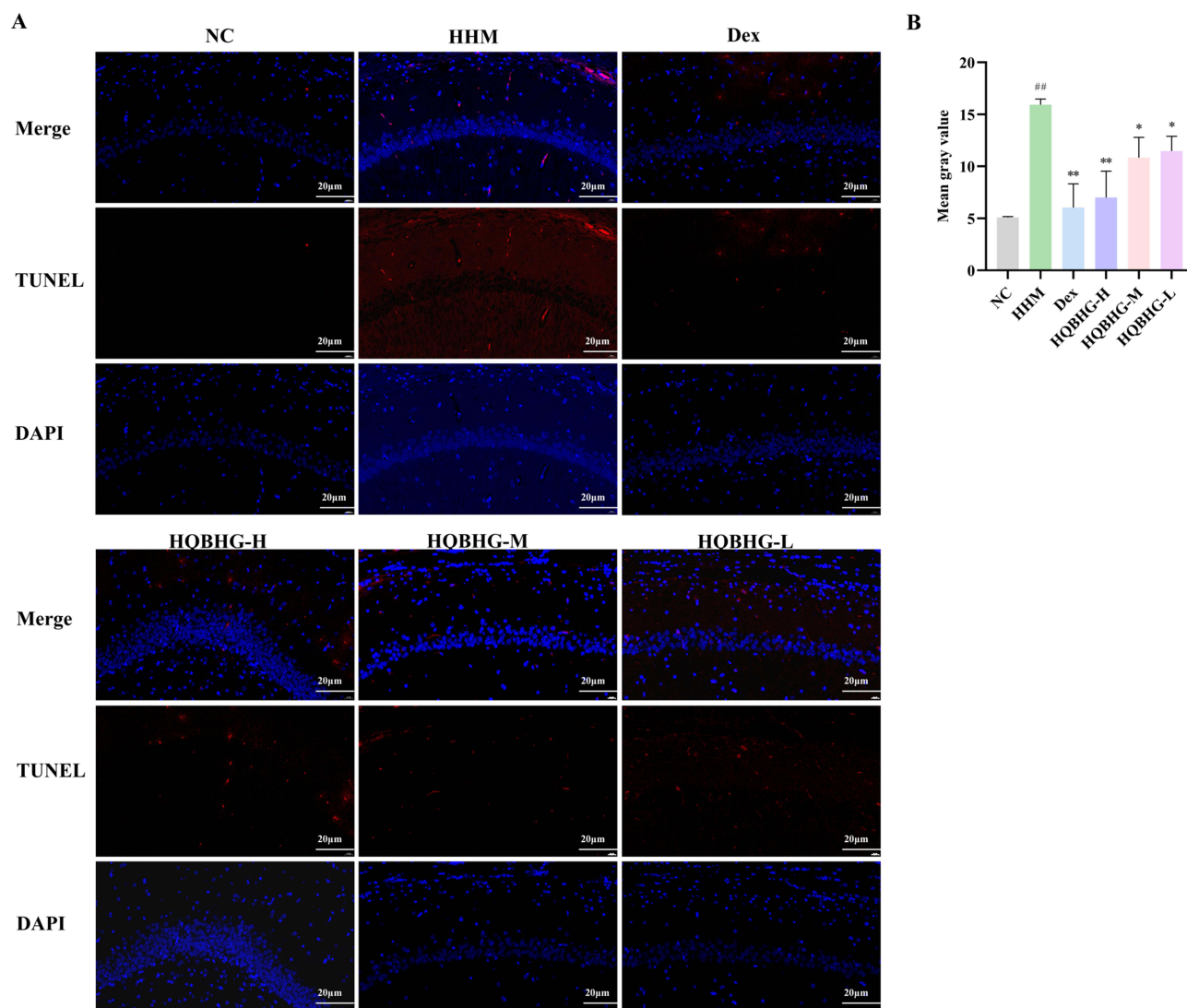


Figure 5 HOBHG attenuates apoptosis in the CA1 region of rat hippocampus. **(A)** TUNEL staining analysis of HOBHG's effect on apoptosis in rat hippocampal CA1 region cells (n=3). **(B)** Statistical diagram of TUNEL staining in the hippocampal CA1 region of rats across groups (n=3). No-treatment Control group (NC); Hypobaric hypoxia model (HHM) group; Dex (5 mg/kg) group; HOBHG-H (4.42 g/kg) group; HOBHG-M (2.21 g/kg) group; HOBHG-L (1.105 g/kg) group. The mean standard deviation represents the data. ## $P < 0.01$ compared to the control group. * $P < 0.05$ and ** $P < 0.01$ compared to the HHM group. (magnification 400 \times).

Results of Transcriptomic Analysis of HOBHG's Possible Role in HACE Rats

DESeq2 identified genes with an adjusted P -value < 0.01 and Fold Change ≥ 2 as differentially expressed. There were significant differences between the principal components of the NC, HHM, Dex, and HOBHG-H groups (Figure 8A). Compared with the NC group, a total of 623 differential gene expressions were found in the model group, of which 136 were up-regulated and 487 were down-regulated, while a total of 628 differential gene expressions were found in the HOBHG-H, of which 411 were up-regulated and 217 were down-regulated (Figure 8B and C). The Venn diagram showed that 218 genes were associated with HOBHG potentially acting on HACE (Figure 8D). To identify potentially affected HOBHG pathways, by including DEGs in KEGG enrichment studies of differentially expressed gene pathways, we were able to derive the following key signaling pathways. We selected the top 20 most important pathways for mapping (Figure 8E). We found that "PI3K/AKT signaling pathway", "Ras signaling pathway", "MAPK signaling pathway", "Rap-1 signaling pathway", "Wnt signaling pathway", "calcium signaling pathway", "phospholipase D signaling pathway", "cGMP-PKG signaling pathway" and "HIF-1 signaling pathway" were significantly enriched. These results suggest that HOBHG may have an attenuating effect on HACE by affecting these pathways.

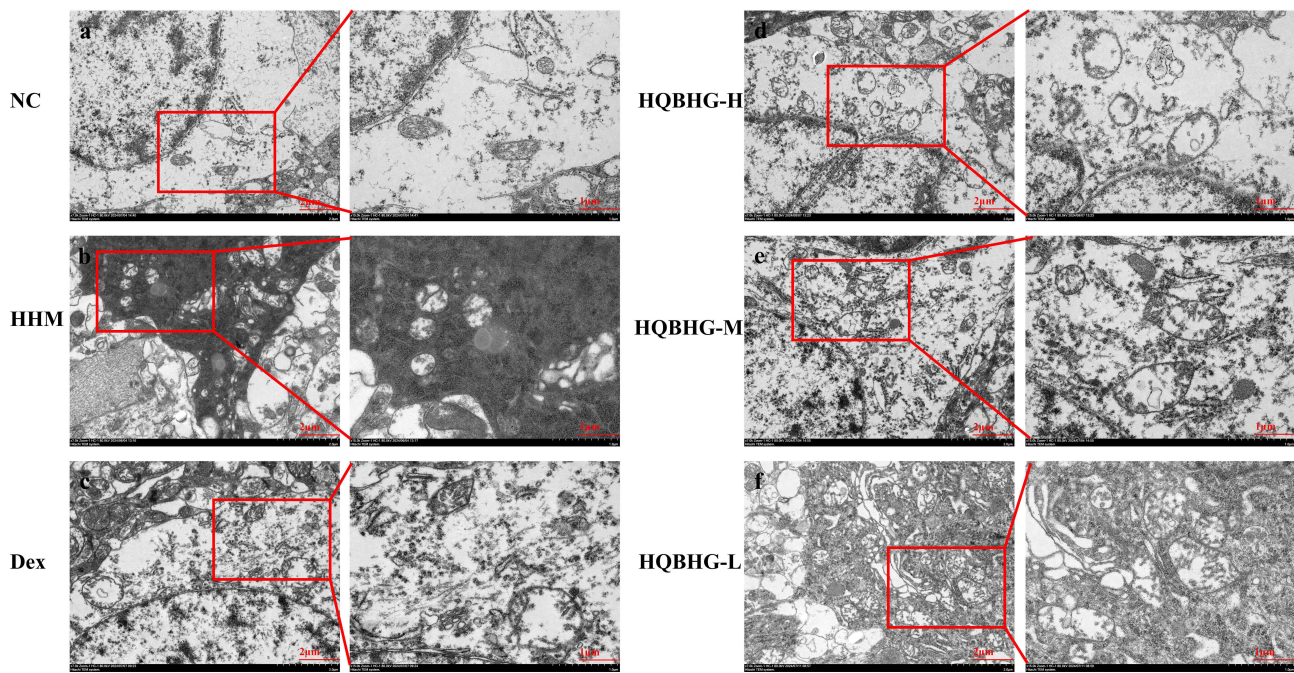


Figure 6 HQBHG ameliorates ultrastructural changes in brain neuronal cells of HACE rats. No-treatment Control group (NC); Hypobaric hypoxia model (HHM) group; Dex (5 mg/kg) group; HQBHG-H (4.42 g/kg) group; HQBHG-M (2.21 g/kg) group; HQBHG-L (1.105 g/kg) group (magnification 7000 × and 15000 ×).

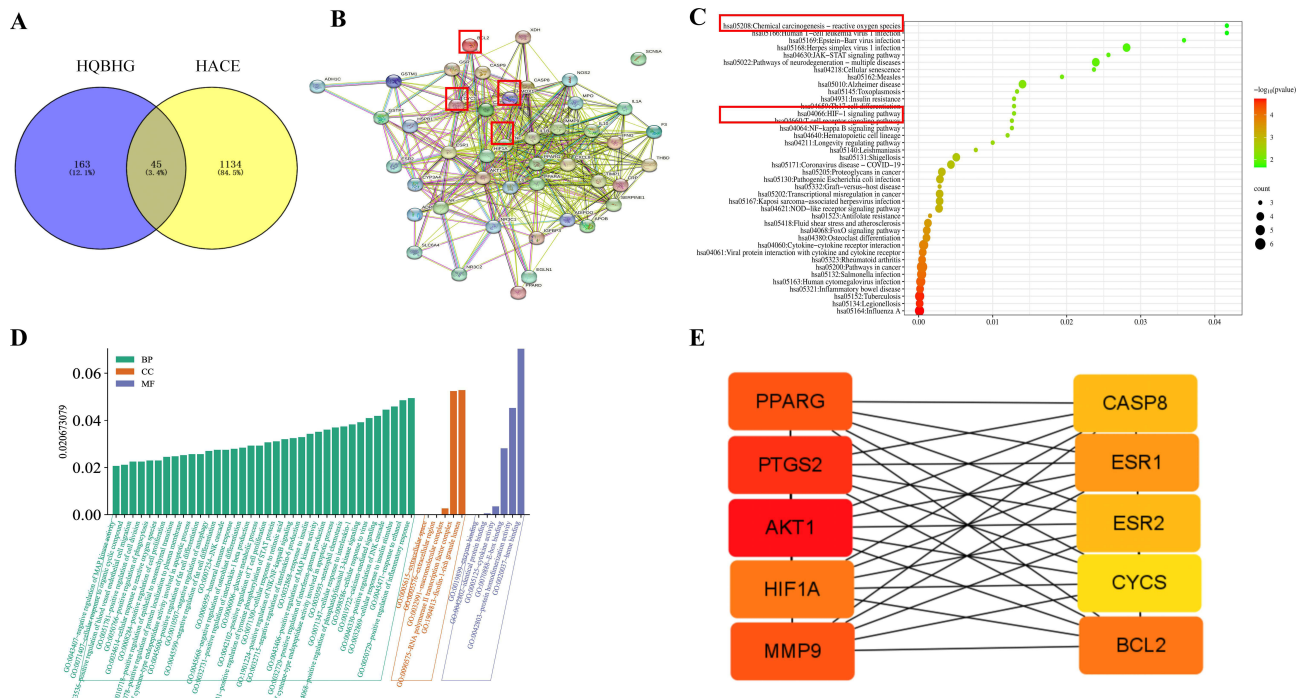


Figure 7 Analysis of the pharmacology of the HQBHG-HACE network. (A) Venn diagram. (B) PPI network of intersecting targets. (C) KEGG pathway enrichment analysis. (D) GO function enrichment analysis. (E) The MCC algorithm screens for key genes.

HQBHG Enhances Ng2 Expression in the CA1 Region of the Cerebral Hippocampus of HACE Rats

Although there is regional variability in Ng2 expression in the brain, the hippocampus is the most sensitive region of the brain to hypoxia. By immunofluorescence, it was found that in the CA1 region of the rat brain hippocampus, the

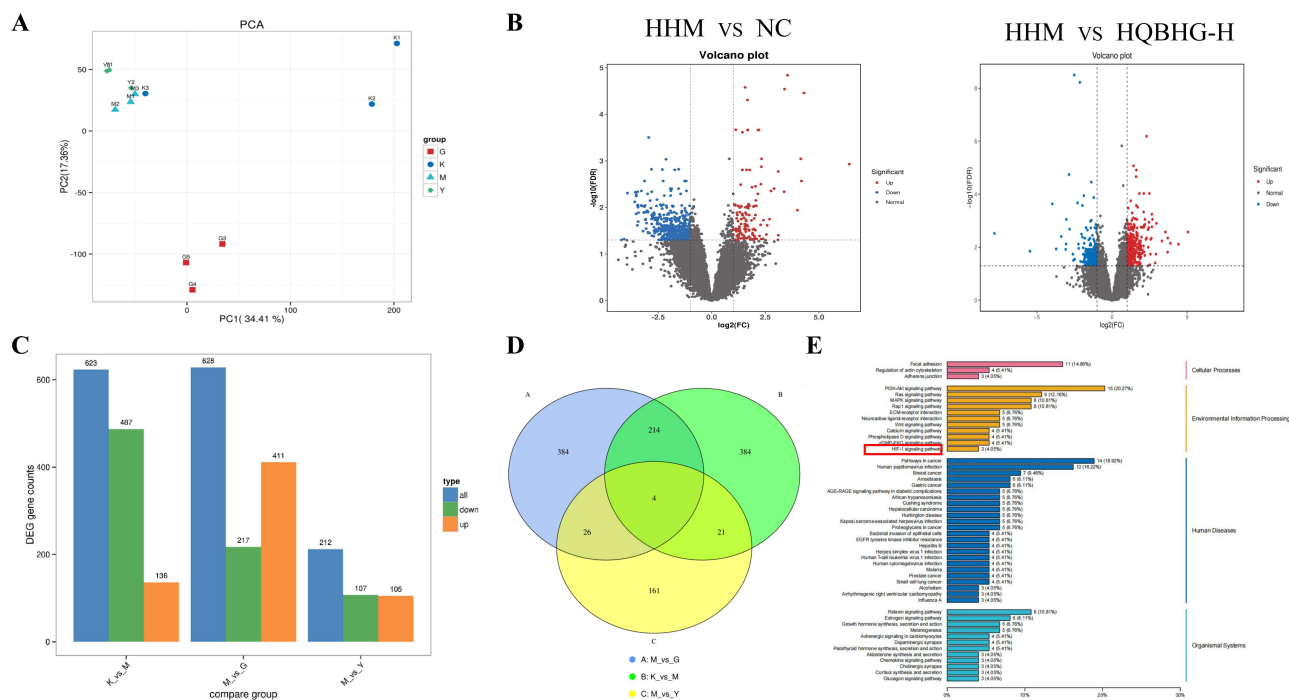


Figure 8 Transcriptomic analysis of HQBHG action on HACE. **(A)** Principal Component PCA plot. **(B)** Volcano plot of differentially expressed genes in HHM vs NC, HHM vs HQBHG-H. **(C)** Histogram of differentially expressed genes in HHM vs NC, HHM vs HQBHG-H, and HHM vs Dex. **(D)** Venn diagram of differentially expressed genes. **(E)** KEGG enrichment analysis of HHM vs HQBHG-H differentially expressed genes. (G:HQBHG-H;K:NC;M:HHM;Y:Dex.).

expression was lower in the NC group; compared with the NC group, the fluorescence expression of Ngb in the brain tissue of the rats in the HHM group was significantly enhanced, and the intervention of HQBHG did not diminish the expression of Ngb in the brain of the rats, and the expression was enhanced instead (Figure 9)

Effect of HQBHG on mRNA and Protein Expression of Genes Related to HIF-1 α /p53/Caspase-3 Signaling Pathway in Brain Tissues of HACE Rats

HIF-1 α and p53 can mediate downstream Caspase-3-induced apoptosis. To explore the specific mechanism of HIF-1 α /p53/Caspase-3 in HACE, their pathway-related gene and protein expressions were verified by qRT-PCR and Western Blot. Compared with the NC group, the brain tissues of rats in the HHM group showed significantly higher HIF-1 α , p53, Bax, Bad & Caspase-3 mRNA and protein expression, and significantly lower Bcl-2 mRNA and protein expression. In contrast, the expression of all these targets showed the opposite result after treatment with HQBHG (Figures 10 and 11).

Discussion

Hypobaric and hypoxic conditions are the main causes of HACE.¹⁴ Its pathogenesis involves multiple pathological factors, and oxidative stress and apoptosis play a key role in the development of HACE. Hypoxia can lead to enhanced anaerobic metabolism and increased oxidative stress in brain tissue, accompanied by damage to brain tissue lipids, proteins, and DNA,¹⁵ which can cause damage to the blood-brain barrier, changes in the morphology and structure of brain tissue,¹⁶ and apoptosis of brain cells, which poses a threat to the life and health of people at high altitude. Therefore, it is imperative to study the mechanism of action of HACE for prevention and treatment.

Chinese medicine believes that plateau hypoxic brain injury is closely related to insufficient Qi, no source of Qi and blood biochemistry, and loss of nourishment of the brain marrow, etc., and the insufficiency of Qi due to hypoxia runs through the whole course of the disease.¹⁷ Free radicals are attributed to phlegm dampness, and the accumulation of free radicals due to weak promotion of Qi deficiency causes oxidative damage. Inflammation corresponds to fire-heat and dryness, and dryness is a Yang evil that easily injures fluids, resulting in Yin deficiency. Deficiency of Qi and Yin can lead to Yang deficiency. Given the Chinese medical mechanism of high altitude cerebral edema with Qi deficiency and

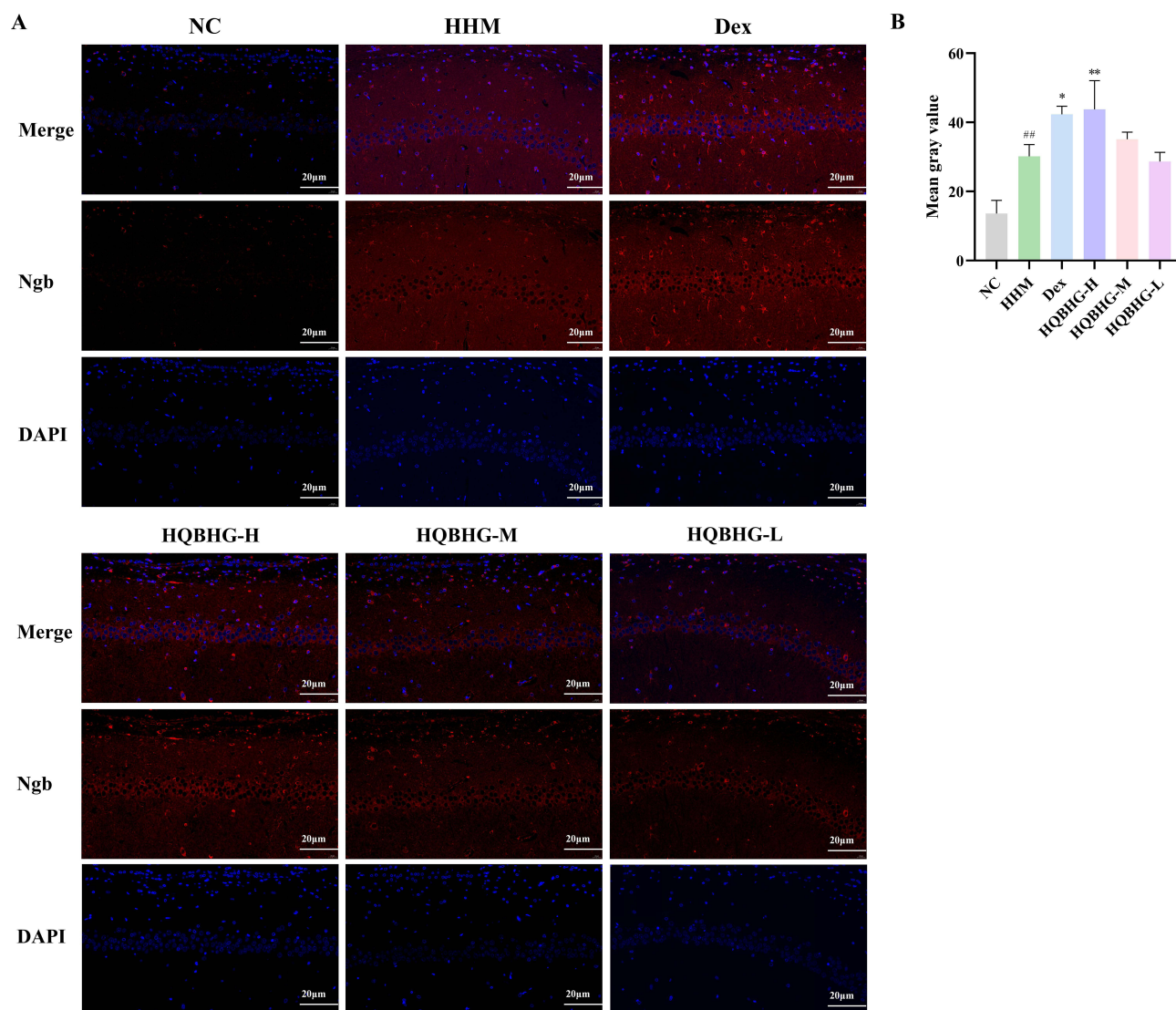


Figure 9 HQBHG promotes Ngb expression. **(A)** Effect of HQBHG on NGB protein expression levels in the rat hippocampal CA1 region (n=3). **(B)** Statistical diagram of NGB immunofluorescence staining in the hippocampal CA1 region of rats across groups (n=3). No-treatment Control group (NC) ; Hypobaric hypoxia model (HHM) group; Dex (5 mg/kg) group; HQBHG-H (4.42 g/kg) group; HQBHG-M (2.21 g/kg) group; HQBHG-L (1.105 g/kg) group. (n = 3). The mean standard deviation represents the data. ###*P* < 0.01 compared to the control group. **P* < 0.05 and ***P* < 0.01 compared to the HHM group. (magnification 400×).

phlegm obstruction and blood stasis, the prevention and treatment of Chinese medicine mostly follow the treatment principle of benefiting Qi and nourishing Yin, drying dampness, and resolving phlegm, so the group chose the modified Huangqi Baihe Granules (HQBHG) to carry out the related research. HQBHG is made from the folk medicine “Huangqi Baihe Porridge” and Dunhuang medical prescription “Astragalus and Cistanches Soup”, In this formula, Astragalus replenishes Qi, Chenpi regulates Qi, if Qi is sufficient, it can promote powerful, phlegm and dampness can be removed easily, and it can play the role of antioxidant; lily, yellow essence, Chinese wolfberry, etc., nourish Yin to eliminate dryness and remove Yin deficiency. The whole formula has the efficacy of benefiting Qi and nourishing Yin, drying dampness, and resolving phlegm, which is in line with the etiology and pathogenesis of high-altitude hypoxic brain injury. Based on this, we investigated the potential mechanism by which HQBHG exerts anti-apoptotic and anti-oxidative stress effects in the prevention of HACE, and provided an experimental basis for the multi-target and multi-pathway intervention of this compound in plateau disease.

The composition of the TCM compound is complex, and its specific active components acting on HACE are not clear. To reveal the specific mechanism of action of HQBHG in effectively alleviating HACE, the active ingredients were

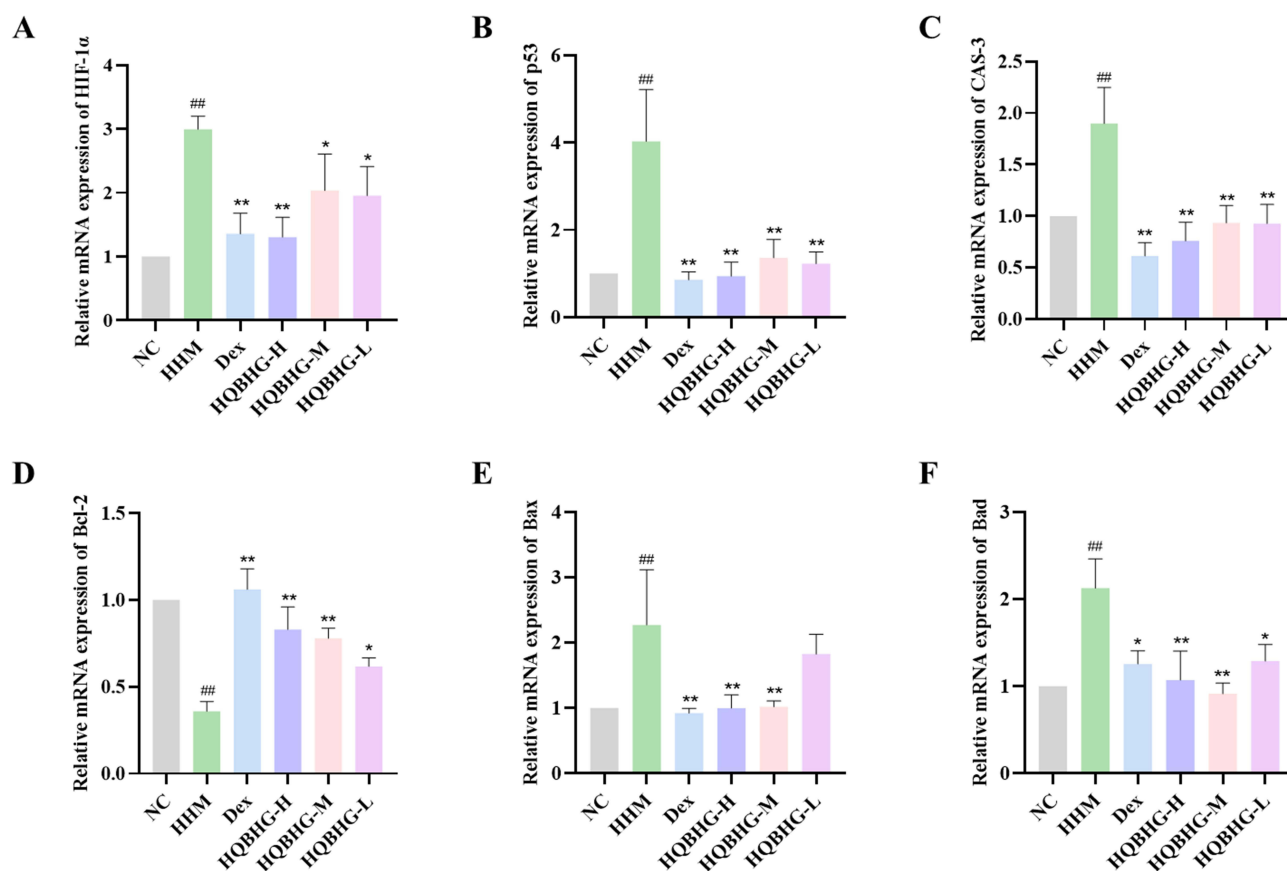


Figure 10 HQBHG regulates the expression of genes related to the HIF-1 α /p53/Caspase-3 signaling pathway. (A–F) Changes in HIF-1 α , p53, Caspase-3, Bcl-2, Bax, Bad mRNA expression (n = 3). No-treatment Control group (NC); Hypobaric hypoxia model (HHM) group; Dex (5 mg/kg) group; HQBHG-H (4.42 g/kg) group; HQBHG-M (2.21 g/kg) group; HQBHG-L (1.105 g/kg) group. The mean standard deviation represents the data. ##*P* < 0.01 compared to the control group. **P* < 0.05 and ***P* < 0.01 compared to the HHM group.

identified and analyzed in this study, and 15 major chemical components were obtained. Most of these components belong to the terpenes, phenolic acids, flavonoids, and alkaloids. Among them, Chlorogenic acid methyl ester, Luteolin-7,3'-di-O-glucoside derived from luteolin, Astragaloside VI derived from *Astragalus membranaceus*, and 6-DeoxyCatalpol derived from *Cistanches*, all of these constituents have anti-inflammatory, antioxidant, and neuroprotective effects. It is therefore hypothesized that these components may be the main basis for the effectiveness of HQBHG in alleviating HACE.

Disruption of the blood-brain barrier (BBB) is a major cause of HACE.¹⁸ A combination of oxidative stress,¹⁹ inflammatory responses,²⁰ and other factors can lead to increased brain barrier permeability,²¹ increased brain water content, toxic substances crossing the barrier and disrupting the normal brain tissue structure, all of which can lead to apoptosis of brain cells. To observe the changes in BBB, changes in blood-brain barrier permeability were evaluated with Evans Blue stain. After being exposed to HH, the EB staining solution successfully penetrated the brain tissue, and the brain W/D was also significantly increased, and the normal morphology and structure of the brain tissue were altered and accompanied by a decrease in neuronal activity. HQBHG-intervened rats showed reduced W/D, decreased EB staining content, and reduced pathological brain tissue damage. After hypobaric hypoxia induction, oxidative stress^{22,23} occurred and there was a decrease in the production of superoxide dismutase (SOD) and reduced glutathione (GSH) and an increase in malondialdehyde (MDA) content in rat brain tissue. Whereas, the intervention of HQBHG resulted in the alteration of these oxidative products and antioxidant enzymes in HACE rats. The above data suggest that HQBHG can alleviate the HACE condition in rats by restoring blood-brain barrier permeability and anti-oxidative stress.

Because stimulated by hypoxia, oxidative stress leads to intracellular ROS accumulation and increased Bax disrupts mitochondrial membrane integrity,²⁴ increases Cyt-c release, and activates Caspase-3 to initiate downstream apoptosis. HH

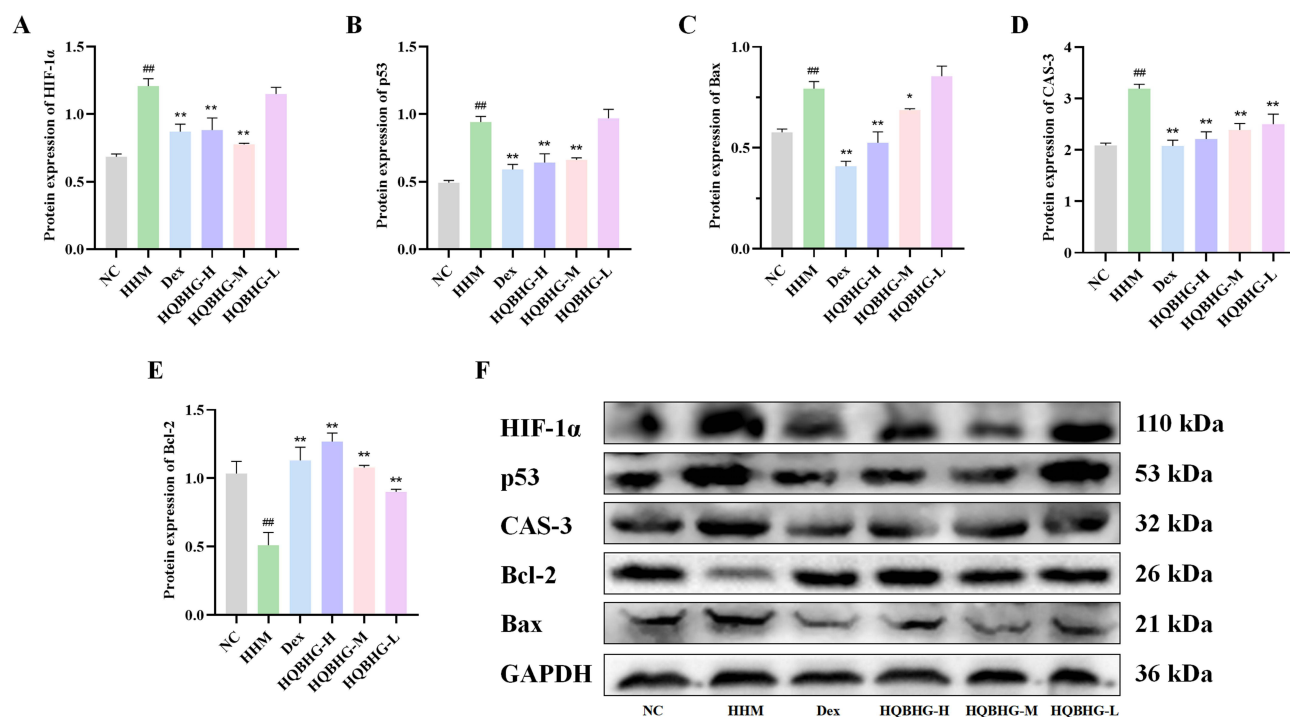


Figure 11 QHBHG regulates HIF-1 α /p53/Caspase-3 signaling pathway-related protein expression. (A–E) Changes in protein expression of HIF-1 α , p53, Caspase-3, Bcl-2 and Bax (n = 3). (F) Western Blot strip graph. No-treatment Control group (NC); Hypobaric hypoxia model (HHM) group; Dex (5 mg/kg) group; QHBHG-H (4.42 g/kg) group; QHBHG-M (2.21 g/kg) group; QHBHG-L (1.105 g/kg) group. The mean standard deviation represents the data. ^{###}P < 0.01 compared to the control group. *P < 0.05 and **P < 0.01 compared to the HHM group.

exposure caused apoptosis and mitochondrial edema in rat brain cells as observed by TUNEL staining and transmission electron microscopy. The intervention of QHBHG effectively restored mitochondrial damage and reduced the occurrence of apoptosis.

To explore the specific mechanism of QHBHG inhibiting oxidative stress and apoptosis in rat HACE, the relevant pathways and targets of QHBHG that may act on HACE were enriched by network pharmacology and transcriptomics sequencing analysis. Both results showed a close relationship with reactive oxygen species, the HIF-1A pathway, and BCL2. For this purpose, immunofluorescence, Western Blot, and qPCR were used to study the above oxidative stress and apoptosis-related genes or targets involved in HACE.

HIF-1 α (hypoxia inducible factor-1 α , HIF-1 α) is a widespread hypoxia-inducible factor in the human body, which is highly expressed in ischemia/hypoxia and is widely involved in inflammation, apoptosis, autophagy, oxidative stress and energy metabolism,²⁵ and decreasing the expression of HIF-1 α can attenuate a wide range of brain injuries.^{26,27} The data of the present study showed that the expression of HIF-1 α gene and protein was significantly elevated in the brain tissues of HH-exposed rats, which was reduced by the intervention of QHBHG. p53 acts as a nuclear transcription factor that regulates a range of physiological and pathological processes, including apoptosis, growth arrest, and senescence.²⁸ During ischemic injury, rapid accumulation of p53 in brain tissue activates neuronal apoptosis through transcription-dependent and non-dependent programs; p53 can also rapidly translocate to mitochondria and interact with Bcl-2 family proteins to activate the mitochondrial apoptotic program.²⁹ Based on this, we explored the relationship between HIF-1 α and p53. It was shown that the p53 gene is a downstream regulator of HIF-1, and hypoxic activation of HIF-1 α activates p53 leading to downstream apoptosis and oxidative stress.³⁰ Meanwhile, it has also been shown that p53 can regulate HIF-mediated senescence and autophagy³¹ in iron neurons, suggesting a bidirectional regulation of p53 and HIF. The results of the present study showed that gene and protein expression of HIF-1 α and p53 were also significantly elevated under HH induction, and oxidative stress and apoptosis occurred to aggravate brain injury. QHBHG may protect the brain from oxidative stress and apoptosis-related injury by inhibiting HIF/p53 expression.

Neuroglobin (Ngb) is an evolutionarily conserved member of the globin family and is expressed primarily in neurons.³² The exogenous neuroprotective protein Ngb is upregulated in response to ischemic-hypoxic injury and

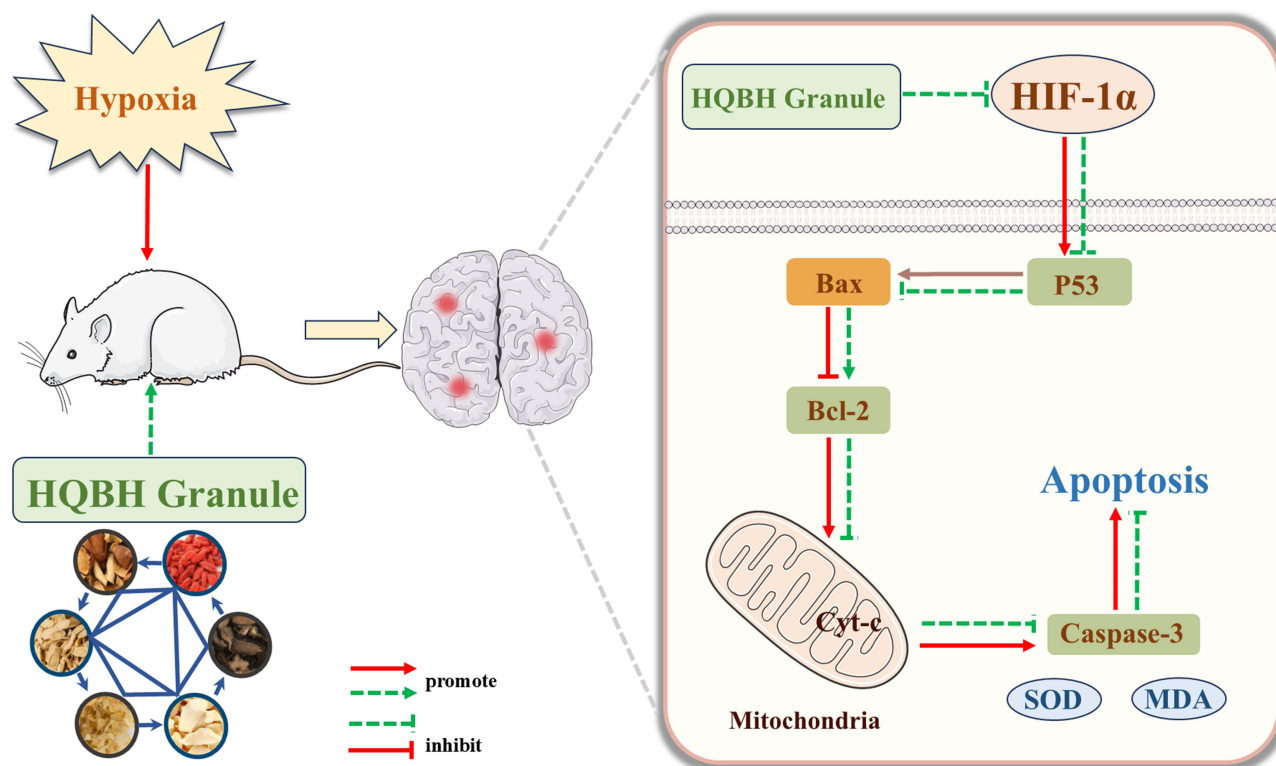


Figure 12 HQBH mechanism of action diagram.

shows protective effects in ischemic-hypoxic brain injury.^{33,34} It has been demonstrated that isoquercetin improves ischemic cerebral perfusion injury and found an upregulation of Ngb,³⁵ and dexmedetomidine improves CIR injury also found an upregulation of Ngb.³⁶ Although in some studies, HIF has a homeostatic effect on Ngb, Ngb has an uncertainty with time in ischemic-hypoxic brain injury, so we only studied it as an indicator of brain injury improvement. Upregulation of Ngb was also observed in our study with the intervention of Astragalus and Lily granules, but there was no isoexpression relationship with HIF-1 α .

The above results indicated that HQBH could attenuate HACE in rats by inhibiting oxidative stress and apoptosis, whereas inhibition of apoptosis mediated by the HIF-1 α /p53/Caspase-3 signaling pathway was the key molecular mechanism underlying the preventive effect of HQBH on HACE (Figure 12), and revealed the multi-target and multi-pathway pharmacological characterization and material basis of the traditional Chinese medicine HQBH. Although existing basic research observations suggest that HQBH can improve HACE-related oxidative damage and apoptosis index, the potential toxicity and safety of HQBH remains a key issue. In the present study, we did not conduct behavioral experiments and more in-depth investigation on the relationship between Ngb and HIF-1 α /p53, but only observed mRNA and protein expression. We should continue to validate the CO-IP and other experiments, and evaluate the bioavailability and safety of HQBH by blood tests, liver function, kidney function and other indexes. The conclusion of this study can only prove that HQBH can attenuate HACE through the HIF-1 α /p53/Caspase-3 pathway, but no in-depth exploration of the related mechanism was done for the observed changes in Ngb. Knockdown experiments of Ngb should be followed up to verify the mechanism of its action in HACE. Although HQBH shows good promise in the prevention and treatment of HACE, its clinical applicability needs further study. At present, HQBH in the prevention and treatment of HACE only remains in basic research, but we consider putting it into clinical studies and supplementing the pharmacokinetic evaluation in the near future. However, the potential regulation of HACE by HQBH through other pathways needs to be further investigated.

Conclusion

In summary, we confirmed the elucidation of the potential mechanism of HQBHG in alleviating HIF-1 α /p53/Caspase-3 signaling pathway-mediated apoptosis in HACE treatment and laid the research foundation for the clinical application of HQBHG intervention and treatment of altitude sickness.

Abbreviations

Bax, Bcl-2 Associated X protein; HH, Hypobaric hypoxia; Bcl-2, B cell lymphoma 2; HIF-1 α , Hypoxia-inducible factor-1 α ; BBB, Blood-brain barrier; HQBHG, Huangqi Baihe Granules; CYCS, Cyt-c, Cytochrome C; Ngb, Neuroglobin; CAS-3, Cysteiny aspartate specific proteinase 3; MDA, Malondialdehyde; Dex, dexamethasone; ROS, Reactive oxygen species; GSH, glutathione, r-glutamyl cysteinyl +glycine; SOD, Superoxide dismutase; EB, Evans Blue; TCM, Traditional Chinese medicine; HACE, High Altitude Cerebral Edema; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; HE, Hematoxylin and Eosin; W/D, Wet/Dry.

Data Sharing Statement

Data will be made available on request.

Ethics Statement

Animal Ethics All methods were performed in accordance with the ARRIVE guideline report and the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Gansu University of Traditional Chinese Medicine (Lanzhou, China).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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