

Ginsenoside rb2 regulates tissue autophagy in the ischemic penumbra area and delays BMSC senescence by improving the ischemic-hypoxic microenvironment

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Abstract

Introduction: Ischemic cardiovascular disease (CVD), is associated with high morbidity and mortality. Panax notoginseng (PNS) has some advantages in improving ischemic cardiovascular diseases, but the mechanism of action of its active ingredient ginsenoside Rb2 is not clear.

Methods: In this study, network pharmacology, molecular docking, and LC-MS/MS were used to evaluate the relationship between PNS and its main active constituent and autophagy. In vivo and in vitro investigations were then used to further confirm the results.

Results: In this investigation, we discovered that the primary active component of PNS, ginsenoside Rb2, might increase tissue autophagy in myocardial infarction rat models' ischemic semidark zone. In the cell model, ginsenoside Rb2 boosted the proliferation rate and decreased the apoptosis rate of bone marrow mesenchymal stem cells (BMSCs). And alleviated oxidative stress, delayed aging, raised GATA4mRNA, lowered cTNTmRNA levels, and protected BMSCs from the harm brought on by ischemic-hypoxic injury.

Discussion: Our research initially established an ischemic-hypoxic cell model, which may be provided as a reference for related research. And clarified the functions of ginsenoside Rb2 in the ischemic-hypoxic microenvironment, which may be conducive to the exploration of therapeutic targets of ischemic CVD.

1. Introduction

Ischemic CVD is a common and frequently occurring disease globally. Its fatality rate ranks first among all types of diseases, which is a great threat to human life and health(Bai et al., 2020). Studies have shown that in the process of aging, atherosclerosis, vascular stenosis caused by plaque rupture or thrombus, and local micro circulatory disorders are secondary to ischemic and hypoxic injury. Ischemia and hypoxia in heart and brain tissue lead to the release of cytotoxic compounds such as proinflammatory cytokines, proapoptotic cytokines and reactive oxygen species, which not only worsen the local microenvironment of heart and brain tissue but also lead to an abnormal microenvironment for cell survival(Xie et al., 2020). Modern medicine mainly utilizes acute thrombolysis, through interventions and drugs, to treat ischemic and hypoxic injuries. Although this can save some dying cells in the infarcted area, many patients cannot benefit from this approach because of the narrow treatment window and the time limit of thrombolytic therapy. At the same time, the necrotic cells in the infarcted area cannot be regenerated and repaired, and the regeneration of scar tissue is a difficult problem in the current treatment. Therefore, researchers gradually turn to cell regeneration therapy to provide better treatment options for patients with ischemic-hypoxic injury.

BMSCs transplantation may be an effective method for the treatment of stroke(Steinberg et al., 2019). However, due to the unique hypoxia and oxidative stress in the ischemic environment, the ability of stem cells to home to the damaged tissue is weakened, and the possibility of external implantation and long-term survival is small. After intravenous or arterial injection of mesenchymal stem cells (MSCs), it has been observed that the vast majority of MSCs are trapped in the lungs within 48 hours(Wang et al., 2018).

Some studies have demonstrated that 14 days after caudal vein injection, only 4% of BMSCs appeared in the ischemic brain tissue of rats, and less than 10% of BMSCs differentiated and expressed neuronal markers after transplantation(Cheng et al., 2018). This seriously hinders the ability of BMSCs to promote angiogenesis and repair damaged tissue and limits their clinical application(Han et al., 2018; Wang et al., 2022).

One research group found that the main therapeutic effects of ginsenoside Rb1 were anti-platelet and anti-vascular remodeling activities(Xiang et al., 2020). Ginsenoside Rb1 has been focused on for the mechanism of prevention and treatment of atherosclerotic embolism(Wang et al., 2021; Xiao et al., 2022). As another active component of PNS, ginsenoside Rb2 has many pharmacological activities, such as anti-apoptosis, anti-oxidative stress, anti-fatigue, anti-inflammatory and immune improvement activities. In the fight against ischemic cardiomyopathy, ginsenoside Rb2 can effectively dilate blood vessels, increase blood oxygen volume, restore coronary blood flow and enhance myocardial contractility(Kim et al., 2019; Wang et al., 2020). Gao et al. (2015) found that ginsenoside Rb2 regulates the Ras-EPK1/2 signaling pathway and can effectively inhibit dexamethasone-induced apoptosis of BMSCs. Kim et al. (2019) established a rat model of ischemic brain injury. Ginsenoside Rb2 can obviously repair damaged cells and protect nerves in rats. It has been shown that ginsenoside Rb2 may treat ischemic-hypoxic injury by promoting blood circulation and relieving blood stasis, but its mechanism of regulating ischemic-hypoxic microenvironment and interfering with the functional activity of BMSCs is not clear.

In order to clarify the defense and mechanism of action of ginsenoside rb2 against ischemic-hypoxic injury, this study combined the outcomes of network pharmacology, molecular docking, and LC-MS/MS, and confirmed them through in vivo and in vitro experiments.

2. Materials and methods

2.1 Screening of the main active ingredients and targets by network pharmacology

Chemical compounds were identified from the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP) (<https://tcmsp-e.com/>) database and BATMAN- TCM database (<http://bionet.ncpsb.org/batman-tcm/>).In addition, the databases of CNKI and PUBMED were used to supplement other compounds. The screening criteria were: drug-likeness (DL) value of ≥ 0.18 and oral bioavailability (OB) of $\geq 30\%$ (Xu et al., 2012). The targets of active compounds in PNS were searched using the TCMSP target module, and the target protein name was convert to official symbol formats of gene targets using Perl(<http://www.perl.org/>) and UniProt (<http://www.UniProt.org/>) databases. And then, we obtained the main actice ingredients and the predicted PNS targets and used Venny 2.1.0 (<http://bioinfo.cnb.csic.es/ tools/venny/index.html>) to construct a Venn diagram.The main actice ingredients and the targets were imported into the STRING database (<http://string-db.org>) .The species was limited to "Homo sapiens" in the operation interface and setted the confidence score ≥ 0.4 . Cytoscape 3.8.2 (<https://www.cytoscape.org/>) was used to construct a network of potential key targets and to perform a systematic analysis of the network parameters.

2.2 Docking analysis of active components into blood and target proteins

According to the basis of our preliminary study, We detected the levels of ginsenoside Rb1, ginsenoside Rb2, ginsenoside Rg1 and ginsenoside Rg2, in the drug-containing serum of PNS rats as blood-entering components. And we searched for the basic information of the four blood entry components in TCMSP database. Therefore, we used these components for molecular docking with autophagy, cardiac development, and myocardial infarction-related targets.

2.3 Animals and treatment

In this study, the animals were divided into two parts. A total of 30 male SPF SD rats (body weight: 180 ± 10 g), the certificate number was SCXK (Yu) 2015-0005. And the other 30 male SPF SD rats (body weight 200 ± 10 g), the certificate number was SXCK (Yu) 2017-0001. All animals were provided by the Experimental Animal Center of Medical College of Zhengzhou University. The animals were raised in the Experimental Animal Center of the Third Affiliated Hospital of Henan University of Traditional Chinese Medicine. This experiment was approved by the Ethics Committee of Henan University of Traditional Chinese Medicine, ethics code: DWLL2018030051, in line with the guiding principles of the Ethics Committee. In the IVC system, the temperature was maintained at 21-25°C, with a humidity of 38–60%, ventilation of 22 times/h, and a 12 h light/dark cycle. The rat had free access to standard animal feed and filtered water. The adaptive period lasted for one week prior to the formal experiment.

2.3.1 Preparation of acute myocardial infarction model

The rat model of myocardial infarction was established by the Olivetti method (Olivetti et al., 1991). Left anterior descending (LAD) coronary artery occlusion was used to cause acute myocardial ischemia (AMI) in rats. The rats were anesthetized with 10% chloral hydrate in 0.3 ml/100 g (5 mg chloral hydrate crystal + 50 ml normal saline). After ligation, the pulsation of the left ventricular anterior wall decreased, the amplitude of R wave in the limb lead increased significantly, and then the ST segment of lead I and aVL were shown to be elevated, indicating that the model of acute myocardial infarction was successfully established. The heart tissue was removed from the rat plate by thoracotomy. Twenty-four hours after operation, the surviving rats were randomly divided into three groups: control group, ischemic-hypoxic group (HI group) and ginsenoside Rb2 group.

2.3.2 Ginsenoside Rb2 administration in the animal experiments

Ginsenoside Rb2 was provided by Chengdu Keloma Biotechnology Co., Ltd. China. (batch number: 11021-13-9). Preparation of it was based on the preliminary experiment with the human and animal dose exchange algorithm. Ginsenoside Rb2 intraperitoneal injection amount was 40 mg/kg, the rat body weight was set as "n", and the quantity of injected raw drug = . The concentration of the ginsenoside Rb2 solution was 10 mg/ml, which was prepared in normal saline, and the injection volume (ml) was determined by the following formula $\text{volume} = \frac{\text{dose}}{\text{concentration}}$. Rats were intragastrically administered 1 ml for

every 100 g of weight. The ginsenoside Rb2 was stored in a 2~8 °C refrigerator. The rats in the control group and the HI group were given 1 ml normal saline per 100 g of weight until the rats were sacrificed.

2.3.3 Determination of the mRNA expression of autophagy genes Beclin-1 and mTOR by RT-PCR

Total RNA was extracted from tissue by a total RNA extraction kit. 1 µg of total RNA was transcribed into cDNA in a total volume of 20 µl using the cDNA DyNAmo kit (Thermo, Germany). Primers for PCR were purchased from Shanghai Shenggong Bioengineering Co., Ltd. Amplification was performed at 95 °C with 3 min, followed by 95 °C with 5' and 60 °C with 34' for 40 cycles. The expression of mRNA in each group was determined. The relative expression of mRNA was calculated by the $2^{-\Delta\Delta C_t}$ method. The sequence of the PCR primers is shown in the table below (Table 1).

2.3.4 Western blot analysis

The myocardial infarction tissue was lysed by RIPA reagent containing protease inhibitors, and the protein concentration was determined by the BCA reagent method. The protein concentration was calculated based on the standard curve, the sample protein was denatured, the supernatant was collected, and the PAGE gel rapid preparation kit was used for sample preparation and gel filling. The 5% skimmed milk powder solution was used to incubate with the PVDF membrane. Based on the dilution multiple of the primary antibody, 5% skimmed milk powder was added to dilute the antibody, Beclin-1 (GeneTex, USA) LC3B (GeneTex, USA). The antibody was allowed to fully bind to the protein by incubation overnight. After washed the rabbit secondary antibody was diluted at a dilution ratio of 1: 5000. Finally, the membrane was developed and fixed in the darkroom. The protein band gray level was analyzed by Imagepro 6.0, and the protein expression level was reflected by the ratio of target protein gray value to the β -actin gray level.

2.4 Extraction, Culture, isolation and purification of BMSCs

BMSCs were flushed from the femurs and tibiae of SD rats with DMEM/F12 culture medium (containing 10% FBS). Cells were collected into a 25 cm² culture bottle, which was incubated at 37 °C with 5% CO₂ and a saturated humidity. The cells were then incubated in constant temperature incubator. The fluid was changed for the first time after 48 hours. An anti-CD105⁺ antibody with a working dilution of 1:50 was labeled with FITC and was incubated with the cells. After shaking gently, the cells were incubated for 30 min in a 37 °C and 5% CO₂ incubator with saturated humidity. After flow cytometry sorting, a cell suspension was prepared, and a T25 culture flask was inoculated with 1×10^{10} cells, which were cultured in a 37 °C incubator. After 24 hours, the liquid was changed, the suspension cells were removed, complete medium containing 10% FBS was added, and the medium was changed every 48 hours. The cells were then subcultured to P3~P5 for the experiments.

2.4.1 Drugs

Ginsenoside Rb2 was provided by Chengdu Keloma Biotechnology Co.,Ltd. China. (batch number:11021-13-9),with four concentrations used in the subgroups: 15μmol, 30μmol,45μmol, and 60μmol.10%FBS-containing complete medium (Cyagen, China) was added, and each concentration volume was adjusted to 20 ml of drug-containing serum for the experiment.

2.4.2 Cell culture and ischemic-hypoxic treatment

Based on methods outlined in a previous study(Zhang et al., 2020) and other references(Cremers et al., 2014; Han et al., 2016; Han et al., 2018; Xing et al., 2013; Zhang et al., 2018). BMSCs were divided into four groups. The control group was cultured with complete medium (containing 10% FBS) in a 37 °C incubator with 5% CO₂.For hypoxic and ischemia trials, the hypoxia group was cultured with complete medium. The hypoxia ischemia group (HI group) was cultured with DMEM medium (containing 4.5g/L glucose, L-glutamine, and no sodium pyruvate) (Solarbio,China,12100), and the ginsenoside Rb2 group was cultured with serum containing ginsenoside Rb2 in a H35 hypoxia workstation(Don Whitley, UK)(37 °C,94% N₂,1% O₂,5% CO₂).All the groups were assessed at 8 time points: 6 h, 12 h, 24 h, 36 h, 40 h, 44 h, 48 h and 60 h.(Table 2)

2.4.3 Determination of survival rate, apoptosis rate and reactive oxygen species level of BMSCs in the ischemic-hypoxic microenvironment

1 Cell viability assay

P3-P5 BMSCs were inoculated in a 96-well plate. Each group was cultured under ischemic and hypoxic conditions for 6 h, 12 h, 24 h, 36 h, 40 h, 44 h, 48 h and 60 h. Then, 10μl of CCK-8 solution (Dojindo China)was added to the cells, which were incubated in a 37 °C incubator for 2 hours. The absorbance (A) was measured at 480 nm by enzyme-linked immunosorbent assay, and a blank control well was set up at the same time. The cell survival rate was calculated with the following formula: cell survival rate (%) = (A experimental group-A blank control) / (A control group-A blank control) × 100%. The experiment was repeated 3 times.

2 Annexin V-FITC/PI apoptosis detection

P3-P5 generation cells were inoculated in a 6-well plate. The cells were removed at each time point, digested for 1 min with 0.25% trypsin (without EDTA) after PBS washing, and 1 ml complete medium was added to terminate digestion, after which the cells were collected by centrifugation at 1000 r/min for 5 min. AnnexinV-FITC and PI(CoWin Biosciences,China) solution was then added to the cells, followed by gentle mixing and incubation for 15 min away from light at room temperature. Then, 400 μl PBS was added and mixed gently with the cells, followed immediately by detection by flow cytometry(Becton,Dickinson and Company,USA). The experiment was repeated 3 times.

3 ROS assessment by flow cytometry

Cells of the P3-P5 generation (2×10^5) were inoculated into 6-well plates. After washing with PBS, the cells were digested with 0.25% trypsin (excluding EDTA) for 1 min, followed by addition of 1 ml complete medium to terminate digestion. The cells were then centrifuged at 1000 r/min for 5 min to collect the cells, after which the cell concentration was adjusted to 1×10^5 cells/mL. A DCFH-DA fluorescent (Elabscience, China) probe (1 ml) was added and incubated for 40 min in a 37 °C incubator, followed by washing with buffer 3 times to fully remove the fluorescent probe that did not enter the cells. The cells were resuspended in 400 μ l buffer and assessed by flow cytometry. Each experiment was repeated 3 times.

4 ROS assessment by fluorescence microscopy

The P3-P5 generation of BMSCs was inoculated into the 6-well plate at a density of 2×10^5 cells per well. The cells were taken out at each time point and treated according to the instructions. The DCFH-DA fluorescent probe (1 ml) was added to the cells, which were put into the normal incubator to incubate for 40 min. The cells were washed with buffer gently to fully remove the DCFH-DA probe that did not enter the cells, and then 400 μ l buffer was added to the cells, followed by observation of the cell morphology under a confocal fluorescence microscope (Olympus, Japan). Each experiment was repeated 3 times.

2.4.4 Effects of ginsenoside Rb2 on cell viability, apoptosis rate and ROS of BMSCs in the ischemic-hypoxic model

Cells established in the ischemic and hypoxic model were treated with serum containing different concentrations of ginsenoside Rb2 (15 μ mol/L, 30 μ mol/L, 45 μ mol/L, and 60 μ mol/L). The cell survival rate was determined by the CCK-8 method, the apoptosis rate and average fluorescence intensity were determined by flow cytometry, and the expression of ROS in cells was observed by an inverted fluorescence microscope.

2.4.5 Detection of mRNA expression of the myocardial-specific genes GATA4 and cTNT by RT-PCR

The total RNA of cells was extracted by Trizol reagent, and the steps of reverse transcription and quantitative PCR fluorescence detection were the same as those outlined above. The sequence of the PCR primers is shown in the table below (Table 3). The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression of the mRNAs.

2.5 Statistical analysis

The data obtained were analyzed and processed by SPSS26.0 statistical software. The metrological data were expressed as , and the normal distribution was analyzed by one-way ANOVA. GraphPad Prism6 software was used for graphs and statistical analysis of data to determine statistical significance.

3. Results

3.1 Network pharmacology

Following the search, 118 PNS compounds were discovered. 1420 targets and 2058 targets linked to autophagy were assigned to the recovered compounds. After importing 1420 PNS targets and 2058 autophagy targets into Venn, a Venn diagram was created and 319 overlapping genes were found. The PPI network had 7301 edges and 316 nodes. To visualize and analyze the PPI network, Cytoscape 3.8.2 was used. The hub gene of PNS therapeutic autophagy was identified by scanning relevant literature (Guo et al., 2022; Shi et al., 2017) as BECN1, MTOR, MAP1LC3B, GATA4, and TNNT2, and the hub gene network in the PPI network was obtained using CytoHubba in Cytoscape. The hub gene network that was discovered included 159 targets and 4395 edges. Therefore, BECN1, MTOR, MAP1LC3B, GATA4, and TNNT2 were hub genes in the PPI network because more than half of the intersecting genes interacted with the aforementioned hub gene.

3.2 Molecular docking results

We analyzed the four blood components of PNS based on LM-MS/MS in the preliminary stage. Molecular docking was performed with key targets beclin1, LC3B, mTOR, GATA4, cTNT between AutoDock Vina. The binding energy $-5.0 \text{ kcal} \cdot \text{mol}^{-1}$ is usually considered to indicate a better docking result between molecules. The results of this study showed that the binding energies of the four blood components and the five core target proteins were all less than $-5.0 \text{ kcal} \cdot \text{mol}^{-1}$, suggesting good binding ability between them, and a molecular docking model was drawn using Pymol. (Figure 2) (Table 4)

3.3 Assessment of the mRNA expression of autophagy genes Beclin-1 and mTOR by RT-PCR

3.3.1 Tissue infarcted area Compared with the control group, the mRNA expression of Beclin-1 and mTOR in the ischemia and hypoxia group increased ($P < 0.05$). The ginsenoside Rb2 group showed increased mRNA expression of Beclin-1 and decreased mRNA expression of mTOR in the ischemia and hypoxia group ($P < 0.05$). (Figure 3 A)

3.3.2 Tissue infarction with a marginal zone Compared with the control group, the mRNA expression of Beclin-1 and mTOR in the ischemia-hypoxia group decreased significantly ($P < 0.05$), and the ginsenoside Rb2 group showed increased mRNA expression of Beclin-1 and decreased mRNA expression of mTOR compared with the ischemia-hypoxia group ($P < 0.05$). (Figure 3 B)

3.4 Protein expression of autophagy-associated genes Beclin-1 and LC3B as determined by Western blotting Compared with the control group, the protein expression of Beclin-1 and LC3B in the ischemia-hypoxia group decreased significantly, and in the ginsenoside Rb2 group compared with the ischemia-hypoxia group, the protein expression of Beclin-1 and LC3B increased significantly ($P < 0.05$). (Figure 3 D)

3.5 Assessment of BMSCs cell viability, apoptosis rate, ROS at each time points

3.5.1 Cell viability Firstly, there were significant differences in the ischemia and hypoxia group when compared with the control group and the hypoxia group at each time point ($P < 0.05$). After 40 hours of ischemia and hypoxia, the cell activity was the lowest (24.86%), which a large number of cells began to die. (Figure 4.A)

3.5.2 Apoptosis rate There were significant differences in the ischemia and hypoxia group when compared with the control group and the hypoxia group at each time points ($P < 0.05$). The apoptosis rate of the BMSCs at 40h in the hypoxia group was the highest (92.77%), and a large number of cells were observed to die gradually.(Figure4.B,C)

3.5.3 ROS There were significant differences between the ischemia-hypoxia group and the control group at each time point ($P < 0.05$). As observed by fluorescence microscopy, at 40 h, the fluorescence intensity of ROS in cells was higher, and the number of cells increased significantly.(Figure 5

3.6 Effect of different concentrations of ginsenoside Rb2 on the survival rate,apoptosis rate,ros at 40 h

3.6.1 Survival rate After 40 hours, compared with the ischemia-hypoxia group, ginsenoside Rb2 could significantly increase the cell activity, with a difference that was statistically significant ($P < 0.05$). (Figure6 A)

3.6.2 Apoptosis rate Compared with the ischemia and hypoxia group, the apoptosis rate of ginsenoside Rb2 in all concentration groups was significantly lower than that in the hypoxia group ($P < 0.05$). (Figure6 B,C)

3.6.3 ROS The intensity of intracellular fluorescence was observed with a fluorescence microscope. Ginsenoside Rb2 inhibited the production of ROS and led to a brightness of fluorescence that was significantly lower than that at 40h in the hypoxia group, which demonstrated a decrease in the level of intracellular reactive oxygen species accumulation($P < 0.05$). (Figure6 D)

3.7 GATA4 mRNA, cTNT mRNA levels in different groups Firstly,the mRNA expression of GATA4 in the hypoxia group and the HI group decreased significantly ($P < 0.05$), while in the ginsenoside Rb2 groups increased significantly compared with ischemia-hypoxia group ($P < 0.05$). Secondly,the mRNA expression of cTNT in the hypoxia group and HI group increased significantly ($P < 0.05$), while in the ginsenoside Rb2 groups decreased significantly compared with the ischemia-hypoxia group ($P < 0.05$) .(Figure5 E)

4. Discussion

With the increase of age, the incidence of atherosclerosis continues to increase, and vascular stenosis, blood clots and fresh thrombi formed by plaque falling off result in a sudden interruption of coronary blood flow or a disturbance of blood supply to the brain. Due to sudden severe ischemia and hypoxia, tissue regional ischemic necrosis occurs in the blood supply area, which leads to a series of critical diseases such as acute myocardial infarction or acute ischemic stroke(Xie et al., 2020).

Our research group has been committed to clinical and experimental research on the treatment of ischemic-hypoxic injury through Traditional Chinese Medicine. It has been concluded that stasis is common in ischemic and hypoxic diseases, and activating blood circulation and relieving blood stasis is an effective method for the treatment of acute cardio-cerebrovascular diseases.

This study was based on the network pharmacology, LM-MS/MS results and molecular docking, we found that ginsenosides Rb2 have good binding ability to autophagy, cardiac development, and myocardial infarction targets. Therefore, we established in vivo and in vitro models to validate the results. For investigated the relationship between the ginsenosides Rb2, ischemic-hypoxic microenvironment and BMSCs.

A rat model of myocardial infarction was established in vivo, and the infarcted area and infarcted marginal zone were compared. In the cardiovascular system, mTOR signaling pathway connects intracellular and extracellular signals. As an intermediate regulatory hub of physiological and pathological processes, it is a known negative regulation pathway of autophagy (Heras-Sandoval et al., 2020; Samidurai et al., 2018). Beclin-1 is the first described mammalian autophagy protein, and its expression level can reflect whether autophagy is activated. Beclin-1, as the initiator of autophagy, has a protective effect on the myocardium during ischemia, but due to the ischemia-reperfusion response caused by oxidative stress, Beclin-1 may inhibit the vesicle treatment in the later stage of the autophagy cascade reaction, resulting in cell death (Shi et al., 2019). In our experiment, we compared the autophagy genes in the infarcted area with those in the infarcted marginal zone and found that autophagy was stimulated in the HI group. Ginsenoside Rb2 inhibited the mRNA expression of mTOR in the infarcted area, enhanced the expression of the Beclin-1 autophagy protein, and effectively increased the level of autophagy in the infarcted area, which was consistent with the conclusion that mTOR acts as a negative regulation pathway of autophagy.

The occurrence of autophagy includes the formation of precursors, the extension of precursors to wrap the substrate to form autophagosomes, and the fusion of autophagosomes and lysosomes to complete substrate degradation. During the occurrence of autophagy, LC3 changes from a non-lipidated type (LC3B-I type) to a lipidated type (LC3B-II type). The lipidated type shows a higher migration rate during electrophoresis, and the LC3B-II/LC3B-I ratio represents autophagy renewal. Some studies have proved that LC3B not only is an important regulatory factor of autophagy but is also related to apoptosis induced by oxidative stress (Lv et al., 2021; Satyavarapu et al., 2018). Our study found that the relative expression of Beclin-1 and LC3B protein in the ginsenoside Rb2 group was higher than that in the HI group and that the transformation of LC3B-II type in the ginsenoside Rb2 group was significantly increased, suggesting that ginsenoside Rb2 can effectively activate autophagy and promote autophagy transformation. According to the results, it was found that 7 days after operation, autophagy was not completely initiated in the HI group in the marginal zone of infarction, but the level of autophagy was significantly improved after drug treatment. The possible explanation for this phenomenon is that the selection of time nodes in the model group may affect the stage of the myocardial ischemia. Ginsenoside Rb2 inhibited the activity of the mTOR signaling pathway, had a protective effect on myocardium and improved the survival rate. However, mTOR is phosphorylated at a certain stage, which leads to its activation or inhibition. The change in phosphorylated mTOR after myocardial injury is a dynamic process, which may be the reason for different autophagy levels in the infarcted zone and the infarcted marginal zone in different studies (Li et al., 2020). The development of autophagy in the rat model of myocardial infarction may be more detailed if the time points of 14 days or 28 days were added. The follow-up study can add the verification

of the key signaling proteins upstream and downstream of the intracellular signaling pathway to provide a more abundant experimental demonstration for the molecular mechanism of ginsenoside Rb2 in regulating ischemic and hypoxic microenvironments.

With regard to establishing the ischemic-ischemic cell model, the establishment of the oxygen fraction is key. Studies on the evaluation of stem cells under hypoxia and normoxia have confirmed that their ability to proliferate in hypoxic microenvironment is stronger than that of stem cells in a 21% oxygen concentration, and normoxic concentrations accelerate stem cell senescence (Schofield, 1978). Bader et al. (2015) found that culturing cells in an environment of 1% O₂, 5% CO₂ and glucose-free DMEM medium for 24 hours simulated hypoxia. It was found that the immunophenotype and karyotype of cord blood MSCs did not change. After ischemia and hypoxia, the number and metabolic activity of cells increased, and the release of apoptotic factors decreased, promoting the transformation of cord blood MSCs into human umbilical vein endothelial cells. This experiment confirmed that hypoxic preconditioning did not change the interval or the basic characteristics of safety and stability of mesenchymal stem cells. Therefore, in this experiment, the cell model of ischemia and hypoxia was established under the experimental conditions of 1% O₂ and 5% CO₂.

The assessment of BMSCs activity in a ischemic-hypoxic microenvironment showed that at 6 h and 12 h, the cell viability of the hypoxia group and the ischemia-hypoxia group increased compared to that of the control group, which may be related to the fact that BMSCs can activate autophagy, induce endogenous protective proteins and activate extracellular signal-regulated kinases under transient and non-lethal hypoxia (Hu et al., 2019; Wang et al., 2014). At 24 h, the cell viability in the hypoxia group and the ischemia-hypoxia group was significantly lower than that in the control group, and the assessment of BMSCs apoptosis and oxidative stress in the ischemia-hypoxia microenvironment showed that the apoptosis rate in the hypoxia-hypoxia group and the ischemia-hypoxia group increased significantly at 6 h, but there was no significant difference between 36 h and 48 h in the ischemia-hypoxia group, which may be related to the gradual adaptation of BMSCs to the ischemic-hypoxic environment, and dynamic observations were made at 40 h and 44 h. Compared with the control group, the cell viability was the lowest, the apoptosis rate was the highest, and the cell injury caused by oxidative stress was the most obvious in the ischemia and hypoxia group at 40 hours. From the experimental results, it can be seen that the level of oxidative stress changed significantly in the hypoxia group and that the level of oxidative stress in the 12-hour and 24-hour hypoxia group was even higher than that in the ischemia-hypoxia group, which may be due to the maintenance of glycolysis metabolism in BMSCs for a short time, which can minimize the production of ROS, but long-term hypoxia activates the inflammatory response, which leads to the release of tissue inflammatory factors. Ginsenoside Rb2 effectively reduced the level of oxidative stress in the ischemia and hypoxia group, which may indicate that ginsenoside Rb2 plays a key role in regulating the inflammatory microenvironment.

For the assay of different concentrations of ginsenoside Rb2 interfering with BMSCs. The cell activity, apoptosis rate, and oxidative stress levels were measured at the same time point for 40h. This was used to determine the protective effects of different concentrations on BMSCs in the ischemic-hypoxic

microenvironment. Our study found that ginsenoside Rb2 could increase the activity of BMSCs, decrease the apoptosis rate of BMSCs and improve the level of oxidative stress after 40 hours of ischemia and hypoxia. Among the concentrations tested, 15 μ mol/L showed the best effect, which was statistically significant. The experimental results from several different detection methods show that the survival rate in the ginsenoside Rb2 group was higher than that in the HI group. The senescence of BMSCs indicates the decline of cell function, and aging BMSCs can affect changes in the microenvironment around senescent cells through secretory functions and then participate in regulating the structural and functional remodeling of the whole tissue and organ. This part of the experiment showed that the tolerance of BMSCs treated with ginsenoside Rb2 to ischemic and anoxic environments was significantly enhanced. Drugs for promoting blood circulation and relieving blood stasis play a key role in promoting the proliferation of BMSCs, reducing the apoptosis rate and delaying the senescence of BMSCs by improving the ischemic and hypoxic microenvironment.

Subsequently, we selected two myocardial specific indexes to assess. GATA4, which is present during many stages of embryonic and cardiac development, is a key regulator of the expression of heart structural genes in BMSCs and other stem cells and promotes the effective differentiation of BMSCs into cardiomyocyte-like cells(Lorenzano et al., 2019). It has been found that co-culture of GATA4 and exocrine molecules secreted by BMSCs can increase the expression of cardiomyocyte-related markers, reduce apoptosis and improve cardiac function after myocardial infarction under the conditions of ischemia and hypoxia(Jain et al., 2019). The differentiation of BMSCs into cardiomyocytes can be divided into two stages: early differentiation and late differentiation. GATA4 is the myocardial marker of early differentiation. As a late myocardial marker, cTNT is a specific contractile protein. cTNT can be released into the blood only after injury. It is often used as a specific biochemical marker to diagnose the degree of myocardial damage in patients with acute myocardial infarction(Barberi & van den Hondel, 2018; Sun et al., 2020). Our experimental results show that ginsenoside Rb2 promoted the expression of myocardial development-related genes in rats with myocardial infarction, which may be one of the mechanisms of promoting myocardial tissue repair and healing after myocardial infarction. The parallel verification results showed that the effect in the 15 μ mol/L ginsenoside Rb2 group was more significant. GATA4 is not only a specific gene of myocardial development but also an important molecule regulating the secretion and autophagy of senescent cells. Damaged DNA can activate GATA4 and then activate the expression of a variety of downstream genes, mediate the expression of a variety of cytokines, and participate in tissue structure and function remodeling. It was also proved that ginsenoside Rb2 can effectively slow the senescence of BMSCs. It should be pointed out that if more indicators or parallel verification methods were used, this experiment would be even more comprehensive. At the same time, the continuous assessment of myocardial marker proteins, cyclin or fibroblast-related protein could demonstrate more macroscopically and carefully the repair ability of ginsenoside Rb2 in myocardial injury after myocardial ischemia and hypoxia and help to understand the change of disease course, which needs to be improved in the future.

5. Limitations

This study has some limitations. Firstly, bioactive chemicals and target information were found in published works and existing databases; thus, the reliability and accuracy of the prediction depended on the quality of the data. Secondly, it may complete the experiment if BMSCs can be transplanted into rats to observe the relevant indexes.

6. Conclusion

In summary, ginsenoside Rb2 has a protective effect on BMSCs in the ischemic- hypoxic microenvironment. And its mechanisms include reducing the production of oxygen free radicals, inhibiting inflammation and reducing the rate of apoptosis. Ginsenoside Rb2 increases the level of autophagy in ischemic tissue, and its mechanism may be achieved by inhibiting the mTOR signaling pathway. Ginsenoside Rb2 effectively achieves its anti-endogenous cellular aging actions by "removing blood stasis" from ischemic areas, "regenerating and repairing" damaged tissues, and improving the pathogenesis of "stagnation" in ischemia-hypoxic diseases.

Abbreviations

Abbreviation	Full name
AMI	acute myocardial ischemia
ANOVA	analysis of variance
BCA	bicinchoninic acid
BMSCs	bone marrow mesenchymal stem cells
CVD	cardiovascular disease
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DMEM	dulbecco's modification of eagle's medium dulbecco
EDTA	ethylene diamine tetraacetic acid
FBS	fetal bovine serum
FITC	fluoresceine isothiocyanate
HI group	hypoxic-ischemic group
LAD	left anterior descending
MSCs	mesenchymal stem cells
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	polyimide
PNS	panax notoginseng
PVDF	polyvinylidene difluoride
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
SD	sprague dawley
TCM	traditional chinese medicine
TCMSP	traditional chinese medicine analysis platform

Declarations

Ethics statement

The animal study was reviewed and approved by the Third Affiliated Hospital of Henan University of Chinese Medicine

Author contributions

JS Z and F X: Supervision, Review & Editing ,SQ W,XS H and Hao Z: Conceptualization, Methodology,Writing-Original Draft,Formal analysis,Hao Z:Investigation,Resources,Hui Z :Data Curation.CM Z:Project administration.All the authors approved the submitted manuscript for publication and other aspects of the work.

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Data availability statement

The data for bioinformatic analysis that support the findings of this study are available in TCMSP ,BATMAN-TCM,Perl and UniProt databases.at <https://tcm-sp-e.com/>, <http://bionet.ncpsb.org/batman-tcm/>, <http://www.perl.org/>, <http://www.UniProt.org/> .Data for experiment that support the results of this study are available from the corresponding author upon reasonable request.

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Tables

Table 1 PCR primer sequence

Primer	Sequence 5'-3'	Amplified fragment length
Beclin-1	F 5'- cggctcctattccatcaaaa -3'	85 bp
	R 5'- ccacttgagattcgctcagca -3'	
mTOR	F 5'- ggcttctgaagatgctgtcc -3'	153 bp
	R: 5'- gagttcgaagggcaagagtg -3'	
β -actin	F 5'-gtcgtaccactggcattgtg-3'	181 bp
	R: 5'-ctctcagctgtggtggtgaa-3'	

Table 2 Cell culture conditions of BMSCs in the ischemia and hypoxia model

Group	Culture medium	Cultivation condition
Control group	10% FBS complete medium	37 °C 5% CO ₂
Hypoxia group	10% FBS complete medium	37 °C 94% N ₂ 1% O ₂ 5% CO ₂
HI group	DMEM/F12 high-glucose serum-free medium	37 °C 94% N ₂ 1% O ₂ 5% CO ₂
Ginsenoside Rb ₂ group	Serum containing ginsenoside Rb ₂	37 °C 94% N ₂ 1% O ₂ 5% CO ₂

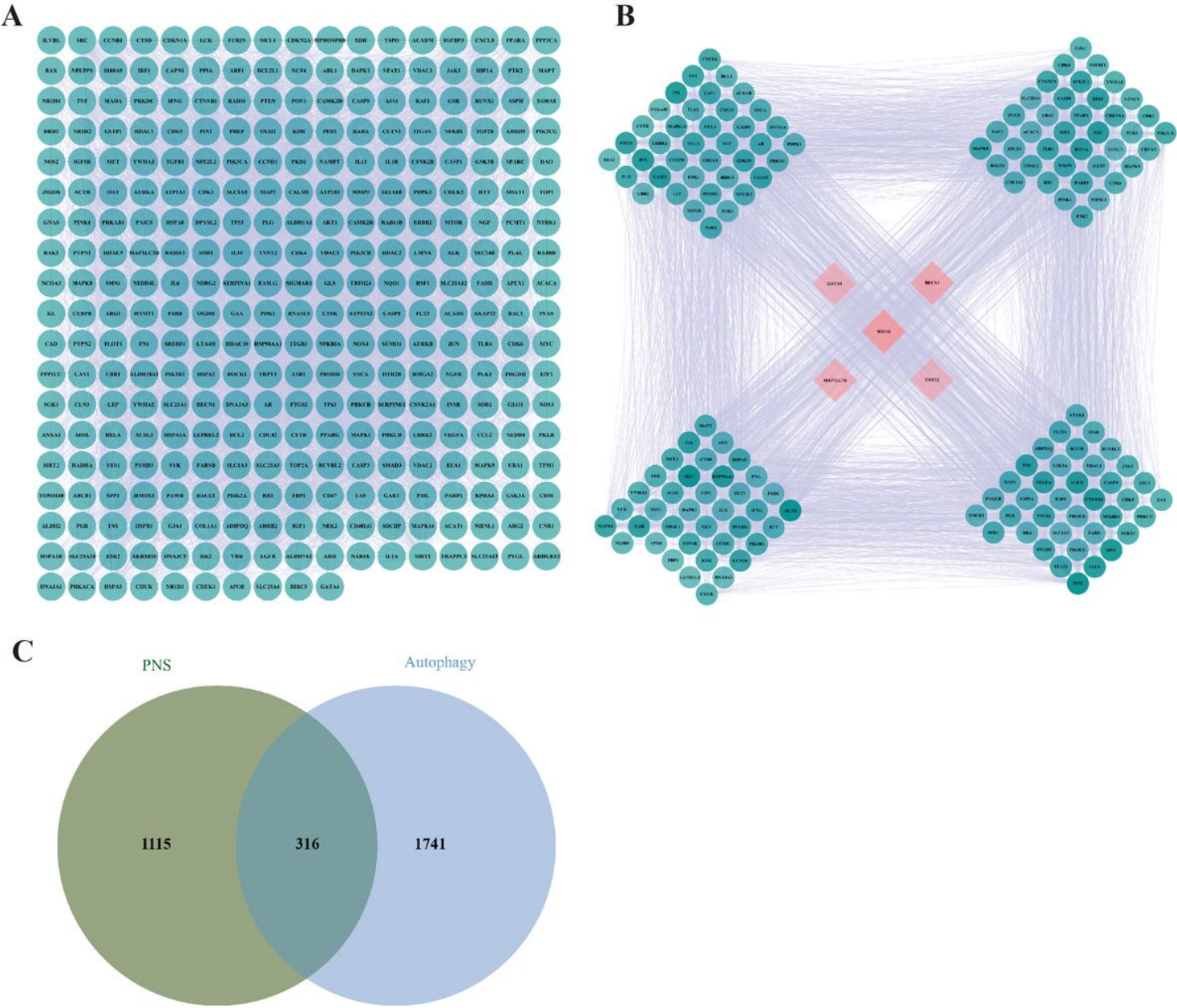
Table 3 PCR primer sequences

Primer	Sequence 5'-3'	Amplified fragment length
GATA4	F 5'- gcagcagcagtgaagagatg -3'	70 bp
	R: 5'- ctgctgtgcccatagtgaga -3'	
cTNT	F 5'- cgtgaggaggaggagaacag -3'	101 bp
	R: 5'- tgagccttctggatgtaccc -3'	
β -actin	F 5'-gtcgtaccactggcattgtg-3'	181 bp
	R: 5'-ctctcagctgtggtggtgaa-3'	

Table 4 Docking and binding capability of blood components and targets

Compound	Binding energy Kcal·mol ⁻¹				
	Beclin1	LC3B	mTOR	GATA4	cTNT
Ginsenoside Rb1	-7.2	-7.9	-9.9	-8.1	-7.5
Ginsenoside Rb2	-6.6	-8.2	-9.7	-7.9	-7.6
Ginsenoside Rg1	-6.3	-6.2	-9.7	-7.6	-6.5
Ginsenoside Rg2	-6.7	-7.4	-10.3	-7.5	-8.6

Figures



(A)The process of topological screening for the PPI network.(B)The core targets and non-core targets network.(C)PNS and autophagy crossover genes by Venn.



(A).LC-MS/MS chromatogram of ginsenoside Rb2 reference substance.

(B). LC-MS/MS chromatogram of ginsenoside rb2 Drug-containing serum.(C) The binding energy molecular docking models of ginsenoside Rb2-Beclin1.(D) Ginsenoside Rb2-LC3B.(E) Ginsenoside Rb2-mTOR.(F) Ginsenoside Rb2-GATA4.(G) Ginsenoside Rb2-cTNT

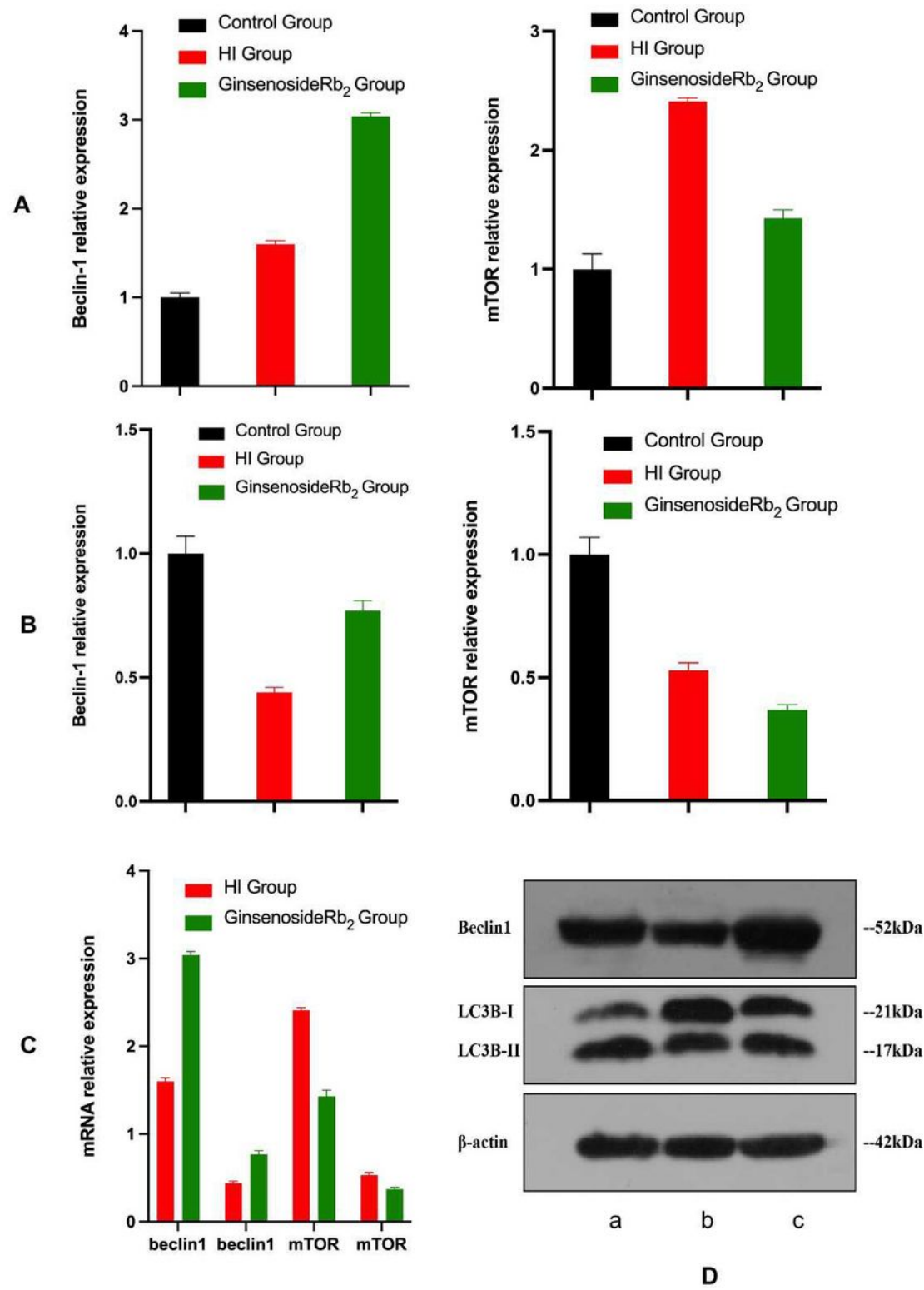


Figure 3

(A) Changes in relative mRNA expression of Beclin-1 and mTOR in tissue infarcted areas.(B) Changes in relative mRNA expression of Beclin-1 and mTOR in tissue infarction with a marginal zone.(C) Comparison of the relative mRNA expression of Beclin-1 and mTOR between infarcted areas and infarcts with a marginal zone (a is an infarcted area, b is an infarct with a marginal zone). (D) Effect of ginsenoside Rb2 on relative protein expression of Beclin-1 and LC3B in rats with myocardial infarction.

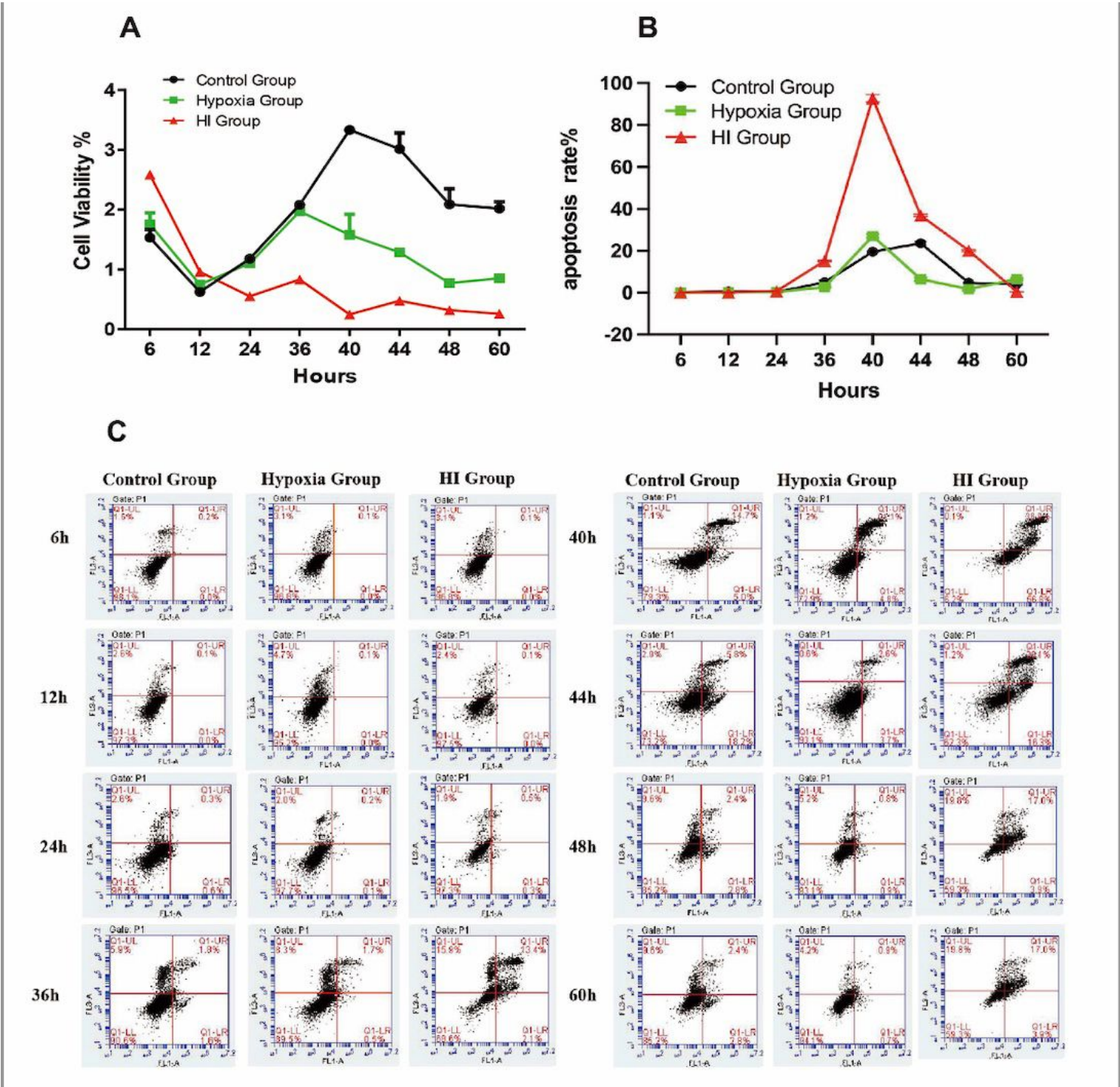


Figure 4

(A) BMSCs survival rate at each time point.(B,C) Assessment of apoptosis rate of BMSCs at different time points.

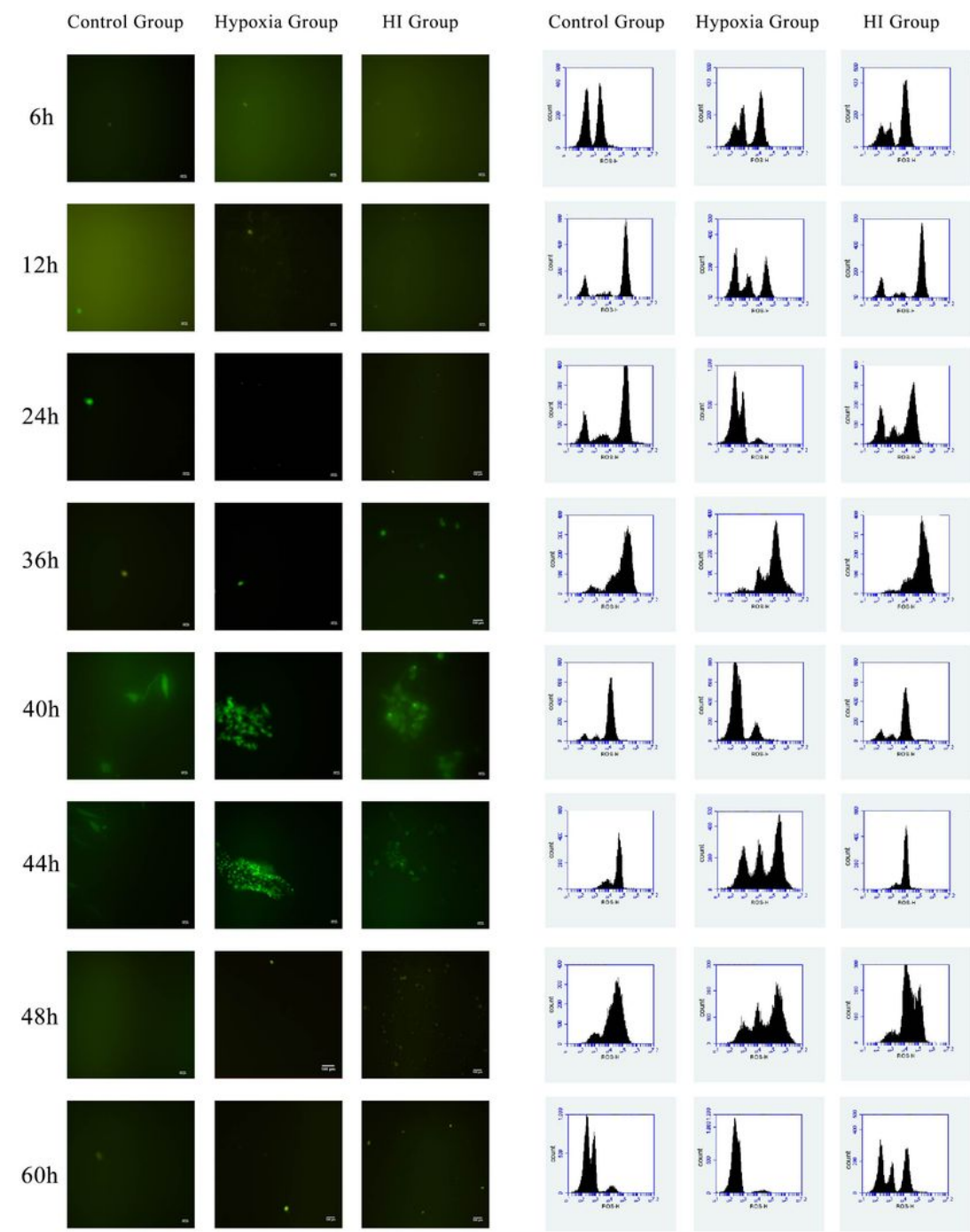


Figure 5

Assessment of ROS levels at each time point

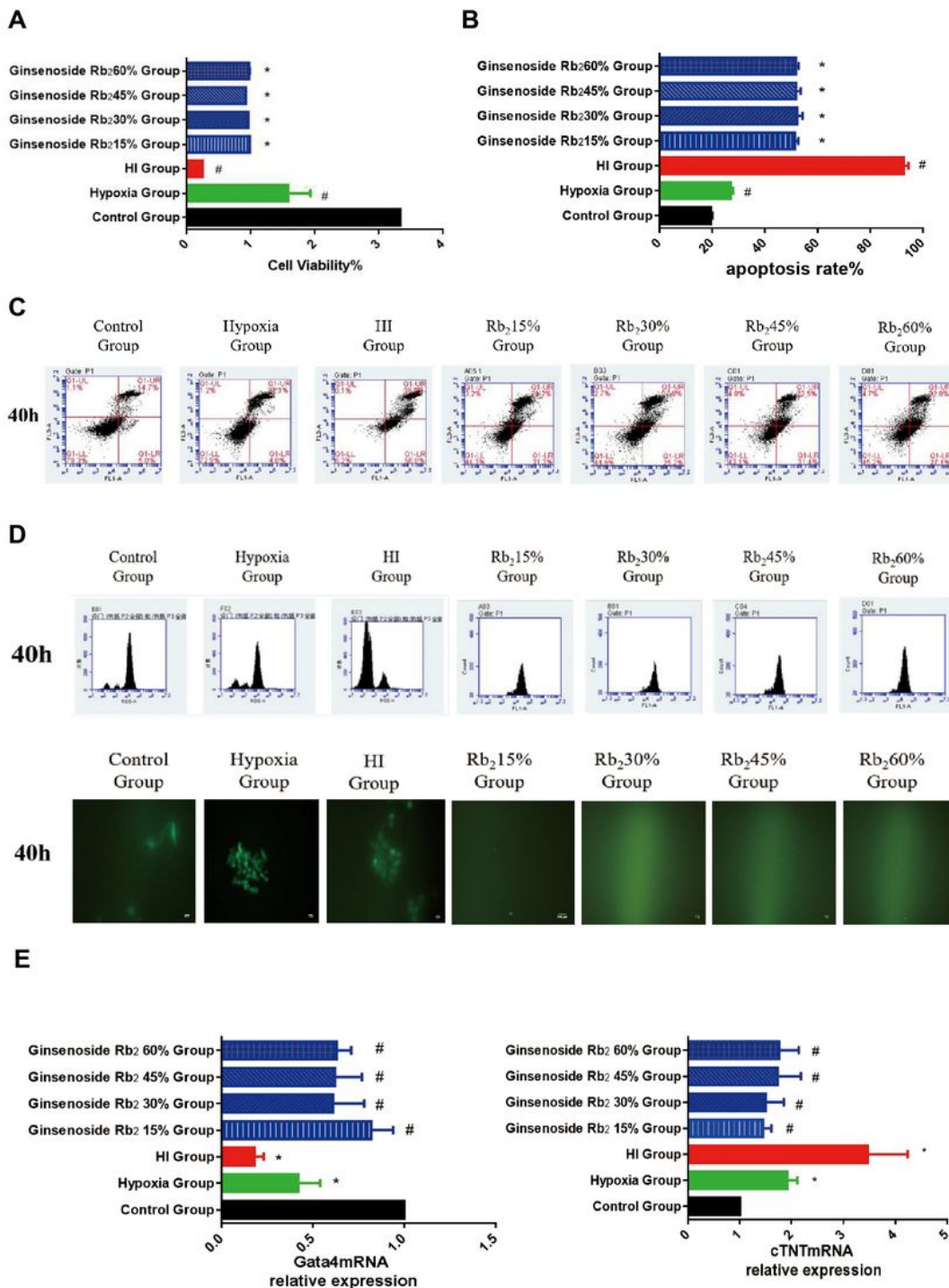


Figure 6

(A) Changes in the 40-hour survival rate after treatment with ginsenoside Rb₂ at different concentrations (compared with control group#, $P < 0.05$; compared with HI group, $P < 0.05$). (B,C) Assessment of the apoptosis rate after treatment with ginsenoside Rb₂ at different concentrations for 40 h by flow cytometry (compared with control group#, $P < 0.05$; compared with HI group, $P < 0.05$). (D) Flow cytometry and confocal fluorescence microscopy were used to observe the changes of oxidative stress in BMSCs treated

with ginsenoside Rb2 at different concentrations for 40 hours. (E) Changes in the relative mRNA expression of GATA4 and cTNT after treatment with different concentrations of ginsenoside Rb2 for 40h(compared with control group#, $P < 0.05$;compared with HI group, $P < 0.05$).