Effects of Borneol on Apoptosis in Hypoxia/Reoxygenation H9C2 Rat Cardiomyocytes

Hui Zhang[1], Jin Wen[1], Jian-wu Zhang[1], Hai-yi Xu[1], Ting Liu[1], and Yang-you Li[1]

[1]North Sichuan Medical College [Search North Sichuan Medical College]

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Abstract

Effective therapies to reduce ischemia/reperfusion and hypoxia/reoxygenation injury are currently lacking. Borneol has a clear protective effect on ischemia-reperfusion injury, and its protective effect on myocardial hypoxia/reoxygenation and its mechanism have remained to be elucidated. This study investigated the protective effects of borneol on H9C2 cardiomyocytes during hypoxia/reoxygenation injury and to explore the underlying molecular mechanisms. H9C2 rat cardiomyocytes were cultured, the MIRI model was established by hypoxia for 5h and reoxygenation for 24h. The cells were divided into control group, hypoxia/reoxygenation group (H/R group), (low-, medium-, high-) dose borneol group. Borneol administration groups were pre-treated for 12h with different doses of borneol prior to hypoxia/reoxygenation, 24h after reoxygenation, the cells or culture medium were collected for testing. The results showed that borneol has no cytotoxicity and can enhance the viability of cardiomyocytes, significantly increased the SOD content, significantly decreased LDH leakage in cell culture medium, and reduced evidently the apoptosis rate, down-regulated the expression of Bax and Caspase-3. In conclusion, this study demonstrated that borneol has a definite protective effect on hypoxia/reoxygenation-induced injury of rat cardiomyocytes, and its protective mechanism can be related to the inhibition of apoptosis by reducing the expression of Bax and Caspase-3.

Introduction

Cardiovascular disease is one of the main causes of death, with its incidence rate and mortality ranking first in the world. According to statistics, cardiovascular diseases account for 30% of the global death toll and cause more than 17 million deaths every year [1]. Cardiovascular disease is a chronic disease, including myocardial infarction, atherosclerosis, angina pectoris, coronary heart disease, arrhythmia, heart failure and cardiomyopathy [2]. In China, the incidence rate, disability rate and mortality rate of ischemic heart diseases such as acute myocardial infarction (AMI), which account for a large proportion, are increasing, and the prevalence rate is getting younger [3]. After the occurrence of ischemic cardiomyopathy such as AMI, timely recovery of blood supply is the key to rescue patients and protect cardiomyocytes. With the clinical application of coronary artery bypass graft (CABG) and percutaneous coronary intervention (PCI), while saving patients, myocardial ischemia-reperfusion injury (MIRI) caused by blood flow re penetration to cardiomyocytes further damages cardiomyocytes. The development of modern medical technology has timely rescued patients with ischemic heart disease, but reducing MIRI is also an urgent problem for patients with ischemic heart disease [4].

Apoptosis is a programmed cell death process accompanied by morphological changes [5]. More and more studies have shown that apoptosis plays an important role in the pathological process of cardiovascular diseases. Apoptosis can be caused by many factors, such as hypoxia, energy deficiency, induction of endogenous signals, etc. After the occurrence of MIRI, apoptosis can be initiated by a variety of stimuli such as hypoxia and energy deficiency, oxidative stress, DNA damage, etc. After apoptosis is activated, apoptosis can be carried out through a variety of apoptotic pathways, and the activation of apoptosis will run through the entire MIRI process [6]. Bax and Caspase-3 play an important role in cell apoptosis. Studies have shown

that the fusion of different anti apoptotic and pro apoptotic proteins in the Bcl-2 protein family affects the apoptosis process of cells. Among them, Bcl-2 protein can block the apoptosis process initiated by Bax, and the ratio of Bcl-2/Bax also regulates and reflects the progress of apoptosis [7,8]. Kaempferol and butyric acid increased p-PI3K、p-Akt、p-GSK-3β, inhibit the apoptotic pathway after Caspase-3, and activate PI3K/AKT/GSK-3β signaling pathways are beneficial to the survival of cardiomyocytes [9,10]. The excessive death of apoptotic cells is the key factor of cell pathological damage after myocardial infarction. Therefore, effective inhibition of cell apoptosis after MIRI is one of the research directions to alleviate MIRI.

Borneol is a terpene compound, it is obtained by artificial synthesis or natural product extraction. Borneol has a variety of pharmacological effects. Camphor essential oil is a by-product of natural crystalline borneol, in the acute inflammation model of mice, it inhibits the inflammatory factor IL-1β And IL-6, which showed anti-inflammatory activity in vitro and in vivo [11]. Kononova [12], Shi [13] and others showed that borneol and its derivatives have inhibitory effects on Marburg virus and influenza A virus. In addition, borneol may promote the distribution and absorption of drugs through the action of biological barrier. It can enhance the permeability of various membranes, including gastrointestinal mucosa and skin, and even blood-brain barrier [14-18]. Borneol can promote the absorption of Ligustrazine and increase blood flow during ischemia [19]. In the process of studying the pharmacokinetic characteristics of borneol modified tanshinone IIA liposomes in rats, ye x et al found that borneol modified liposomes could significantly increase the maximum plasma concentration and area under the curve of tanshinone IIA during cerebral ischemia-reperfusion [20]. For ischemia-reperfusion injury, borneol can reduce ischemia-reperfusion injury by regulating apoptosis or autophagy. The combined use of tetramethylpyrazine phosphate and borneol can alleviate cerebral ischemia-reperfusion injury in rats by regulating apoptosis and autophagy [21], and has neuroprotective effects on cerebral ischemia-reperfusion injury [22]. As one of the effective active ingredients of Guanxinshutong capsule, borneol has cardioprotective effect during myocardial ischemia injury and calcium overload inhibition [23].Therefore, we speculate that borneol has a protective effect on myocardial ischemia-reperfusion injury. In this study, we used hypoxia/reoxygenation H9C2 rat cardiomyocyte model to simulate the clinical myocardial ischemia-reperfusion injury, and explored the protective effect of Borneol on hypoxia/reoxygenation induced cardiomyocyte injury and its mechanism related to inhibition of apoptosis.

Materials and methods

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [24].

Experimental reagents

H9C2 rat cardiomyocytes (Procell, batch No.: CL-0089), borneol were purchased from Sichuan Qingshen Kanghua Pharmaceutical Co., Ltd.(batch No.:20190511), fetal bovine serum (Every green, batch No.: 20050503), DMEM high sugar culture medium (boster, batch No.:PYG0021), DMEM sugar free culture medium (Solarbio, batch No.:90013), and trypsin (boster, batch No.:PYG0015), Bax(Cell signaling,batch No.:14796), Caspase-3(Cell signaling,batch No.:14220) and β-Actin(Proteintech, batch No.:10021293) antibody, WB primary and secondary antibody diluent (boster, batch No.:16A07A17), BCA protein assay kit (Dingguo, batch No.:BCA02), RIPA strong lysate (biosharp, batch No.:21210310), total superoxide dismutase (T-SOD) test kit (hydroxylamine method), lactate dehydrogenase (LDH) kit (microplate method) were purchased from njjcbio(batch No.:20210114), Annexin V-FITC/PI double staining apoptosis detection kit (keygen, batch No.:20210112), CCK8 Kit (dojindo, batch No.:SH949), ECL chemiluminescent substrate Kit (biosharp, batch No.:2022.4.06).

Experimental methods

Cell culture and establishment of H/R model

H9C2 rat cardiomyocytes were cultured in DMEM high glucose medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and in a constant temperature incubator with 5% CO_2 at 37 . When cells grew to 70–80% confiffluence, they were pretreated with bornel (8, 16, 32 μg/ml) for 12h (a time point with better

effiffifficacy in cardiomyocytes protection). Hypoxia was induced by culturing H9C2 cells in DMEM without glucose or serum in an atmosphere of 100% N_2. Following hypoxia treatment for 6 h, the H9C2 cells were returned to normoxic conditions (air with 5% CO2) for 24 h. Experiments were performed using cells that were to be subjected to this hypoxia and reoxygenation treatment.

Cytotoxicity test

H9C2 rat cardiomyocytes were pre-treated with 0,4, 8, 16, 32, 64, 128, 256 μg/ml bornel for 12h, 24h, 36h respectively. The viability of H9C2 cells was measured by using a cell counting kit 8 (CCK-8) cell viability assay.

10% CCK-8 reagent was added into each well, followed by incubation for 2 h. The absorbance was measured utilizing a microplate reader at 450 nm. The experiment was repeated six times. The survival rate of H9C2 cells at different pre administration times was calculated. Cell survival rate = (experimental wells blank wells) / (control wells blank wells) * 100%

Cell viability assay

H9C2 rat cardiomyocytes were divided into 5 groups: Control group, hypoxia/reoxygenation group (H/R), H/R+low-dose borneol (8 μg/mL) group (H/R+8-borneol), H/R+medium-dose borneol (16 μg/mL) group (H/R+16-borneol) and H/R+high-dose borneol (32 μg/mL) group (H/R+32-borneol). Cells were grouped according to this method in subsequent experiments. H9C2 rat cardiomyocytes were pre-treated with 8, 16, 32 μg/mL bornel for 12h prior to hypoxia and reoxygenation treatment. H9C2 cells without bornel or hypoxia/reoxygenation treatment served as a control. The viability of H9C2 cells was measured by using a CCK-8 cell viability assay. 10% CCK-8 reagent was added into each well, followed by incubation for 2 h. The absorbance was measured utilizing a microplate reader at 450 nm.

Detection of myocardial injury markers

H9C2 rat cardiomyocytes were divided into 5 groups as mentioned above. The total superoxide dismutase (T-SOD) content was measured after the cells were collected and homogenized by using kits. Cell culture medium was collected and lactate dehydrogenase (LDH) content in culture medium was detected by using kits.

Flow cytometry

Cell apoptosis was detected with the Annexin V-FITC / PI fluorescence double staining apoptosis assay kit by flow cytometry. H9C2 rat cardiomyocytes were divided into 5 groups as mentioned above. The cells from each of the 5 groups were washed with PBS twice and incubated with trypsin at 37@C for 2 min. Following digestion, the cell suspension was centrifuged at 3000 r/min at room temperature for 5 min. The cell pellet was resuspended with PBS and the centrifugation and resuspension steps were repeated twice. The cells were blocked with 10% bovine serum albumin for 30 min at room temperature. Before the test, incubate with annexin V/PI dye solution for 10 min at room temperature in the dark. The apoptosis rate was determined using annexin V-FITC apoptosis detection kit.

Western Blot Analysis

The Western blots were employed to determine the expression of Bax and caspase-3. H9C2 rat cardiomyocytes were divided into 5 groups as mentioned above. The cells of each group were collected 24h after reoxygenation, and total proteins of each group were extracted by using protein extraction RIPA buffffer with protease inhibitor. Protein concentration was estimated by using the BCA protein assay Kit. Proteins samples were separated via SDS-PAGE and transferred to NC membranes. The membranes were blocked with solution containing 5% skim milk powder for 2 h at room temperature and then incubated with primary antibodies (1:1000) at 4 overnight. After washing, membranes were incubated with secondary antibody (1:4000) at room temperature for 1 h. Blots were visualized with ECL^{TM} reagents, and the signals were captured with chemiluminescence detection system. The density of the band was analyzed with Image J

software. The relative expression of Bax, caspase-3 and β-actin was expressed by the ratio of the gray values of actin bands.

Statistical analysis

SPSS 22.0 software and graphpad prism 8.0 were used for data analysis and statistical processing. The data were expressed as mean ± SEM. One way ANOVA was used for comparison between groups. A value of $P<0.05$ was considered as statistically significant.

Results

Effect of different concentrations of Borneol on normal cell survival rate at different time of pretreatment

Compared with the control group, borneol with 4˜128 μg/ml and pretreatment for 12 h and 36 h has a tendency to promote cell growth (Fig. 1). Pretreatment for 12 hours had the strongest effect on cell viability, therefore, borneol pretreatment for 12 hours was used as the way of administration in subsequent experiments. The viability of H9C2 cells was significantly increased after pretreatment for 12 h with 8μg/ml and 128 μg/ml borneol (P <0.01) (Fig. 1). Therefore, it is appropriate to choose 8,16, and 32 μg/ml as low, medium and high doses.

Fig. 1

Effects of different doses of Borneol on the viability of hypoxic/reoxygenated cells

Compared with that in the control group, the viability of H9C2 cells was significantly decreased after hypoxia/reoxygenation treatment (P <0.01; Fig. 2). However, the viability of H9C2 cells treated with 16 or 32 μg/ml borneol prior to hypoxia/reoxygenation was markedly increased compared with that in the H/R group (P <0.01; Fig. 2).

Fig. 2

Effects of Borneol on SOD and LDH in hypoxia/reoxygenation H9C2 cells and culture medium

Compared with control group, the injury caused a significant decrease in SOD content and an increase in LDH overflow in the H/R group (P <0.01; Figs. 3). However, compared to the H/R group, the SOD content in the cells of each treatment group increased significantly and the LDH overflow decreased significantly after borneol treatment (P <0.01; Figs. 3).

Fig. 3

Effects of Borneol on apoptosis of hypoxic/reoxygenated cardiomyocytes

The apoptotic rate of rat cardiomyocytes was significantly increased after hypoxia/reoxygenation treatment as compared with that in the control group (P <0.01; Figs. 4). Borneol pre-treatment significantly protected H9C2 cells against apoptosis after hypoxia/reoxygenation (P <0.01). The results suggest that borneol may inhibit the apoptosis of hypoxia/reoxygenation cells.

Fig. 4

Effects of Borneol on the expression levels of proteins Bax and Caspase-3

The result displays the expression levels of the pro-apoptotic proteins Bax and caspase-3 in cells without or with hypoxia/reoxygenation exposure in the absence and presence of 8, 16, and 32 μg/ml borneol. In rat cardiomyocytes, hypoxia/reoxygenation insult, as in ischemia/reperfusion insult, up-regulating Bax and caspase-3. Borneol can decrease the up-regulated Bax and cleaved caspase-3. Borneol 8 μg/ml and 32μg/ml can significantly decrease the up-regulated Bax (p <0.05; Figs. 5), and borneol 16 μg/ml and 32μg/ml can significantly decrease the up-regulated caspase-3 (p <0.01; Figs. 5). These results confifirmed that borneol may antagonize the apoptosis induced by hypoxia/reoxygenation insult.

Fig. 5

Discussion

In the process of establishing hypoxia/reoxygenation cardiomyocyte model, it was found that the survival rate of cells after hypoxia for 6h was significantly decreased, indicating that the model was successful. After borneol treatment, compared with H/R group, the cell survival rate of each treatment group had an upward trend. The leakage of LDH indicates that the cell energy metabolism is impaired or redox imbalance may occur. The leaked LDH can damage the membrane lipid by generating peroxide and generating peroxidation damage. However, due to the stable nature of LDH, it can reflect the degree of cell damage, and the increased permeability of the plasma membrane leads to the rapid overflow of LDH, which is a key feature leading to cell damage [25]. Compared with H/R group, LDH leakage of each treatment group decreased significantly, suggesting that borneol alleviated hypoxia/ reoxygenation induced injury.

In the hypoxia environment, the cellular oxidative metabolism process is abnormal, and many free radicals will be produced, resulting in oxidative stress reaction, leading to DNA damage, lipid peroxidation and protein oxidation [26]. The oxidative stress reaction will be initiated by the accumulation of excessive oxygen free radicals, leading to further damage. However, the antioxidant system in cells can neutralize free radicals to protect cells from the damage of oxygen free radicals and reduce the damage of cells [27,28]. Superoxide dismutase (SOD) can protect cells from free radical damage. The experiment showed that the SOD content of each treatment group was significantly higher than that of H/R group. Similarly, transgenic mice overexpressing wild-type human SOD1 attenuated brain injury after transient ischemia/reperfusion [29]. Increasing the content of SOD is beneficial to the protection of injury. The increase of SOD content in each group pretreated with Borneol also confirmed the protective effect of Borneol on hypoxia/reoxygenation induced injury.

During the execution of apoptosis, Bcl-2 protein family located on the mitochondrial pathway regulates myocardial survival and death in MIRI [30,31]. The Bcl-2 protein family can be divided into pro apoptotic proteins and anti apoptotic proteins according to different domains [32]. The increase of Bcl-2/Bax ratio indicates the inhibition of apoptosis process and contributes to the survival of infarcted myocardium. The upregulation of Bcl-2 also further inhibited the caspase cascade and the opening of mitochondrial osmotic transition pore, inhibiting the activation of Caspase-3 [33]. At the same time, inhibition of apoptosis has an important impact on the degree of myocardial injury and prognosis. In the rat model of myocardial ischemia-reperfusion in vivo, ethyl pyruvate (EP) inhibits the apoptosis of cardiomyocytes by reducing the expression of Bcl-2-related X protein and cleaved caspase-3 and plays a role in protecting the myocardium [34]. At the same time, the results of this experiment showed that after borneol treatment, the cell apoptosis rate of each treatment group was significantly decreased, and the expression of Bax and Caspase-3 in the borneol high-dose group was significantly decreased.

The protective effect of Borneol on cerebral ischemia-reperfusion has been widely confirmed, and borneol derivatives also have protective effects on H/R cardiomyocytes. In this context, we confirmed that borneol can reduce the damage of cardiomyocytes in the case of cell damage caused by H/R. The protective effect can be achieved by reducing LDH overflow, increasing the content of SOD in cells and reducing the expression of Bax and caspase-3.

Conclusion

In conclusion, our results demonstrate that borneol has a definite protective effect on hypoxia/reoxygenation-induced injury of rat cardiomyocytes, and its protective mechanism can be related to the inhibition of apoptosis by reducing the expression of Bax and Caspase-3.

Contributions Yang-you Li contributed to conception of the study and perform the analysis with constructive discussions. Hui Zhang and Jing Wen contributed to the experiment and performed the data analyses and wrote the manuscript. Jiang-wu Zhang revised the manuscript and analysis with constructive discussions. Hai-yi Xu and Ting Liu performed the experiment and revised the manuscript.

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Declarations

Consent to Publish All authors read and approved the final manuscript and agreed to submit it for consideration for publication.

Conflict of Interest The authors declare that they have no conflict of interest.

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