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Hypoxic Preconditioning Attenuated Simulated Ischemia/Reperfusion Injury in Cultured Bone Marrow Stromal Cells

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Abstract

Aim: Bone marrow stromal cells (BMSCs) are a promising candidate for cell therapy in ischemic stroke. However, the majority of BMSCs are readily lost after transplantation because of apoptosis due to ischemia-reperfusion (I/R) injury. In the present study, we aimed to evaluate the effects of hypoxia preconditioning (HP) on simulated I/R injury in cultured BMSCs.

Methods: Four generations of BMSCs were used as subjects. BMSCs were divided into 6 groups, including normal group, I/R group(I/R followed 0 h HP), 2 h-HP group(I/R followed 2 h HP), 4 h-HP group(I/R followed 4 h HP), 6 h-HP group (I/R followed 6 h HP) and 8 h-HP group(I/R followed 8 h HP) 2 h-HP group(I/R followed 2 h HP), 4 h-HP group(I/R followed 4 h HP), 6 h-HP group (I/R followed 6 h HP) and 8 h-HP group(I/R followed 8 h HP). BMSCs were subjected to HP by exposing the cells to hypoxia (2%O_2). After 12 h of reoxygenation, BMSCs were suffered from 3 h ischemia (<0.5% O_2 and serum deprivation) and subsequent reperfusion (I/R). Cell viability, hypoxia-inducible factor (HIF) 1-α, caspase-3 and apoptosis were tested using MTT, ELISA and immunofluorescence staining respectively.

Results: There were no changes in cell viability after 2 h to 8 h HP in cultured BMSCs as compared to the normal group. But HP upregulated the content of HIF-1α in BMSCs and protected the BMSCs from later I/R injury, as evidenced by increased cell viability and decreased Caspase-3 release and apoptosis, especially in 8 h-HP group

Conclusion: HP attenuated the apoptosis induced by I/R injury via co-regulating the expression of HIF-1α and Caspase-3 in BMSCs, and thereby might play a beneficial effect on cell therapy.

Keywords: Hypoxic preconditioning (HP); Cell viability; Hypoxia-inducible factor (HIF) 1-α; Apoptosis; Ischemia/reperfusion (I/R) injury

Abbreviations: ANOVA: analysis of variance; BMSCs: Bone marrow stromal cells; ELISA: enzyme-linked immunosorbent assay; HP: hypoxia preconditioning; HIF: hypoxia-inducible factor; I/R: ischemia-reperfusion; PBS: phosphate buffered solution

Introduction

Ischemic stroke is one of the leading causes of death and disability in the world, but there are currently few effective clinical therapies for it. Alternatively, cell-based therapy has provided a promising hope to enhance tissue repair and functional recovery after stroke [1-3]. Bone marrow stromal cells (BMSCs) can be easily obtained from patients themselves without ethical or immunological problems and can proliferate massively in vitro. After transplantation, its can promote the neurogenesis and angiogenesis in ischemic brain [4]. However, the cell therapy is limited by the poor survival of the transplanted cells in brain lesion [5]. Studies confirmed that a large number of transplanted cells died in ischemic brain because of hypoxia, oxidative stress, inflammatory mediators and the deficiency of trophic factor [6,7]. Thus, it is imperative to identify therapies that can improve the viability of the stem cells in the hazardous ischemic tissue. The physiological oxygenated environment for BMSCs in bone marrow not exposed to atmospheric oxygen is thought to range from 2-8% [8]. Recent study showed hypoxic preconditioning(HP) could decrease apoptosis induced by anoxia in cultured hippocampus neurons [9]. So in this study, we wanted to study whether HP might attenuate the apoptosis of BMSCs from stimulated ischemic/reperfusion (I/R) injury. We elaborated cell viability, hypoxia-inducible factor (HIF) 1-α, apoptosis and Caspase-3 appreciate in this protection mechanism. Furthermore, the proper time of HP involved was also studied.

Materials and Methods

Isolation and culture of BMSCs

BMSCs were isolated as previously described (Deng et al., 2004) [10]. Briefly, we humanely killed male Sprague-Dawley rats (3-4 weeks old), bone marrow was obtained from the rat femurs and tibias. Cells were centrifuged at 1000 rpm for 5 min and suspended in Dulbecco's modified Eagle medium with low glucose (L-DMEM, Invitrogen, Carlsbad, CA) supplemented with 12.5%% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). Cell cultures were kept in humidified 95% air-5% CO_2 at 37°C. After the cells were incubated for 3 d, the culture medium was changed. Once the BMSCs reached 80% confluence, the adherent cells were digested using 0.25% trypsin with EDTA. At passage 4 (P4), cells were harvested for experiments.

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Hypoxic Preconditioning (HP) and experimental protocols

Cultured BMSCs were divided into 6 groups(n=5/group): (1) the normal control group, BMSCs were placed in normal culture condition(21%O_2 and normal cultured medium) during the all experiment; (2) I/R group: BMSCs were cultured in L-DMEM without FBS and exposed to hypoxia for 3 h (<0.5% O_2) in an airtight chamber (NAPCO 7101 FC-1), followed by 24 h of reoxygenation (21%O_2 and normal cultured medium). There was no HP treatment before I/R injury. (3) 2 h-HP group: 2 hours HP treatment before I/R injury. (4) 4 h-HP group, 4 hours HP treatment before I/R injury. (5) 6 h-HP group: 6 hours HP treatment before I/R injury. (6) 8 h-HP group: 8 hours HP treatment before I/R injury. BMSCs were subjected to HP by exposing the cells to hypoxia (2%O_2), after 12 h of reoxygenation, BMSCs were suffered from 3 h I/R injury. The oxygen level in the chamber was monitored with an oxygen analyzer; some assays were carried out immediately after HP, others assays were administered followed the I/R attack. We use a graphical to depict our study in briefly (Figure 1).

Cell viability assay

The cells were seeded in 96-well plates at a density of 5×10^4 cells/well at passage 4. After HP and I/R procedure, the media was replaced with 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)(Sigma-Aldrich, USA)-containing DMEM, and cultured for 4 h in a CO_2 incubator respectively. An equal volume of solubilization solution (10% SDS, 0.01 M HCl) was added, and the plate was incubated at 37°C overnight to solubilize formazon crystals. When dissolved in SDS, functional mitochondrial succinate dehydrogenase in cells can convert MTT to formazan that generates a blue color. After the purple formazan producer was dissolved, the intensity was measured at an absorption wave length of 570 nm and 630 nm with a microplate reader.

ELISA for HIF-1α and Caspase-3

BMSCs in 6-well plates were covered with 2 ml of culture medium and experienced 2 h, 4 h, 6 h and 8 h HP respectively, culture medium were withdrawn after HP, HIF-1α levels were measured in these samples with an ELISA kit for rat HIF-1α (CUSABIO, China) according to the manufacturer's instructions. The protein extract was obtained from the supernatant of BMSCs.

Hoechst 33342 fluorescent stain

When cells sufficiently spread on the slides, the cells were hed with cold phosphate buffer sodium (PBS, pH 7.0) solution and fixed in 4% methanol for30 min. After washing with PBS for 5 min, the cells were incubated with 0.1% acetic acid for 30 s, and then washed again with PBS. Cells were thoroughly air-dried at room temperature, and then stained with Hoechst 33342 for 10 min, followed by washing with distilled water for 1 min and again air-drying at room temperature. Finally, the BMSCs were observed under fluorescence microscope and the apoptotic cells were photographed.

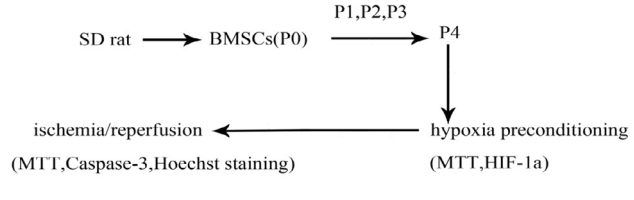

Figure 1: Experiment procedure.

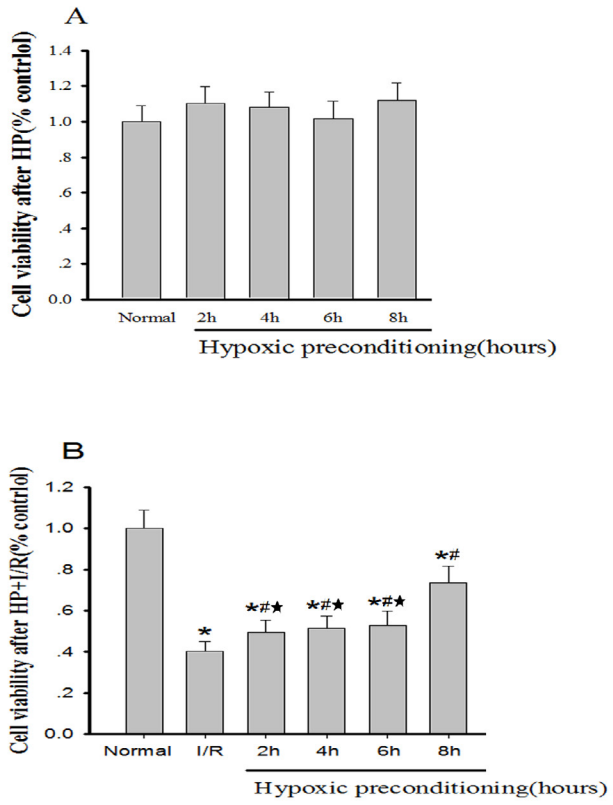

Figure 2: Cell viability measured by MTT assay. (A): Cell viability measured after HP, there was no statistic difference among all groups. (B): Cell viability measured after I/R injury, Y axis value=OD value(570-630 nm)(HP groups)/OD value(570-630 nm)(normal group)%, *p<0.01, vs. normal group , #p<0.01, vs. I/R group, *p<0.01, vs. 8 h HP group.

Statistical analysis

All measurements were performed blindly. Results were presented as mean ± SD. Statistical analyses were performed using SPSS 17.0 software. The statistical differences between different groups were evaluated with one-way analysis of variance (ANOVA) and post hoc test LSD (Fisher's Least Significant Differences) for multiple comparisons. A value of P<0.05 was considered statistically significant.

Results

Effect of HP on BMSCs viability

There were no statistical differences in the cell viability among the normal culture and HP treated BMSCs groups (Figure 2A). Increasing exposure to 2% O_2 for 12 to 24 h resulted in duration-dependent apoptosis (data are not shown here). This suggested that BMSCs exposed to hypoxia from 2 h to 8 h remained fully viable similar to normoxia control BMSCs. But cell viability was decreased after subsequent I/R (<0.5% O_2-3 h, without FBS, followed by reoxygenation) injury. However, HP treated BMSCs reduced the injury, especially 8 h HP before I/R insult presented an outstanding protection (Figure 2B).

HP induced additional HIF-1α expression in BMSCs

To further understand the mechanism of the protection of HP on anoxia, we tested the HIF-1α expressions in normal and HP treated BMSCs. We found the contents of HIF-1α, a survival factors for some

cell lines in response to hypoxia, in the BMSCs was significantly upregulated after HP($P<0.05$), and 8 h-HP showed a prominent role($P<0.01$) (Figure 3).

HP inhibited the activation of Caspase-3

Caspase-3 is released by damaged degenerating cells and plays a key role in the mechanism of apoptosis. Here we tested the production of activated caspase-3 in BMSCs. As shown in Figure 4, the contents of activated caspase-3 were obviously increased in I/R group, but HP powerfully inhibited the harmful reaction ($P<0.05$). Similarly, 8 h-HP showed a significant antiapoptosis role ($P<0.01$, vs. I/R group).

HP decreased the expression of apoptotic body

Hoechst 33342 staining was used to observe apoptotic body. Only a few cells in normal culture group showed positive staining in the nuclei(apoptotic cell), while as a large number of cells in I/R group exhibited strong staining in the nuclei, but the apoptotic density was obviously decreased in 8 h-HP I/R injury group (Figure 5).

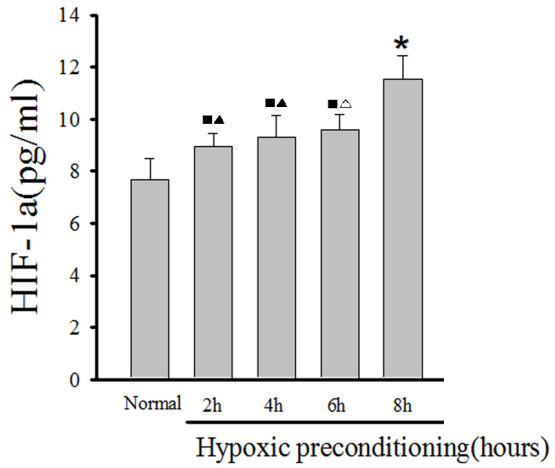

Figure 3: The content of HIF-1a in cultured BMSCs after I/R injury. ▪p<0.05, vs. normal group, *p<0.01, vs. normal group, ▲p<0.01, vs. 8 h HP group, △p<0.05, vs. 8 h HP group

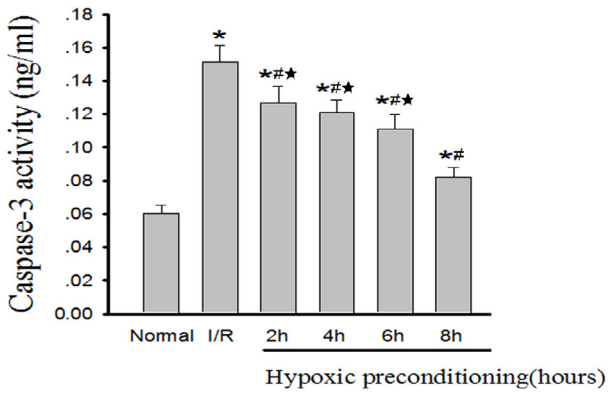

Figure 4: The content of active Caspase-3 in cultured BMSCs after I/R injury. *p<0.01, vs. normal group, #p<0.01, vs. I/R group, △p<0.01, vs. 8 h HP group.

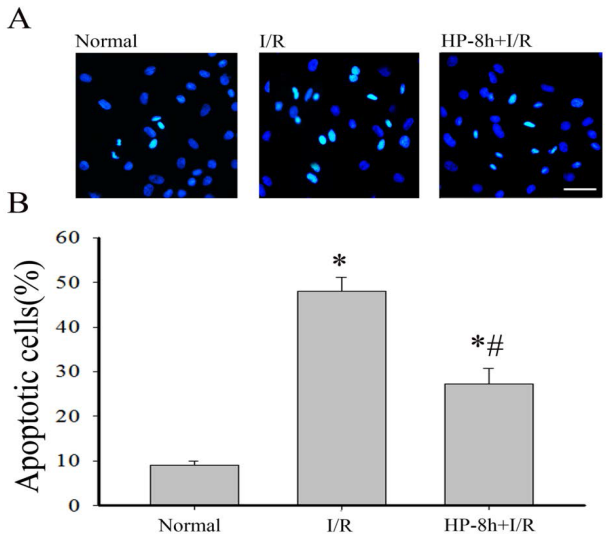

Figure 5: Influence of HP on BMSCs apoptosis after I/R injury. A: Hoechst 33342 staining in the cultured BMSCs, the strong staining nucleus represent the apoptosis cells. B: The percent of apoptotic cells in normal group, I/R injury without HP group and I/R injury with 8 h-HP group. *p <0.01 vs. normal group, #p <0.01 vs. I/R group. scale bar indicates 200 μm.

Discussion

Transplantation of BMSCs is a potential therapy for ischemic stroke, however, a constant outcome that dramatically impairs the efficacy of the cell therapy is the limited number of stem cells surviving after graft in brain lesion [11,12]. Thus the valid defensive measures have been sought to prevent the engrafted cells from ischemic injury for a long time. A large number of data have been reported that the application of brief, transient periods of non-lethal hypoxia (hypoxic preconditioning, HP) before a subsequent lethal episode of hypoxia markedly increase the endurance against followed severe anoxia and delay the development of programmed cell death [13-15]. Thus, HP might provide a new and hopeful strategy for cell therapy in ischemic diseases through the transformation of gene expression and activation of intracellular signaling pathways [16-20]. In vitro experiment, HP protected the myocytes from succedent hypoxia/reoxygenation injury by decreased apoptosis and LDH release and increased cell viability [21]. *In vivo*, HP was shown to be neuroprotective against ischemic brain injury through the upregulation of pro-survival and endogenous regeneration [22,23]. In the present study, we firstly simulated the physiological oxygenated tension (2%O_2) of BMSCs as the HP condition in cultured BMSCs and analysed the optimal time duration and mechanism of HP in reducing I/R injury. We found 2 h to 8 h HP did not arouse any harmful effect on cell viability. In addition, the contents of HIF-1a in BMSCs were up-regulated followed HP, when cells subsequently suffered from I/R injury, the decreased caspase-3 release and increased cell viability were seen in HP groups as compared to I/R without HP group.

HIF-1a is a transcription factor specifically activated by hypoxia, in proper low oxygen pressures, the level of HIF-1 a is regulated to maximal involving multiple mechanisms of control at the levels of mRNA expression, protein stability, nuclear translocation and transactivation activity [24], and play a protective role in the anti-apoptosis after ischemia [25,26]. Caspase-3, one member of the caspase family, is in the downstream of Bax, and particularly believes to be most commonly involved in the execution of apoptosis induced by

many stimuli, including cleaving DNA repair molecules, depredating the anti-apoptosis proteins, cleaving extra cellular matrix protein and other related molecules [27,28]. Wang et al reported that apoptosis suppression by HP correlated with the prevention of mitochondrial dysfunction and promotion of ERK and Akt phosphorylation in hypoxia and reoxygenation injury [29], and Akt signaling pathway was also found to play a critical role in the prevention of apoptotic cell death by inhibiting caspase release [21]. Moreover, the over expression of Bcl-2 and maintenance of MMP in HP cells presumably involves in the protective effects [30,31]. In this study, the up-regulated expression of HIF-1a protected the BMSCs from subsequent I/R injury as evidenced by decreased caspase-3 release and increased cell viability in HP groups, 8 h HP represented a prominent role in antiapoptosis after I/R injury.

A proper HP time is also important for defending ischemia, prolonged HP failed to exert protective effects and even seemed deleterious in some cells [29]. In our system, sublethal hypoxia 2 h to 8 h before critical insult time-dependently attenuated apoptosis and resulted in discrepant cell viability, 8 h-HP presented a distinguished activeness. The underlying mechanism needs further research.

In conclusion, HP could protect BMSCs from critical anoxia via up-regulating HIF-1a content and decreasing caspase-3 release and apoptosis in cultured BMSCs. A suitable HP time might develop a more considerable protection, which would produce a beneficial effect on cell therapy.

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