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Combined preconditioning with hypoxia and Hydrogen Peroxide Improved efficiency of Mesenchymal Stem Cell in cell culture

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ABSTRACT

Preconditioning with hypoxia or ROS such as hydrogen peroxidase improved long term survival of Mesenchymal Stem Cells. But combined therapy with both of them is not investigated enough in cell culture. Therefore, the present study investigates the protective effects of co-preconditioning of the cells with H_2O_2 and hypoxia in cell culture. Bone Marrow MSCs were cultured and divided in 6 groups; control, groups I and II exposed to H_2O_2, group III treated with $COCL_2$, Groups IV and V treated withbothH2O2and$COCL_2$ during 6, 12, 24 and 48h. Then, all groups were underwent to lethal dose of H_2O_2 (300 μM) for 24 hours followed by 24h recovery. Afterward, MTT assay and trypan blue staining were conducted to evaluate the cell proliferation and viability. Also, TUNEL was done to study the cell apoptosis. Cell proliferation and viability after preconditioning with H_2O_2 and hypoxia were considerably increased compared to the control group after 6, 12, 24 and 48 hours ($P < 0.01$). Furthermore, cell proliferation and viability in groups IV and V were remarkably more than control and others preconditioning groups ($P < 0.01$). Apoptosis was significantly decreased in preconditioned groups compared to the control group after 12 ($P < 0.01$), 24 ($P < 0.05$) and 48 hours ($P < 0.01$). Also, cell apoptosis was decreased significantly in groups IV and V after 12, 24 and 48 hours compared to control and other preconditioning groups ($P < 0.01$). This study clearly demonstrated that preconditioning with H_2O_2 and $COCL_2$ can improve BMMSCs proliferation and viability and also have inhibitory effect on cell apoptosis.

Keywords: Bone Marrow Mesenchymal Stem Cells, hypoxia, oxidative stress, co-preconditioning.

INTRODUCTION

MSCs are adult pluripotent cells, which can be found in several tissues such as bone marrow, adipose tissue, peripheral blood, pulp, kidney, synovial membrane and fluid and even saphenous vein[1]. They easily propagated *in vitro* and have immunological compatibility and low risk of malignancy[2].It has been shown that the actual number of MSCs for tissue regeneration is very low after cell therapy because of less survival rates and proliferation[3].Normally, Stem cells are located in places called Niche that maintain their Properties[4]. After isolation and transplantation to a new microenvironment, they face to some problems that decrease their efficiency[5]. Different microenvironments during cell propagation in culture systems or in injured tissue after injection, they should protect themselves from detrimental effects of thermal shock, food shortage, free radicals, ischemia and hypoxia[6]MScs gradually decrease in number because of cell death and their regenerative capability dramatically mitigate[7].Currently, MSCs based therapy is promising but its therapeutic properties need more attention. Recently, some strategies are being designed to enhance stem cells efficiency before application in in

vivo[8]. However, most of researches in this field are in preclinical stage and require more considerations. Preconditioning of MSCs as a new modality is encouraging in preclinical and clinical application. Preconditioning might induce some pathways in the cells that result in more survival rate and less apoptosis[9, 10]. Treatment of cell with agents such as heat shock, food shortage, free radicals mainly H2O2, ischemia and hypoxia might increase the expression of protective genes and proteins in these cells[11]. In many studies Hydrogen peroxide(H_2O_2)was applied *in vitro* to induce a form of cellular damage similar to oxidative stress[12].Oxidative stress begins when the amount of free radicals are more than the ability of the cells to scavenger them[13]. At low concentrations, H_2O_2 could activate various enzymes like phosphatases and cell signaling. However, at high concentrations, it could lead to irreversible cell damage similar to a strong oxidative stress[12].Oxygen is essential for aerobic metabolism in all mammalian cells to maintain function and homeostasis. The cells are sensitive to hypoxia and respond to it[14]. It is confirmed that Mesenchymal stem cell normally reside in 2-8 % oxygen in their niche[15]. For propagation of these cells in culture system 20% oxygen is used[16]. Therefore, oxygen should consider carefully when the cells culture in in vitro. Furthermore, hypoxia induced proliferation of hematopoietic bone marrow stem cells[17] which are self-renewing precursor cells of non-hematopoietic stromal tissues, are currently under intense investigation for cardiac repair[18]. In addition, MSCs can suppress local inflammation [19]. Hypoxic preconditioning attenuated cortical infarction in the rat brain induced by oxidative stress [20] and protected retinal morphology and function against light induced apoptosis [21]. There are several studies about application of H2O2 and hypoxia each modality alone in cell culture of MSCs[14, 22] but our knowledge about combined therapy that uses both of them at same time is limited. Therefore, present study not only determines the effects of each modality alone but also it determines combined therapy on viability and proliferation of MSCs. The mechanisms through which the modality affects the cells behaviors were investigated.

MATERIALS AND METHODS

Cell culture

BMMSCs were extracted from the femur and tibia of 6 weeks old Wistar albino male rat and suspended in the low glucose Dulbecco's minimum essential medium (DMEM) (Gibco, Invitrogen, CA, USA) with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, CA, USA). Then, MSCs were centrifuged (3000g for 3 min) and re-suspended in DMEM medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells were incubated in 5% CO2 at 37C for 48 h, and the adherent cells were washed twice consecutively in phosphate-buffered saline (PBS) (Gibco, Invitrogen, CA, USA). The cultures were depleted of erythroid progenitor cells through the removal of cells that did not adhere to the culture dishes with medium changes. At 80% confluence, cells were detached with trypsin-EDTA and passaged at a ratio of 1:3. Passage 4 of the cells was used in present study. The medium was changed every 3 days.

Differentiation capability of BM-MSCs

The multi-potency of BM-MSC was confirmed by induction of osteogenic, chondrogenic and adipogenic differentiation using specific differentiation media.

Osteogenic differentiation

BMMSCs were seeded at density2×10^4cells/cm^2 in 24 well plate and then induced to differentiate in an osteogenic induction medium composed of DMEM with 10% FBS (Gibco, Invitrogen, CA, USA), 100 units/ml penicillin and 100 g/ml streptomycin,10nM dexamethasone, 50 µg/ml ascorbic acid, 10 mMβ-glycerophosphatefor 21 days. After21 days Osteoblast differentiation was evaluated by2%Alizarin Red(Sigma) staining. Cells were washed three times with PBS(Gibco, Invitrogen, CA, USA) and fixed for 15-30 min with 4% formaldehyde. The cells were stained with Alizarin Red and examined under microscope.

Adipogenic differentiation

The cells were cultured at a density of 2×10^4 cells/cm2 in24well with adipogenic Induction Medium including of DMEM, supplemented with 10% FBS, 100 units/ml penicillin and 100 g/ml streptomycin, 100nM dexamethasone, 50µg/mlindomethacin for 10 days.Fat droplets were examined under microscope.

Chondrogenic differentiation

The cells were cultivated at a density of 2×10^4 cells/cm2 in24wellplate. The cells were cultured under Chondrogenic induction medium for 21 days. After21 days, the cells were stained with Alcian blue (Sigma). Briefly, the wells were washed with PBS and fixed with 4% paraformaldehyde for 15-30min. The cells were stained with Alcian blue for 30 min and examined under microscope.

Preconditioning of the cells with H_2O_2

The cells cultured in 96well plates in 10^4concentration and treated with different doses of H_2O_2 (0, 5, 10, 15, 20, 30, 40, 50, 60, 70,80,90, 100 µM)during 6, 12, 24 and 48 hours. Then the cells exposed to lethal dose of H_2O_2 (300 µM) for 24h followed by 24h recovery. Cell proliferation was examined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide(MTT). First, 100µl of the culture medium was removed and 15µl of MTT (5mg/ml) was added to each well. The plates were incubated at 37˚C in a 5% CO_2 for 3 hours. Then 200µl DMSO was added to dissolve the formazan crystals and. Optical density (OD) was spectrophotometric ally measured at 570 nm using an ELISA reader (TECAN/ sunrise, Magellan program, Austria).Cell proliferation percent was calculated using the following formula:

$$OD = \frac{\text{OD of exprimental group}}{\text{OD of control}} \times 100$$

Preconditioning of the cells with$COCL_2$

The cells cultivated in 96well plates and treated with different doses of $COCL_2$(5, 10, 20, 50, 70, 90, 100, 120, 150, 200 µM)for 6, 12, 24 and 48 hours. Then, the cells exposed to lethal dose of $COCL_2$ (200 µM) for 24hours and 24hours recovery of the cells with fresh medium. Afterward, cell proliferation was performed with MTT assay.

BMMSCs Preconditioning with H_2O_2 and$COCL_2$

The cells were seeded in 96well plates and treated with 5or 10µM $H_2O_2$and 120µM cobalt chloride for 6hours. For 12 and 24h, the cells exposed to 5 or 10µM H_2O_2 and 20 µM cobalt chloride and finally for 48h the cells exposed to 5 or 10µM H_2O_2 and 5µM cobalt chloride. In all experiments there was a control group which did not received H_2O_2 and $COCL_2$. Then the different groups received 300 µM lethal dose of H_2O_2 for 24h followed by 24h recovery. BMMSC proliferation was measured by MTT assay and cells viability with Trypan blue staining. Also cells apoptosis examined by TUNEL. The experiments were repeated three times.

Evaluation of cell Viability with Trypan blue

BMMSCs were seeded in a 6-well plate and incubated at 37˚C in 5% CO_2.The cell viability was evaluated after treatment with H_2O_2 and $COCL_2$ by the Trypan blue(0.4%, Sigma-Aldrich, StLouis) dye exclusion method. Dead cells were stained in blue color. Viability percentage was measured according to below formula:

Cell viability (%)= living cells /total number of cells ×100

Detection of apoptosis

Sterile slides were placed on bottom of 6well plates and the cells were seeded on it. After adhesion, the cells preconditioned with H_2O_2 and $COCL_2$ as mentioned previously for 6, 12, 24 and 48h. Apoptosis method was performed using In situ cell death detection kit (Rochea, Canada). Briefly, the cells were washed with PBS (Gibco, Invitrogen, CA, USA) and fixed with4% paraformaldehyde for 60 minutes at room temperature. After three times swashing with PBS, the cells were exposed to blocking solution (3% H_2O_2 in methanol) for 10 minutes in 15-25°C followed by 5 minute incubation with 0.1% Triton x-100 on ice. After three times washing with PBS, cells were resuspended in 50µl of TUNEL Reaction solution for 60 minutes at 37°Cin moisture chamber. Then, after three times washing in PBS, the slides were incubated with 50µl of POD solution for 30 minutes at 37°C in a humidified chamber and finally treated with DAB for 10 minutes in the dark. After washing the slides with PBS, they were observed by light microscope under 40X magnification.

Statistical analysis

The data are expressed as mean ±standard deviation (SD). Statistical comparisons were analyzed by one-way analysis of variance using Tukey test for multiple comparisons by SPSS software version 22. Differences were considered significant at $p<0.05$.

RESULTS

MSC Characterization

MSCs were propagated using standard procedures and sub cultured at least for four passages before treatment with $COCL_2$ and H_2O_2. The BMMSC continued to display a uniform fibroblast-like appearance throughout the culturing process (Figure 1). They had capability to osteogenic, adipogenic and chondrogenic differentiation (Figure 2).

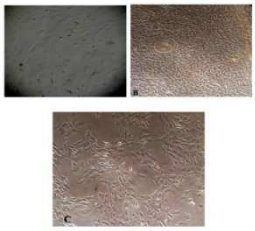

Figure 1.BMMSC after 5 days of primary calture (A) passage 3 (B) passage 4 (C) magnifications100X

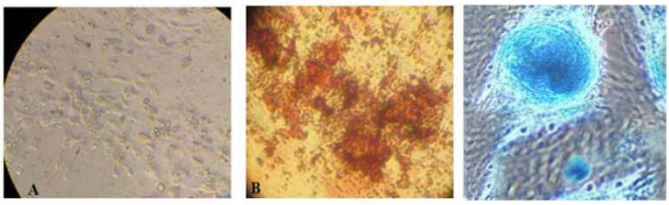

Figure 2.Differentiation property of MSC to adipogenesis (A) osteogenesis(B) and Chondrogenesis.

Morphological study

The current study demonstrated that treatment of the cells with $COCL_2$ did not change the cell morphology and they have a fibroblast-like morphology before and after exposure to $COCL_2$(Figure 3).

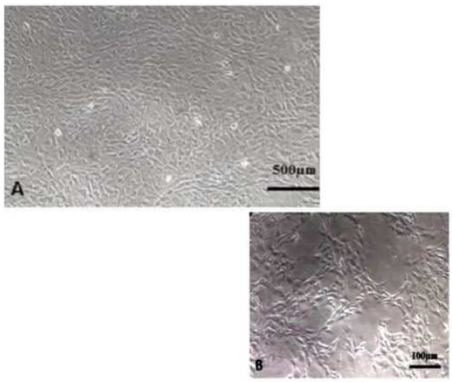

Figure 3.BMMSC have a fibroblast-like morphology before hypoxia preconditioning (A) spindle like cells after treatment with $cocl_2$ with no change in their morphology(B)

Combined preconditioning improved cell proliferation

Cell proliferation in groups IV and V (treated both with COCL2 and H_2O_2)increased significantlyafter6 and 12 hours toward the control and other preconditioning groups ($P < 0.01$).Also, there were significant differences in cell proliferation between all preconditioning and control groups after 24 hour ($P < 0.01$).After 48 hours of treatment cell proliferation in groups II, III, IV and V increased compared to the control ($P < 0.01$). Moreover, a significant increase in cell proliferation was observed in groups IV and V(treated with COCL2+ H_2O_2) compared to control and other preconditioning groups in all 6, 12, 24 and 48hours ($P < 0.01$) (Figure 4).

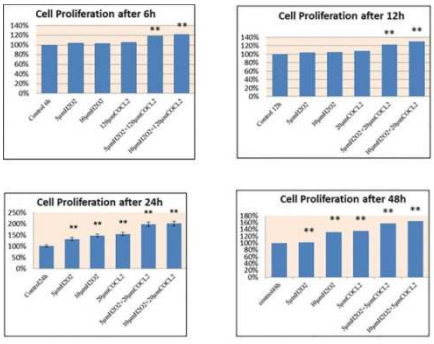

Figure 4.BMMSC$_s$Proliferation under preconditioning with different concentrations of COCL2 and H_2O_2 at 6, 12, 24 and 48 hours. Cell proliferation was significantly increased after preconditioning. Data are mean ± standard deviationA significant difference < 0.01 toward control group.**

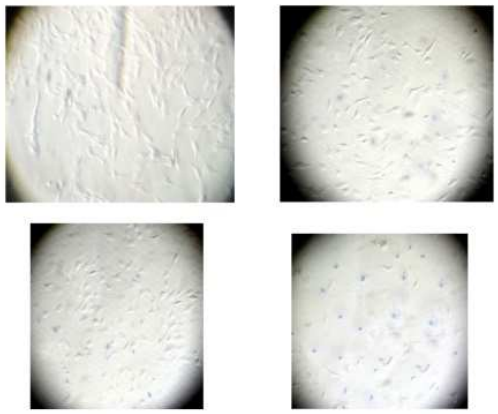

Figure.6. Intact MSC (A), trypan blue staining in control group (B), the cells were stained with trypan blue in combined groups (COCL2 plus H2O2). Blue dye shows the dead cells (C and D).

Hypoxia and H2O2 preconditioning increased cell survival

Cell viability in groups IV and V(treated with $COCL_2$+ H_2O_2) was significantly higher than control and other groups after 6 and 12 hours ($P < 0.01$). Furthermore, after 24h cell viability was significantly increased in group II ($P < 0.05$) and group III ($P< 0.01$).Also there was considerable increased in cell viability in groups IV and V(treated with $COCL_2$plus H_2O_2) toward the control group after 24h ($P< 0.01$). (Figure.**5**). After 48 hour cell viability in group III ($P < 0.05$) and in groups IV and V (treated with $COCL_2$+ H_2O_2) was remarkably increased compared to control group after 48h($P < 0.01$).In addition cell survival in groups IV and V (treated with $COCL_2$+ H_2O_2) was significantly increased compared to control and other preconditioning groups during6, 12, 24 and 48h ($P < 0.01$)(Figure5, 6).

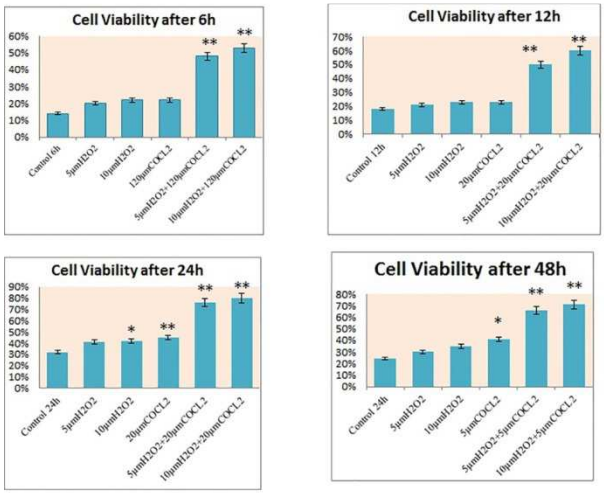

Figure 5.BMMSC$_S$Viability after preconditioning with different concentrations of COCL2 and H_2O_2 at 6, 12, 24 and 48 hours. Cell Viability was significantly increased after preconditioning. Data are mean ± standard deviation. * A significant difference < 0.05, ** A significant difference < 0.01, toward control group.

Co-preconditioning of the cells with hypoxia and H_2O_2 inhibited cell apoptosis

Apoptosis after 6 hour did not show any significant decrease compared to control group ($P < 0.05$). However, cell apoptosis in groups IV and V (treated with $COCL_2$+ H_2O_2) was significantly lower than control group after 12h ($P < 0.01$), 24h ($P < 0.05$) and 48 hour ($P < 0.01$)(Figure7, 8).

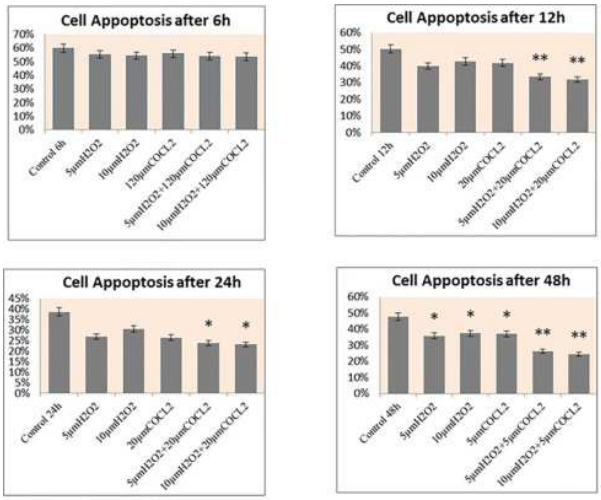

Figure 7. Rate of apoptosis in BMMSC after preconditioning with different concentrations of COCL2 and H_2O_2 at 6, 12, 24 and 48 hours. Cell Apoptosis was significantly decreased after preconditioning. Data are mean ± standard deviation. * A significant difference < 0.05, ** A significant difference < 0.01, toward control group.

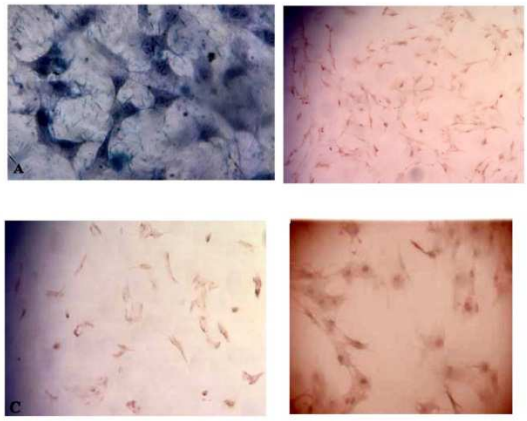

Figure 8 Mesenchymal stem cells after staining with Hematoxylin as negative control group (A). Apoptotic cells in Control group (NP) (B). Apoptotic cells in combined groups preconditioned with COCL2 plus H2O2(C and D). Magnification: 40X

DISCUSSION

MSCs-based therapy has potential value in tissue replacement and regeneration. Currently, there are many clinical trials studies of stem cell therapy[23]. However, application of MSCs in cell therapy has been limited due to some problems such as their low proliferation rate[24], restricted life span, apoptosis and gradual loss of stemness during ex vivo expansion[25].Therefore, we studied the BMMSCs survival, proliferation and apoptosis after their preconditioning under hypoxic and oxidative stress conditions. In the present study, preconditioning of BMMSCs by H_2O_2 and $COCL_2$ at 6, 12, 24 and 48 hours showed significant increase in cell proliferation and viability. Also, cell apoptosis rate was significantly declined compared to the control group after preconditioning. More interestingly, combined preconditioning had more protective effects against cell damage after high dose of ROS. The results also indicated that groups IV and V (treated with $COCL_2$plus H_2O_2) at short-time of experiment (6 and 12 hours) did not show considerable changes in cell survival , proliferation and decreasing cell apoptosis compared to longer-times of experiment (24 and 48 hours).Interestingly, although the cells at 12 and 24 hours was preconditioning with the same dose ofH_2O_2(5 and 10 µM) and $COCL_2$ (20 µM), but results at 24 hour was better than 12 hour($P < 0.01$).It is clear that oxygen tension is an important element in maintenance of MSCs stemness and for determination of their fate [26]. For example, O2 concentration of 0.5–1 % reduces apoptosis, and enhances regenerative capacity of bone marrow derived-MSCs (BM-MSCs) for repairing infarcted myocardium [27]. However, some studies have shown cytotoxic effects of $COCL_2$ (as hypoxia mimetic agent) in concentrations higher than 200 mmol/L for more than 24 hours. Higher concentrations of cobalt and prolonged conditioning (72> h) induced obvious cell death[28]. According to study of Chacko et al, hypoxia treatment of MSC (0.5% O2) for 24–48 hours was not a sufficient stimulus to negatively affect the cells' proliferation status, however a longer exposure (72> h) could induce apoptosis when mesenchymal stem cells are cultured under hypoxic conditions in vitro, their proliferative and self-

renewal capacities are significantly improved[29]. Compared to the routine normoxic culture of 20% O2, long-term culture of human MSCs in 1% O2 has been shown to reduce their proliferative potential[30]. In addition treatment of MSCs with minute Concentrations of H_2O_2 for a short period of time protects MSCs against oxidative damages upon their exposure to high concentrations of ROS(Reactive oxygen species). ROS play essential roles in apoptosis and in the regulation of several transcription factors under both physiological and pathological conditions [22]preconditioning with low concentrations of H_2O_2 strongly increase the survival of MSCs under oxidative stress conditions[31]. Several studies have been reported the alteration of intrinsic signaling pathways of some growth factors, cytokines, and chemokines and the expression levels of their receptors (especially CXCR4) after preconditioning[32].Similarly, short-term exposure of stem cells to hypoxia up-regulates expression of CXCR4 a key factor of stem cell migration and engraftment[33].Hypoxia can increase the protective effect of pMSCs on H_2O_2-treated-caco2 cells through enhancement of their paracrine actions especially IGF-1[34]. IGF-I has been expressed in MSCs to increase osteogenesis for bone repair[35]. It also, increases the survival and migration of stem cells. Several studies showed that with new strategies such as application of novel biomaterials in culture medium, using $COCL_2$, incorporation of peptides on the surface of biomaterials and loading growth factors in scaffolds can increase MSC therapeutic properties[10]. The activity of the PI3K/Akt pathway in MSCs can be stimulated by growth factors or hypoxia[36]. The activation of the phosphoinositide 3 kinase (PI3K)/Akt signaling pathway plays central regulatory roles in MSC survival, proliferation, migration, angiogenesis, cytokine production, and differentiation [37]. Activated Akt promotes MSC proliferation, angiogenesis, survival and migration via downstream target proteins such as mTOR, VEGF, Bcl-2 and Rac-1[38]. Also, Akt can increase MSC survival by blocking Bax, which is a pro-apoptotic protein, and by increasing Bcl-2, which can reduce the level of apoptosis[39]. several studies demonstrated that Stromal cell-derived factor-1 (SDF-1) can increase the resistance of MSCs to apoptosis induced by H_2O_2[40]. Although, there are a plenty of studies strongly encourage of preconditioning of stem cells in preclinical stage, but its application in clinical trials is limited and challenging. Therefore, more studies about their safety and feasibility, dose and time and other factors should be considered.

CONCLUSION

In conclusion, our results showed that when cells were preconditioned with oxidative stress and hypoxia their survival and proliferation strongly increased and their apoptosis also decreased remarkably. Furthermore, duration of exposure of the cells with hypoxia was important. These findings suggest that the efficient strategy to increased BMMSCs survival and protect them against apoptosis is preparation them with exposure to stresses such as hypoxia and ROS as they interface after injection to in vivo systems.

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