

The Effects of Preconditioning on Survival of Mesenchymal Stem Cells in Vitro

Zahra Farzaneh Taban,¹ Shiva Khatibi,¹ Raheleh Halabian,² and Amaneh Mohammadi Roushandeh^{3,*}

¹Department of Biology, Basic Sciences Faculty, Islamic Azad University of Hamadan, Hamadan, Iran

²Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, IR Iran

³Endometrium and Endometriosis Research Center, Hamadan University of Medical Sciences, Hamadan, IR Iran

*Corresponding author: Amaneh Mohammadi Roushandeh, Endometrium and Endometriosis Research Center, Hamadan University of Medical Sciences, Hamadan, IR Iran. E-mail: a.mohammadiroshandeh@umsha.ac.ir

Received 2016 June 27; Revised 2016 July 19; Accepted 2016 July 20.

Abstract

Background: Mesenchymal stem cells (MSCs) survival decreased following in vivo injection and its application has been limited in stem cell therapy. Preconditioning was established as a strategy to increase the cells' efficiency, but more studies are necessary to determine its safety and mechanisms.

Objectives: The aim of this study was to evaluate the effect of preconditioning mesenchymal stem cells with low serum and H₂O₂ on in vitro cell survival.

Methods: Mesenchymal stem cells were cultured and preconditioned with low serum and H₂O₂ for 6, 12, 24 and 48 hours in six groups. Control, group I with 5 μM H₂O₂, group II with 10 μM H₂O₂, group III with 0.5% fetal bovine serum (FBS), group IV: 5 μM H₂O₂ + 0.5% FBS and V: 10 μM H₂O₂ + 0.5% FBS. Cell proliferation was evaluated with the MTT assay.

Results: Cell proliferation in groups IV and V increased significantly compared to the other groups after 6, 12 and 24 hours of treatment (P < 0.05). Also, after 48 hours, cell treatment with 5 μM H₂O₂ + 0.5% FBS led to a significant increase in cell proliferation (P < 0.05).

Conclusions: Our data suggest that preconditioning with 5 μM H₂O₂ + 0.5% FBS and 10 μM H₂O₂ + 5% FBS improved cell proliferation and viability. However, the mechanisms related to protective properties of preconditioning and using this strategy in stem cell therapy requires more research.

Keywords: Mesenchymal Stem Cell, Proliferation, Cell Survival, Preconditioning

1. Background

Mesenchymal stem cells (MSCs) are multi-potent cells, which can differentiate into mesenchymal and trans-differentiate to non-mesenchymal tissues in vitro and in vivo (1). They are found in specialized tissues of the body like the brain, bone marrow, Wharton jelly, pulp and umbilical cord blood (2). Stem cells and especially MSCs produce all types of multicellular tissues in the body via proliferation and differentiation in controlled in vivo environments. These cells are characterized by their easy access, expanding potential in vitro and features that are not easily lost (3). Bone-marrow mesenchymal stem cells (BMSCs) are non-hematopoietic stromal cells with powerful proliferative and differential potential and represent interesting modalities for the establishment of efficient stem cell-based therapy in various diseases. In the recent years, there has been growing interest in MSC application in cell-based therapy (4-6). However, MSCs-based therapy faces some limitations such as low proliferation and survival rate (7-

9) after replacement in an in vivo environment (10-12). Following MSCs transplantation, the cells are faced with factors such as low serum, hypoxia, heat shock, and oxygen free radicals. This environment-induced cell death and apoptosis hours after transplantation (13). There are several strategies to improve survival rate, potency, migration and targeting of MSCs. Some studies have reported that preconditioning of MSCs by cultivation under H₂O₂, hypoxic and serum deprivation as well as sub-lethal stress environment improves the survival and proliferation rates, differentiation and migration capacities (14, 15). Preconditioning using H₂O₂ is the most practical and important protocol applied in preclinical studies. It has been suggested that preconditioning exerts its effects through altering intrinsic signaling pathways of growth factors, cytokines, and chemokines and expression level of their receptors and consequently improves stem cells survival and proliferation rate (16, 17). Treatment of MSCs with low concentrations of H₂O₂ for a short period of time adapts them

to harmful conditions. It protects MSCs against oxidative damage upon their exposure to high concentrations of reactive oxygen species (ROS) and prevents apoptosis induction as well (18). Enhancement of heart function and decrease of myocardial fibrosis have been reported following transplantation of H₂O₂ preconditioned MSCs to infarcted myocardium in mice (19). Interestingly, H₂O₂ preconditioning decreases serum deprivation (SD)-induced apoptosis.

2. Objectives

In the present study, protective properties of co-preconditioning of MSCs with low serum and H₂O₂ were investigated in vitro.

3. Methods

3.1. Mesenchymal Cells Isolation and Culture

Mesenchymal stem cells (MSCs) were obtained from the femoral and tibial bones of six-week-old Wistar Albino male rats. These cells were suspended in low glucose Dulbecco's modified eagle medium (DMEM) medium with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, CA, USA) and incubated at 37°C in a humidified chamber with 5% CO₂. The culture medium was completely replaced every three days and non-adherent cells were removed. Mesenchymal Stem Cells were recognized by their capability to proliferate in culture with an attached well-spread morphology. Once cells were more than 80% confluent, adherent cells were trypsinized and re-plated 1: 3 (passage1). The differentiation potency of MSC was confirmed by induction of osteogenic, chondrogenic and adipogenic differentiation using specific media.

3.2. Differentiation of Bone Marrow Mesenchymal Stem Cells (BMMSC)

The multi-potency of bone marrow mesenchymal stem cells (BMMSC) was confirmed by induction of osteogenic, chondrogenic and adipogenic differentiation using specific differentiation media.

3.3. Osteogenic Differentiation

For osteogenic differentiation, BMMSCs were seeded at density of 2×10^4 cells/cm² to a 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, 10 nM dexamethasone, 50 µg/mL ascorbic acid, 10 mM -glycerophosphate. The cells were incubated for 21 days in 5% CO₂ at 37°C. After 21 days, osteoblast differentiation was

evaluated by 2% Alizarin Red (Sigma) staining. Briefly, Cells were washed three times with Phosphate Buffer Saline (PBS) (Gibco, Invitrogen, CA, USA) and then fixed for 15-30 minutes with 4% formaldehyde. After rinsing three times with distilled water, they were stained with Alizarin red for three minutes. The cells were washed with distilled water several times and examined under the microscope.

3.4. Adipogenic Differentiation

The cells were seeded at a density of 2×10^4 cells/cm² in 24-well plates. After 80% confluency, the cells were exposed to adipogenic induction medium, consisting of DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 g/mL streptomycin, 100 nM dexamethasone, 50 µg/mL indomethacin for 10 days and fat droplets were examined under the microscope.

3.5. Chondrogenic Differentiation

The cells were cultured at a density of 2×10^4 cells/cm² in 24-well plates. Chondrogenic induction medium was added to cells for 21 days. Medium was changed twice a week. After 21 days, the cells were stained with Alcian blue (Sigma). Briefly, the wells were washed with PBS and fixed with 4% paraformaldehyde for 15 - 30 minutes. Then, the cells were stained with Alcian blue for 30 minutes and washed with HCL 0/1 N. The plate was examined under a light microscope.

3.6. Preconditioning of Mesenchymal Stem Cells

The cells were cultured in 96-well plates at concentration of 10⁴ cells/well. The experiment was designed with six groups. The control group received DMEM with 10% FBS, group I received 5 µM H₂O₂, group II received 10 µM H₂O₂, group IV received 0.5% serum and group V received 5 µM H₂O₂ + 0.5% serum. All groups were cultured and incubated for 6, 12, 24 and 48 hours in 37°C with 5% CO₂. Then, all groups were exposed to a lethal dose of 300 µM of H₂O₂ for 24 hours followed by 24 hours of incubation with fresh medium composed of DMEM and 10% FBS as a recovery period. Finally, MTT assay was performed to detect proliferation of the cells in different groups.

3.7. MTT Assay

Cell proliferation evaluation was confirmed by MTT assay. Briefly, 100 µL of the culture medium was removed and 15 µL MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (5mg/ml) was added. After three hours, the wells were replaced with 200 µL of dimethyl sulfoxide (DMSO). The wells were pipetted and

read with a spectrophotometer (ELISA reader, TECAN/ sunrise, Magellan program, Austria) at optical density of $\lambda 570$. Percentage of viable cells was measured by:

$$\frac{OD \text{ of experimental groups}}{OD \text{ of control}} \times 100 \quad (1)$$

3.8. Statistical Analysis

All results were expressed as mean \pm standard deviation. Statistical analyses were performed by one-way analysis of variance (ANOVA) using SPSS software version 20. The differences between means was considered statistically significant when the probability level (P value) was less than 0.05 ($P < 0.05$).

4. Results

4.1. Morphology of Mesenchymal Stem Cells

After one week, the attached cells were found in the flasks. The cells were attached to plastic and their morphology was fibroblast like (Figure 1).

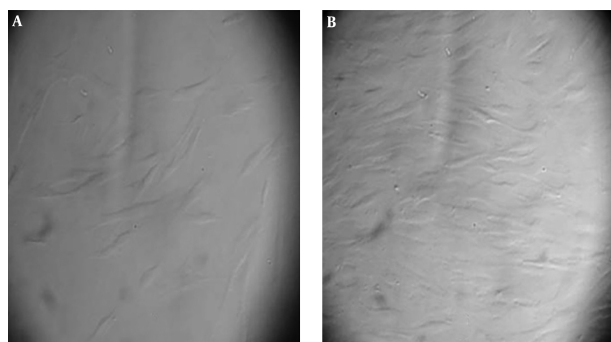


Figure 1. A, The Primary Culture of the Cells After one week and B, at Passage 4

4.2. Differentiation of Mesenchymal Stem Cells

The cells were cultured with differentiation media for 21 days (osteogenesis and chondrogenesis) and 10 days for adipogenesis. As it is illustrated in Figure 2 the MSCs differentiated to bone, adipose and cartilage tissue (Figure 2 A-C).

4.3. Improvement of Cell Proliferation After Preconditioning

Cell proliferation rate of different groups after preconditioning is shown in Figure 3. It is clear that cell proliferation increased in all preconditioned groups compared to the control. Also, it is indicated that cell proliferation in groups IV ($5 \mu\text{M H}_2\text{O}_2 + 5\% \text{ FBS}$) and V ($10 \mu\text{M H}_2\text{O}_2 + 5\% \text{ FBS}$) increased significantly compared to the other groups after

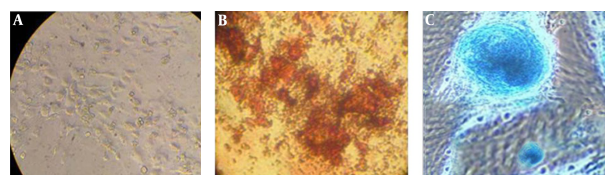
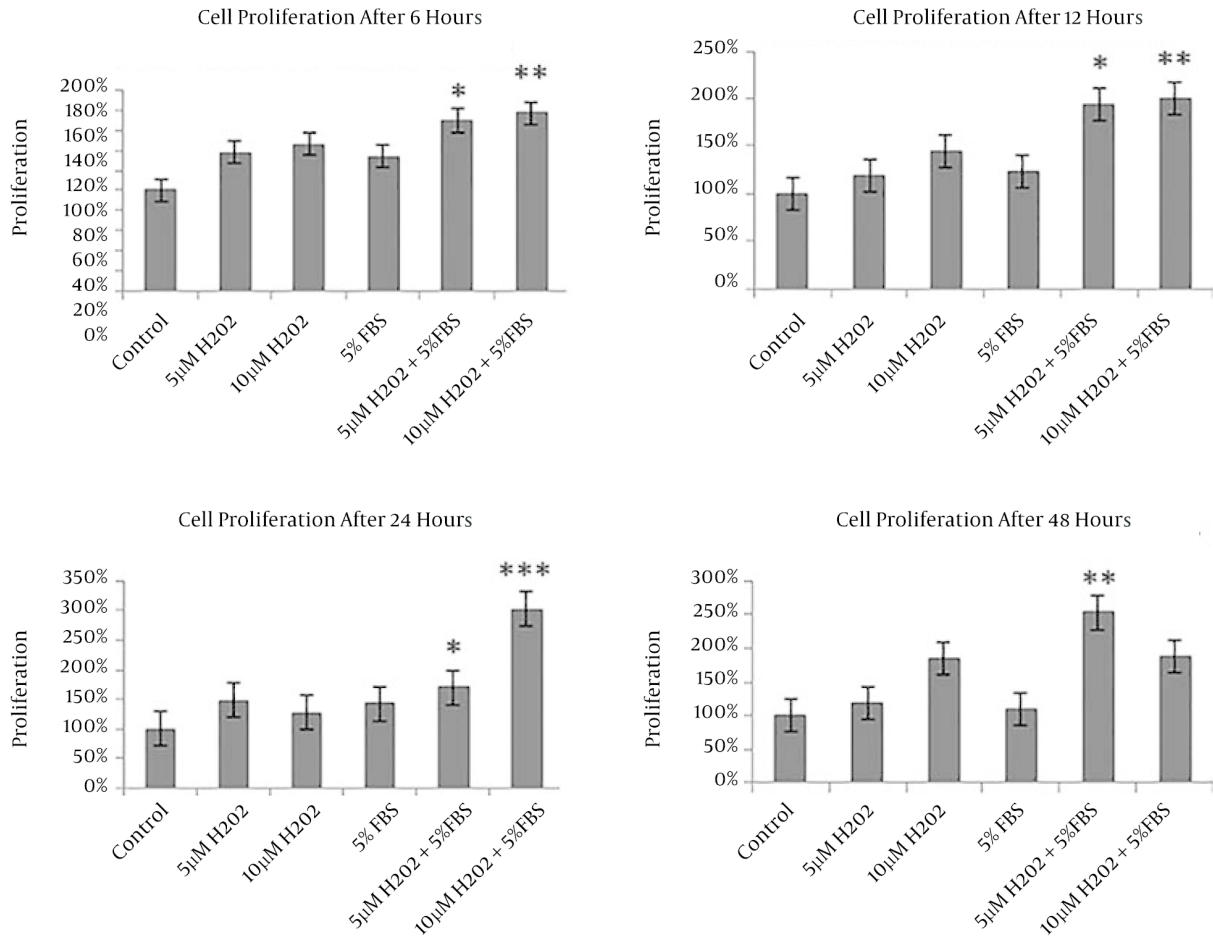


Figure 2. A, The Cells Were Cultured in Differentiation Media for Adipogenesis for Ten Days, B, Osteogenesis for 21 Days and C, Chondrogenesis for 21 Days

6, 12 and 24 hours of treatment ($P < 0.05$). Moreover, significant increase in cell proliferation was observed only in group IV ($5 \mu\text{M H}_2\text{O}_2 + 5\% \text{ FBS}$) towards the other groups after 48 hours ($P < 0.05$) (Figure 3).

5. Discussion

In spite of its benefits, cell therapy for treatment of different diseases has been limited by poor survival ability of MSCs and their cell death due to in vivo ischemic environment (8, 17, 20), therefore, it is essential to discover a novel strategy to improve the efficiency of MSCs for successful stem cell transplantation. In this regard, the present study investigated the MSCs survival ability following preconditioning with cultivation under hypoxic and serum deprivation conditions. The major finding of this study was the improvement of MSCs proliferation after preconditioning with H_2O_2 and serum deprivation. Preconditioning with low concentrations of H_2O_2 and FBS deprivation effectively enhanced the proliferation of MSCs in a dose and time-dependent manner. Several in vitro and in vivo studies reported increase of MSCs survival after preconditioning under hypoxic and serum deprivation conditions (15, 21). Indeed, some studies have shown that rate of successful engraftment and differentiation capacity of the pretreated MSCs was higher than non-preconditioned controls (14, 22). In some studies, higher viability rate and clonogenic capacity was reported after serum deprivation-treated MSCs consequently cultivated in presence of FBS (16, 23). Also, it has been stated that MSCs cultured in serum deprivation conditions show normal morphology (24). However, the detailed mechanisms involved in MSCs protection following preconditioning are not fully determined. It is suggested that, alteration of intrinsic signaling pathways of some growth factors, cytokines, and chemokines and the expression levels of their receptors (especially CXCR4) due to preconditioning might increase their efficiency (25). Preconditioning with H_2O_2 facilitates stem cell differentiation, heart regeneration, and enhancement of early cardiac development by activating the Notch and Wnt signaling pathways, and it also prevents induc-

Figure 3. The Cells Were Cultured in 96-Well Plates to Analyze Their Proliferation by the MTT Assay

The shows shows proliferation rate in different groups after 6, 12, 24 and 48 hours; * shows significance at < 0.05; ** shows significance at < 0.01 level.

tion of apoptosis in MSCs (26). Low H₂O₂ concentration enhances expression of CXCR4 at gene and protein levels and also facilitates the interaction between CXCR4 and its ligand, and stromal cell derived factor-1 α (SDF-1 α) promotes migration capability of MSCs (27).

In spite of its efficiency in preclinical studies, its application in clinical trials needs more attention in regards to safety of preconditioning.

5.1. Conclusions

Our findings confirmed that preconditioning with H₂O₂ as well as serum deprivation improves MSCs proliferation ability.

Acknowledgments

The authors would like to thank Motahareh Mortazavi, for the technical help. There was no conflict of interest amongst the authors.

Footnote

Authors' Contribution: Study concept and design: Amaneh Mohammadi Roushandeh and Raheleh Halabian; acquisition of data: Zahra Farzaneh Taban and Shiva Khatibi; analysis and interpretation of data: Amaneh Mohammadi Roushandeh and Zahra Farzaneh Taban; drafting of the manuscript: Amaneh Mohammadi Roushandeh and Shiva Khatibi; statistical analysis and manuscript preparation and final approval of the manuscript: Zahra Farzaneh Taban.

References

- da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci*. 2006;**119**(Pt 11):2204–13. doi: [10.1242/jcs.02932](https://doi.org/10.1242/jcs.02932). [PubMed: [16684817](https://pubmed.ncbi.nlm.nih.gov/16684817/)].
- Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther*. 2007;**9**(1):204. doi: [10.1186/ar2116](https://doi.org/10.1186/ar2116). [PubMed: [17316462](https://pubmed.ncbi.nlm.nih.gov/17316462/)].
- Nussler A, Konig S, Ott M, Sokal E, Christ B, Thasler W, et al. Present status and perspectives of cell-based therapies for liver diseases. *J Hepatol*. 2006;**45**(1):144–59. doi: [10.1016/j.jhep.2006.04.002](https://doi.org/10.1016/j.jhep.2006.04.002). [PubMed: [16730092](https://pubmed.ncbi.nlm.nih.gov/16730092/)].
- Law S, Chaudhuri S. Mesenchymal stem cell and regenerative medicine: regeneration versus immunomodulatory challenges. *Am J Stem Cells*. 2013;**2**(1):22–38. [PubMed: [23671814](https://pubmed.ncbi.nlm.nih.gov/23671814/)].
- Wei X, Yang X, Han ZP, Qu FF, Shao L, Shi YF. Mesenchymal stem cells: a new trend for cell therapy. *Acta Pharmacol Sin*. 2013;**34**(6):747–54. doi: [10.1038/aps.2013.50](https://doi.org/10.1038/aps.2013.50). [PubMed: [23736003](https://pubmed.ncbi.nlm.nih.gov/23736003/)].
- Murphy MB, Moncivais K, Kaplan AL. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp Mol Med*. 2013;**45**:54. doi: [10.1038/emm.2013.94](https://doi.org/10.1038/emm.2013.94). [PubMed: [24232253](https://pubmed.ncbi.nlm.nih.gov/24232253/)].
- Han SM, Han SH, Coh YR, Jang G, Chan Ra J, Kang SK, et al. Enhanced proliferation and differentiation of Oct4- and Sox2-overexpressing human adipose tissue mesenchymal stem cells. *Exp Mol Med*. 2014;**46**:101. doi: [10.1038/emm.2014.28](https://doi.org/10.1038/emm.2014.28). [PubMed: [24946789](https://pubmed.ncbi.nlm.nih.gov/24946789/)].
- Liu XB, Wang JA, Ogle ME, Wei L. Prolyl hydroxylase inhibitor dimethylxylglycine enhances mesenchymal stem cell survival. *J Cell Biochem*. 2009;**106**(5):903–11. doi: [10.1002/jcb.22064](https://doi.org/10.1002/jcb.22064). [PubMed: [19229863](https://pubmed.ncbi.nlm.nih.gov/19229863/)].
- Yoon DS, Kim YH, Jung HS, Paik S, Lee JW. Importance of Sox2 in maintenance of cell proliferation and multipotency of mesenchymal stem cells in low-density culture. *Cell Prolif*. 2011;**44**(5):428–40. doi: [10.1111/j.1365-2184.2011.00770.x](https://doi.org/10.1111/j.1365-2184.2011.00770.x). [PubMed: [21951286](https://pubmed.ncbi.nlm.nih.gov/21951286/)].
- Li Q, Wang Y, Deng Z. Pre-conditioned mesenchymal stem cells: a better way for cell-based therapy. *Stem Cell Res Ther*. 2013;**4**(3):63. doi: [10.1186/scrt213](https://doi.org/10.1186/scrt213). [PubMed: [23739590](https://pubmed.ncbi.nlm.nih.gov/23739590/)].
- Francois S, Mouiseddine M, Allenet-Lepage B, Voswinke LJ, Douay L, Benderitter M. Human mesenchymal stem cells provide protection against radiation-induced liver injury by antioxidative process, vasculature protection, hepatocyte differentiation, and trophic effects. *BioMed research international*; 2013.
- Xu J, Qian J, Xie X, Lin L, Zou Y, Fu M, et al. High density lipoprotein protects mesenchymal stem cells from oxidative stress-induced apoptosis via activation of the PI3K/Akt pathway and suppression of reactive oxygen species. *Int J Mol Sci*. 2012;**13**(12):17104–20. doi: [10.3390/ijms131217104](https://doi.org/10.3390/ijms131217104). [PubMed: [23443132](https://pubmed.ncbi.nlm.nih.gov/23443132/)].
- Liu NS, Du X, Lu J, He BP. Diva reduces cell death in response to oxidative stress and cytotoxicity. *PLoS One*. 2012;**7**(8):43180. doi: [10.1371/journal.pone.0043180](https://doi.org/10.1371/journal.pone.0043180). [PubMed: [22905226](https://pubmed.ncbi.nlm.nih.gov/22905226/)].
- Jaderstad J, Brismar H, Herlenius E. Hypoxic preconditioning increases gap-junctional graft and host communication. *Neuroreport*. 2010;**21**(17):1126–32. doi: [10.1097/WNR.0b013e328340a77b](https://doi.org/10.1097/WNR.0b013e328340a77b). [PubMed: [20938361](https://pubmed.ncbi.nlm.nih.gov/20938361/)].
- Oh JS, Ha Y, An SS, Khan M, Pennant WA, Kim HJ, et al. Hypoxia-preconditioned adipose tissue-derived mesenchymal stem cell increase the survival and gene expression of engineered neural stem cells in a spinal cord injury model. *Neurosci Lett*. 2010;**472**(3):215–9. doi: [10.1016/j.neulet.2010.02.008](https://doi.org/10.1016/j.neulet.2010.02.008). [PubMed: [20153400](https://pubmed.ncbi.nlm.nih.gov/20153400/)].
- Amiri F, Jahanian-Najafabadi A, Roudkenar MH. In vitro augmentation of mesenchymal stem cells viability in stressful microenvironments. *Cell Stress Chaperones*. 2014;**20**(2):1–15.
- Lu H, Li Y, Sheng Z, Wang Y. Preconditioning of stem cells for the treatment of myocardial infarction. *Chinese Med J*. 2012;**125**(2):378–84.
- Li D, Xu Y, Gao C, Zhai Y. Adaptive protection against damage of preconditioning human umbilical cord-derived mesenchymal stem cells with hydrogen peroxide. *Genetics Molecular Res*. 2014;**13**.
- Pendergrass KD, Boopathy AV, Seshadri G, Maiellaro-Rafferty K, Che PL, Brown ME, et al. Acute preconditioning of cardiac progenitor cells with hydrogen peroxide enhances angiogenic pathways following ischemia-reperfusion injury. *Stem Cells Dev*. 2013;**22**(17):2414–24. doi: [10.1089/scd.2012.0673](https://doi.org/10.1089/scd.2012.0673). [PubMed: [23544670](https://pubmed.ncbi.nlm.nih.gov/23544670/)].
- Swanger SA, Neuhuber B, Himes BT, Bakshi A, Fischer I. Analysis of allogeneic and syngeneic bone marrow stromal cell graft survival in the spinal cord. *Cell Transplant*. 2005;**14**(10):775–86. [PubMed: [16454352](https://pubmed.ncbi.nlm.nih.gov/16454352/)].
- He A, Jiang Y, Gui C, Sun Y, Li J, Wang JA. The antiapoptotic effect of mesenchymal stem cell transplantation on ischemic myocardium is enhanced by anoxic preconditioning. *Canadian J Cardiol*. 2009;**25**(6):353–8.
- Ren H, Cao Y, Zhao Q, Li J, Zhou C, Liao L, et al. Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. *Biochem Biophys Res Commun*. 2006;**347**(1):12–21. doi: [10.1016/j.bbrc.2006.05.169](https://doi.org/10.1016/j.bbrc.2006.05.169). [PubMed: [16814746](https://pubmed.ncbi.nlm.nih.gov/16814746/)].
- Pochampally RR, Smith JR, Ylostalo J, Prockop DJ. Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. *Blood*. 2004;**103**(5):1647–52. doi: [10.1182/blood-2003-06-1967](https://doi.org/10.1182/blood-2003-06-1967). [PubMed: [14630823](https://pubmed.ncbi.nlm.nih.gov/14630823/)].
- Fu WL, Jia ZQ, Wang WP, Zhang JY, Fu X, Duan XN. Proliferation and apoptosis property of mesenchymal stem cells derived from peripheral blood under the culture conditions of hypoxia and serum deprivation. *Chinese Med J Beijing*. 2011;**124**(23):3959.
- Lu HH, Li YF, Sheng ZQ, Wang Y. Preconditioning of stem cells for the treatment of myocardial infarction. *Chinese Med J*. 2012;**125**(2):378–84.
- Boopathy AV, Pendergrass KD, Che PL, Yoon YS, Davis ME. Oxidative stress-induced Notch1 signaling promotes cardiogenic gene expression in mesenchymal stem cells. *Stem Cell Res Ther*. 2013;**4**(2):43. doi: [10.1186/scrt190](https://doi.org/10.1186/scrt190). [PubMed: [23597145](https://pubmed.ncbi.nlm.nih.gov/23597145/)].
- Li S, Deng Y, Feng J, Ye W. Oxidative preconditioning promotes bone marrow mesenchymal stem cells migration and prevents apoptosis. *Cell Biol Int*. 2009;**33**(3):411–8. doi: [10.1016/j.cellbi.2009.01.012](https://doi.org/10.1016/j.cellbi.2009.01.012). [PubMed: [19356708](https://pubmed.ncbi.nlm.nih.gov/19356708/)].