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Research Article

The Role of SMER28 in Increasing the Life of BMSCs after Exposure to Oxidative Stress Induced by Hydrogen Peroxide

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ABSTRACT

Background: Bone Marrow Mesenchymal Stem Cells (BMSCs) have long been of therapeutic importance in tissue engineering and restorative medicine. Recently, introducing the autophagy inducer SMER28 (Small-Molecule Enhancers of Rapamycin) as a molecule for increasing BMSCs viability has received special attention. We aimed to determine the role of SMER28 in increasing the life of BMSCs after exposure to oxidative stress induced by hydrogen peroxide.

Methods: Five female and adult Wistar field samples prepared to extract BMSCs specimens. To prove the mesenchymal origin of BMSCs in the third passage, the expression of CD31 (Cluster of Differentiation 31), CD34, and CD106 were evaluated as the immunocytochemical markers. Trypan blue staining was performed on cells to evaluate the toxicity of H_2O_2 and the protective effect of SMER28.

Results: Pretreatment with SMER28 at a dose of 5 μM increased cell viability. In this regard, cell survival in medium containing SMER28 (5 μM) and H_2O_2 at concentrations of 50, 100, 200, 400 μM were 94%, 86%, 78%, and 43%, respectively. Compared between cells exposed to H_2O_2 with and without SMER28, SMER28 significantly increased cell viability and decreased cell death due to H_2O_2.

Conclusion: Pretreatment with SMER28 at a dose of 5 μM increases BMSCs survival.

Keywords: SMER28 (Small-Molecule Enhancers of Rapamycin), Bone marrow Mesenchymal Stem Cells (BMSCs), Oxidative stress, Hydrogen peroxide

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INTRODUCTION

Due to their accessibility and development potential, Bone Marrow Mesenchymal Stem Cells (BMSCs) have long been of therapeutic importance in tissue engineering and restorative medicine. Recently, it has been shown that not only BMSCs are able to participate directly in repair, but also have the capacity to migrate to distant areas of tissue damage and participate in tissue repair, either directly by differentiation or indirectly through paracrine mechanisms (Chu DT, *et al*., 2020; Dong CL, *et al*., 2019). In addition, they can produce immune-regulating signals that weaken inflammatory and immune responses (Mohanty A, *et al*., 2020). In fact, BMSCs have been clinically studied to treat a wide range of diseases, including bone defects, host graft disease, cardiovascular disease, autoimmune diseases, diabetes, neurological diseases, and liver and kidney diseases (He Y, *et al*., 2022; Ni W, *et al*., 2021; Zhao D, *et al*., 2021). For this reason, these cells are of special importance, but these cells die quickly after transplantation to the lesion area due to the environment of oxidative stress or hypoxic conditions, as well as deprivation of serum (Zhong L, *et al*., 2021; Garcia AL, *et al*., 2021). These cells have a short lifetime after transplantation into a damaged spinal cord or ischemic area in the brain due to oxidative agents and inflammatory cells (Xin W, *et al*., 2021). Therefore, ideas that increase the lifespan of these cells play an important role in increasing the survival of these cells after transplantation. In recent years, large-scale chemical screening has been performed to find new drugs in the regulation of autophagy for the treatment of neurodegenerative diseases, some of which are 50729 compounds that increase and decrease the cytosolic effect of rapamycin (Zheng J, *et al*., 2021). In these large screenings, *Saccharomyces cerevisiae* was first studied to find a molecule that could inhibit the growth inhibition of rapamycin, and this study led to the identification of SMER (Small-Molecule Enhancers of Rapamycin) molecules (Floto RA, *et al*., 2007). SMERs were initially identified as modulators of the effect of rapamycin on yeast growth, but eventually their effect on mammalian neuronal autophagy was also investigated (Sarkar S, *et al*., 2007). SMER is actually a bromo-substituted quinazoline. In this study, the effect of this substance on increasing the life of BMSCS cells was investigated. In fact, in this study, the role of SMER28 in increasing the life of BMSCs exposed to hydrogen peroxide at different concentrations was investigated. In fact, we aimed to determine the role of SMER28 in increasing the life of BMSCs after exposure to oxidative stress induced by hydrogen peroxide.

MATERIALS AND METHODS

Extraction and culture of bone marrow stromal cells

In this study, 5 female and adult Wistar field samples prepared from Pasteur Institute at the age of 6-8 weeks were used. The animals were kept in a 12-hour light and dark period in the standard conditions of the zoo of Qazvin University of Medical Sciences in accordance with the ethical rules. After separation from the lower bones of the lower limbs and washing with saline buffer phosphate, BMSCs were placed in Dulbecco Modified Eagle's Medium: F12 (DMEM/F12) with Fetal Bovine Serum (FBS) (100 μg/mL), FBS 10%, penicillin (100 U/mL) and streptomycin in an incubator with the condition of CO_2 5%, humidity 95% and temperature of 37°C.

Immunohistochemistry assessment

To prove the mesenchymal origin of BMSCs in the third passage, the expression of CD31 (marker of endothelial cells), CD34 (marker of blood stem cells), and CD106 (marker of BMSCs) were evaluated as the immunocytochemical markers. After trypsinization, 5,000 cells were evenly spaced in each of the 6-cell lamellar plate cells. Immunocytochemical steps were performed similar to the recommended protocols. The cells were immersed in 4%

paraformaldehyde solution for 20 minutes. After washing with phosphate buffer, the cells were placed in Triton X 0.3% for 15 minutes. After re-washing with phosphate buffer, the cells were exposed to the initial antibody at 4°C for 24 hours. Primary antibodies included primary antibodies CD31, CD34, and CD106. The cells were washed with phosphate buffer and placed in a secondary antibody conjugated to FITC (1:100; Chemicon), in which the cytoplasm is green, at room temperature for 2 hours. Propium Iodine (PI) dye was used to count the cells, in which the cell nucleus turns red. Cells with a positive immune response were counted under a fluorescent microscope.

Viability test

Prior to the tests, trypan blue was performed on cells to evaluate the toxicity of H_2O_2 and the protective effect of SMER28. The cells were divided into 1000 cells in a well of 96 cells. To evaluate the toxicity of H_2O_2, the durability and survival of BMSCs were measured at different doses of H_2O_2 (0, 50, 100, 200 and 400 μM) for 24 hours. To evaluate the protective effect of SMER28, cells were pretreated with SMER28 (5 μM) about two hours before exposure to H_2O_2. To evaluate cell survival, a volume of cell suspension and an equal volume of trypan blue dye are mixed and cell counts were performed using a neobar slide under a microscope. In this method, the dye penetrated into the dead cells and turned to blue color. Unstained cells represented living cells, which was obtained by counting the total number of cells and stained cells.

Categorization of the cell groups

The cells assessed were finally categorized as:

- Control group (BMSCs cells in the third passage).
- Oxidative stress group (BMSCs in different doses of H_2O_2 (0, 50, 100, 200 and 400 μM) for 24 hours).
- Treatment group (BMSCs cells pretreated with SMER28 at a dose of 5 μM at different doses of H_2O_2 (0, 50, 100, 200 and 400 μM) for 24 hours).

RESULTS

Recognize the nature of BMSCs

BMSCs had a rounded appearance after separation (*Figure 1*), but after 48 hours, they adhered to the bottom of the flask, and after rinsing with PBS, the floating cells that did not adhere to the bottom of the flask were washed (*Figure 2*). There are large populations of different stem cells in the bone marrow, such as endothelial stem cells, blood stem cells, and bone marrow stromal stem cells, which are usually washed away after 48 hours after separation and rinsing with PBS, except for BMSCs. However, to ensure the identity of the BMSCs, the cultured cells were evaluated after the third passage for superficiality and mesenchymal origin by immunocytochemical analysis of surface markers. BMSCs were negative for CD31 antibody, which is a marker of endothelial cells, and CD34, which is a marker of blood stem cells, but the cells responded positively to the CD106 antibody (marker of BMSCs). Fluorescence light is associated with the Fluorescein Isothiocyanate (FITC)-conjugated secondary antibody, which appears green. PI dye is used to count the cells, and the nuclei of the cells emit red light.

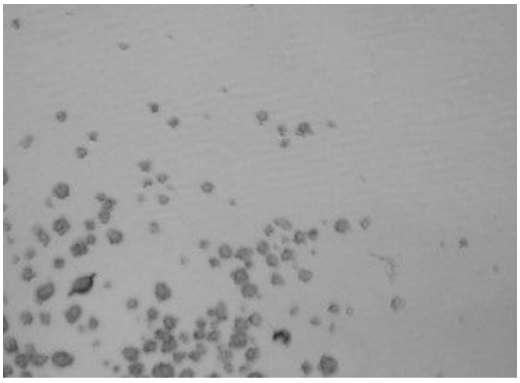

Figure 1: Bone marrow Mesenchymal Stem Cells (BMSCs) isolated from rat femur in flasks containing culture medium. The cells have a spherical and floating appearance

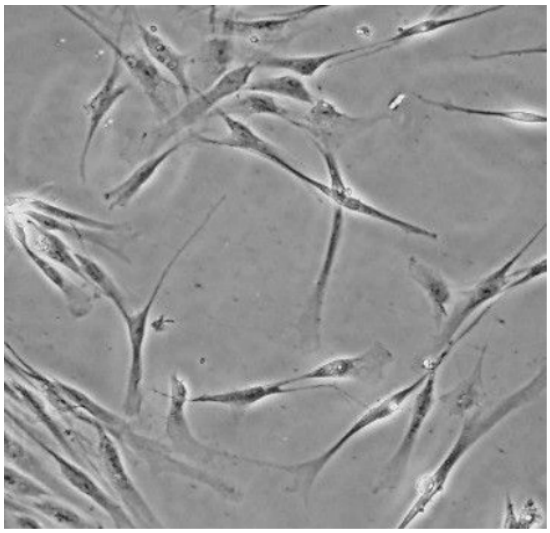

Figure 2: BMSCs cells after the third passage that are attached to the bottom of the flask and have a spindle-shaped and polyhedral appearance

Check the viability of BMSCs

In the viability test review, BMSCs in the third passage without H_2O_2 were considered as the control group. The cells were then treated with H_2O_2 at different concentrations for 24 hours. Viability rate after 24 hours was 88%, 75%, 48%, 38% of the control group after receiving 50, 100, 200, 400 µM of H_2O_2, respectively. The highest cellular death was revealed in the cell groups that received H_2O_2 with the doses of 200 µM and 400 µM, indicating a significant difference as the control group without exposing H_2O_2 ($p<0.001$).

Investigation of SMER28 protective effect

To evaluate the protective effect of SMER28, BMSCs cells were pretreated with SMER28 at a concentration of 5 µM, two hours before exposure to H_2O_2. The results showed that pretreatment with SMER28 at a dose of 5 µM increased cell viability. In this regard, cell survival in medium containing SMER28 (5 µM) and H_2O_2 at concentrations of 50, 100, 200, 400 µM were 94%, 86%, 78%, and 43%, respectively. Compared between cells exposed to H_2O_2 with and without SMER28, SMER28 significantly increased cell viability and decreased cell death due to H_2O_2.

DISCUSSION

Due to the recent discovery of these molecules, little research has been done on their effect only on PC12 (Pheochromocytoma) cell line and *Drosophila*. But the same studies conducted by Floto RA, *et al.* showed that SMERs are good candidates for increasing autophagy. In Alzheimer's disease, SMER 28 is one of the small molecules that cause a significant reduction in the accumulation of Aβ peptide and Amyloid Precursor Protein-C-Terminal Fragments (APP-CTF), and the presence of Autophagy related 5 (ATG5) is essential in this pathway (Floto RA, *et al*., 2007). A study by Sarkar S, *et al*. on PC12 cell line found that four SMERs of 16, 18, 28, and 10 could be good candidates for increasing α-syn A53T cell clearance through autophagy and is able to reduce the amount of α-syn by up to 50% (Sarkar S, *et al*., 2009). In this regard, when rapamycin and 28 SMER are used together, the clearance effect of α-syn A53T is much higher than when used alone. According to the present information, the increase in SMER 28 autophagy is through a non-mammalian Target of Rapamycin (mTOR)-dependent pathway and possibly depends on a downstream composition of the pathway. On the other hand, in the same study, the SMERs mentioned in Huntington's disease increase the autophagy of Huntington's protein. Today, the structural analogues of these SMERs have been identified and tested as new drug candidates for activating autophagy, and according to the results obtained, they are also effective. Due to the above, SMER28 molecule with the highest ability to induce autophagy has a high potential for further studies in the treatment of neurodegenerative diseases. Another advantage of SMER28 is that in sufficient doses to increase autophagy they do not produce any apparent toxicity to the PC12 cell line (Clark AE, *et al*., 2018). In a study conducted by Koukourakis MI, *et al*., it was revealed that first SMER28 increased the rate of autophagy flow leading elevation of the survival rate of liver cells and thus it has a protective effect on stem cells, which is only on normal cells but not on cancer cells (Koukourakis MI, *et al*., 2018). In another study conducted by Darabi S, *et al*., SMER28 has been shown to prevent the destruction of Substantia Nigra pars Compacta (SNC) cells, weaken microgliosis, and improve motor function (Darabi S, *et al*., 2018).

CONCLUSION

According to previous researches and studies on SMER28, in this study, its effect on increasing the viability of stem cells was investigated. The results showed that pretreatment with SMER28 at a dose of 5 µM increased cell survival. Cell survival in medium containing SMER28 (5 µM) and H_2O_2 at concentrations of 50, 100, 200, 400 µM were 94%, 86%, 78%, and 43%, respectively. In other words, compared to cells that were not previously pretreated with SMER28 in the face of oxidative stress induction by H_2O_2 with the same concentrations, the percentage of viable cells in the state of pretreatment with SMER28 could be successfully increased.

DECLARATIONS

Author contributions

SD designed the study planning, SM1 and SM3 collected the data, and SM1 analyzed the data and drafted the paper. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Informed consent

Informed consent was obtained from all individuals included in this study.

Ethical approval

The study was ethically approved by the Qazvin University of Medical Sciences.

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The authors would thanks all the subjects participated in the project.

Consent state

The participants gave written informed consent before sampling.

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