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## H<sub>2</sub>O<sub>2</sub> Damages the Stemness of Rat Bone Marrow-Derived Mesenchymal Stem Cells: Developing a “Stemness Loss” Model

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Statistical Analysis C  
Data Interpretation D  
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**Background:**

The number of patients with spinal cord injury caused by motor vehicle accidents, violent injuries, and other types of trauma increases year by year, and bone marrow mesenchymal stem cell (BMSC) transplants are being widely investigated to treat this condition. However, the success rate of BMSCs transplants is relatively low due to the presence of oxidative stress in the new microenvironment. Our main goals in the present study were to evaluate the damaging effects of H<sub>2</sub>O<sub>2</sub> on BMSCs and to develop a model of “stemness loss” using rat BMSCs.

**Material/Methods:**

Bone marrow-derived mesenchymal stem cells were obtained from the bone marrow of young rats reared under sterile conditions. The stem cells were used after 2 passages following phenotypic identification. BMSCs were divided into 4 groups to evaluate the damaging effects of H<sub>2</sub>O<sub>2</sub>: A. blank control; B. 100 μM H<sub>2</sub>O<sub>2</sub>; C. 200 μM H<sub>2</sub>O<sub>2</sub> and D. 300 μM H<sub>2</sub>O<sub>2</sub>. The ability of the BMSCs to differentiate into 3 cell lineages and their colony formation and migration capacities were analyzed by gene expression, colony formation, and scratch assays.

**Results:**

The cells we obtained complied with international stem cell standards demonstrated by their ability to differentiate into 3 cell lineages. We found that 200–300 μM H<sub>2</sub>O<sub>2</sub> had a significant effect on the biological behavior of BMSCs, including their ability to differentiate into 3 cell lineages, the expression of stemness-related proteins, and their migration and colony formation capacities.

**Conclusions:**

H<sub>2</sub>O<sub>2</sub> can damage the stemness ability of BMSCs at a concentration of 200–300 μM.

**MeSH Keywords:**

**Adult Stem Cells • Hydrogen Peroxide • Mesenchymal Stem Cell Transplantation**

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## Background

The number of patients with spinal cord injuries resulting from motor vehicle accidents, violent injuries, and other types of trauma increases year by year. These injuries often lead not only to lifelong disability, but also to a physical and economic burden for the patient's family and for society [1]. Although in past decades stem cell transplantation has experienced great advances and has been used for muscle regeneration [2,3], osteoporosis [4,5], wound healing [6,7] and blood regeneration [8,9], there are still many problems with this therapeutic approach, including poor survival and limited proliferation of transplanted cells [10]. Oxidative stress plays an important, negative role in the process of stem cell transplantation [11–13]. In the abnormal new microenvironment, increased production of oxidative factors like reactive oxygen species (ROS) creates an unfavorable condition for BMSCs to settle down and grow [14–16]. Oxidative stress often causes ROS accumulation, and ROS can have harmful effects on cells, such as DNA damage, lipid peroxidation, protein oxidation, and oxidation of cofactors, inactivating specific enzymes and leading to pathological consequences. However, ROS does not always play a damaging role, since recent studies have shown beneficial cell physiological effects at lower concentrations [17]. There have also been many studies investigating methods to protect against oxidative stress and to prevent BMSC apoptosis, such as the use of gigantol [18] or preconditioning with far-infrared irradiation [19].

As mentioned above, H<sub>2</sub>O<sub>2</sub> and ROS do not always have a damaging effect, and this depends on the concentration. One study found that H<sub>2</sub>O<sub>2</sub> induced the proliferation of murine bone marrow-derived stromal cells (BMSC) and hematopoietic stem/progenitor cells (HSPC) at a concentration of 10 μM, and that JNK-FOXO3a-catalase signaling played an important role in this process [20]. However, when the concentration of H<sub>2</sub>O<sub>2</sub> exceeds a certain threshold, BMSC apoptosis can occur. Chen and Huang [18] found that when the concentration of H<sub>2</sub>O<sub>2</sub> reached 600 μM, apoptosis of BMSCs occurred at a relatively high rate. There has been scarce research investigating the effects of H<sub>2</sub>O<sub>2</sub> on the stemness of BMSCs, despite the fact that stemness is a crucial BMSC characteristic when they are going to be transplanted. In addition, a model to investigate BMSC "stemness loss" is necessary for scientific research.

## Material and Methods

### Isolation and culture of BMSCs

BMSCs were obtained from healthy male or female Sprague-Dawley rats weighing about 200 g. All the surgical procedures complied with ethical requirements. Briefly, after cervical

vertebrae dislocation, the rats were immersed in 75% alcohol for 10 min. Then, the femurs and humeri were removed under sterile conditions and placed on a culture dish with 75% alcohol. Ten min later, the metaphyses were cut off and the bone marrow canals were washed repeatedly with a sterile syringe filled with F12-DMEM medium. Then, the cells were incubated at 37°C and 5% CO<sub>2</sub> for 2 to 3 days. After several culture medium changes, BMSCs displayed the characteristic adherent growth.

Detection of cell viability and survival state. BMSCs were replated in 96-well plates at a density 1×10<sup>5</sup> cells per well, and the culture medium was plated after synchronization. Cells were then treated with H<sub>2</sub>O<sub>2</sub> of 24 h at various concentrations (0, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM, and 600 μM). Cell viability was detected according to the instructions of the CCK8 assay. Ten cells were treated according to the aforementioned experimental groupings. Cell viability was detected according to the manufacturer's instructions.

### Multilineage differentiation

To assess the multilineage differentiation potential of BMSCs, osteogenic, adipogenic, and chondrogenic differentiation was induced. For osteogenic differentiation, BMSCs were seeded onto 6-well plates at 2×10<sup>4</sup> cells/cm<sup>2</sup> and incubated in osteogenic differentiation medium (Cyagen, USA) until they reached 60–70% confluence. The differentiation media was changed twice a week. After differentiating for 14 days, the BMSCs were collected to extract RNA and protein and were stained with alizarin red solution. For adipogenic differentiation, BMSCs were seeded onto 6-well plates at 2×10<sup>4</sup> cells/cm<sup>2</sup>. When the cells reached 100% confluence, the medium was changed to adipogenic differentiation medium A (Cyagen, USA). Three days later, the medium was changed to adipogenic differentiation medium B (Cyagen, USA). After another 24 h, the medium was replaced with medium A. This cycle was repeated 4 times, followed by incubation in medium B for an additional 7 days. After differentiation, the BMSCs were collected to extract RNA and protein and to stain them with oil red O. For chondrogenic differentiation, BMSCs were seeded onto 6-well plates at 2×10<sup>4</sup> cells/cm<sup>2</sup> and incubated in chondrogenic differentiation medium (Cyagen, USA) until they reached 60–70% confluence. The medium was changed every 2 to 3 days. After differentiating for 3 weeks, the BMSCs were collected to extract RNA and protein and to stain them with alcian blue.

### Colony forming assay

To investigate the effects of H<sub>2</sub>O<sub>2</sub> on colony formation, BMSCs were treated with H<sub>2</sub>O<sub>2</sub> at different concentrations (0 μM, 100 μM, 200 μM, 300 μM). Then, the cells were seeded onto 6-well plates at a density of 500 cells/well. The medium was

changed twice a week. After 2 weeks, the cells were fixed with 4% paraformaldehyde. Fifteen min later, the cells were washed with PBS and stained with 0.1% crystal violet for 15 min. Colonies containing more than 50 cells were counted.

### Wound-healing assay

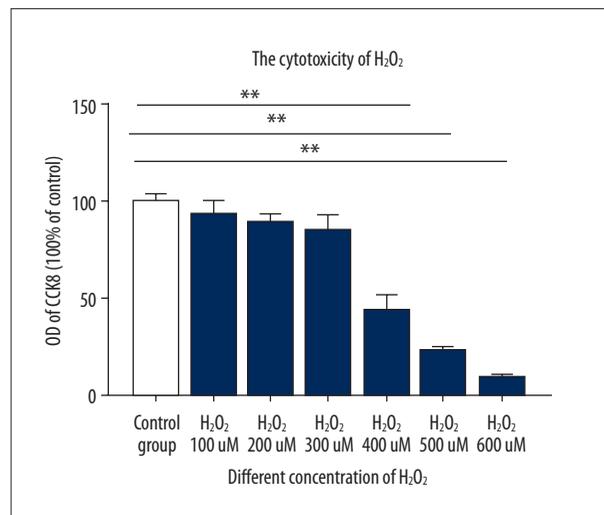
BMSCs were seeded onto 6-well plates containing F12-DMEM complete medium. When the cells reached 100% confluence, a scratch wound was created with a pipette tip, and the medium was replaced with serum-free F12-DMEM. Then, the plates were randomly assigned to the H<sub>2</sub>O<sub>2</sub> or control groups. Photomicrographs were obtained after 24 h.

### Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from BMSCs with Trizol reagent (Invitrogen, USA) 7 days after treatment with H<sub>2</sub>O<sub>2</sub>. The RNA was reversed transcribed into cDNA using a reverse transcription kit (Takara, Japan) following the manufacturer's protocol. After reverse transcription, QRT-PCR was performed with the SYBR Premix Ex Taq II according to the manufacturer's instructions (Takara). The 2<sup>-ΔΔCT</sup> method was followed to analyze the data, and the house-keeping gene GAPDH was used to normalize the levels of mRNA. The primer sequences were as follows: 5'-AAGCCTTCATGTCCAAGCAG-3'(forward) and 5'-TCCGCTAGCTCGTACAATT-3'(reverse) for OC; 5'-TCAGCGTCTATCAGTCC-3'(forward) and 5'-ATTCAAAACGGTTGGGGAGC-3'(reverse) for RUNX2; 5'-ACAAGAAAGACCACCCGAT-3' (forward) and 5'-CGTCTGTTTTGGGAGTGGT-3'(reverse) for SOX-9; 5'-CAGCAGGTTACGTACACTG-3'(forward) and 5'-ACAAGAAACAGACAGGCCCT-3'(reverse) for COL-II; 5'-ACGGTGACAGGAATGTATGA-3'(forward) and 5'-CCAAGACTGTACCCTAAGAGG-3'(reverse) for LPL; 5'-TTTCCTTCAAAGTGGGGTG-3' (forward) and 5'-CATTCCACCACAGCTTGTC-3' (reverse) for AP2; 5'-AGAGGGATGTGGTTCGAGT-3'(forward) and 5'-CTCAGGAAAAGGGACCGAGT-3'(reverse) for OCT4; 5'-CCAAGATGCACAACCTCGGAG-3'(forward) and 5'-CTCCGGGAAGCGTGTACTTA-3'(reverse) for SOX-2; 5'-GGATCTTCTGTGCCACA-3' (forward) and 5'-TCGGGACCAGACAGCTTAG-3'(reverse) for NANOG; 5'-AATCCCATACCA TCTCCAG-3'(forward) and 5'-GAGCCCCAGCCTTCTCCAT-3'(reverse) for GAPDH.

### Western blot analysis

BMSCs were lysed on ice with standard buffer (Beyotime, China) and the total protein content was extracted using a protein extraction kit (Beyotime, China). The cell lysate was centrifuged at 12 000×g for 10 min at 4°C. After protein transfer,



**Figure 1.** Effect of different concentrations of H<sub>2</sub>O<sub>2</sub> on cell survival. We defined the control group (with no H<sub>2</sub>O<sub>2</sub>) as having 100% cell ability, and found when the concentration of H<sub>2</sub>O<sub>2</sub> reached 400 uM, there was significant cell death. When H<sub>2</sub>O<sub>2</sub> reached 600 uM, almost all cells died. At concentrations of 0–300 uM, there was no significant cell death.

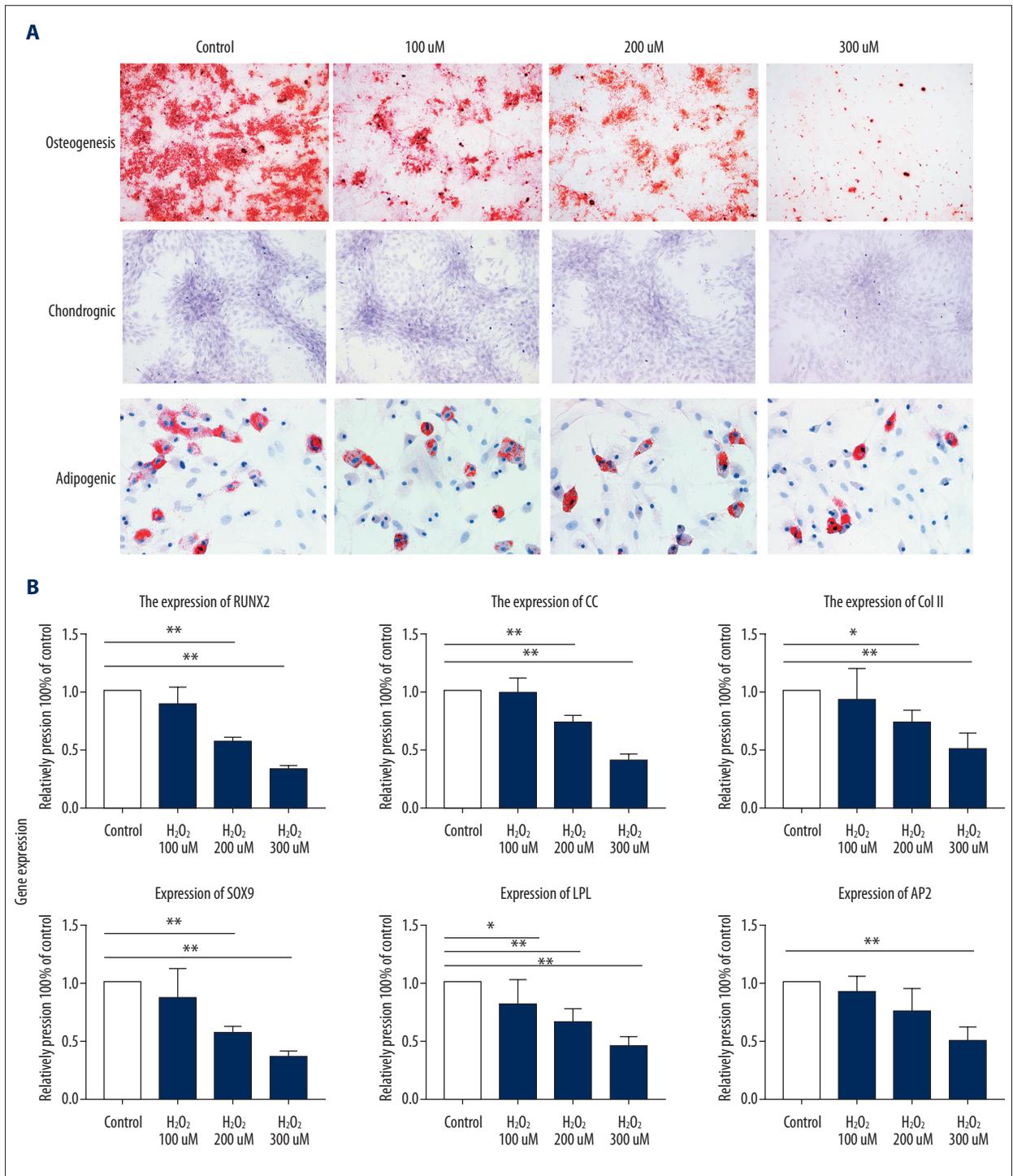
the membranes were blocked with nonfat milk and then incubated overnight at 4°C with rat polyclonal antibodies directed against cleaved Sox2, Oct4, and GAPDH (Abcam, 1: 2000). After several washes, the membranes were incubated with secondary antibodies for 1 h at room temperature. Finally, bound antibodies were visualized using the enhanced chemiluminescence (ECL) method following the manufacturer's instructions (Amersham Biosciences, USA).

### Statistical analysis

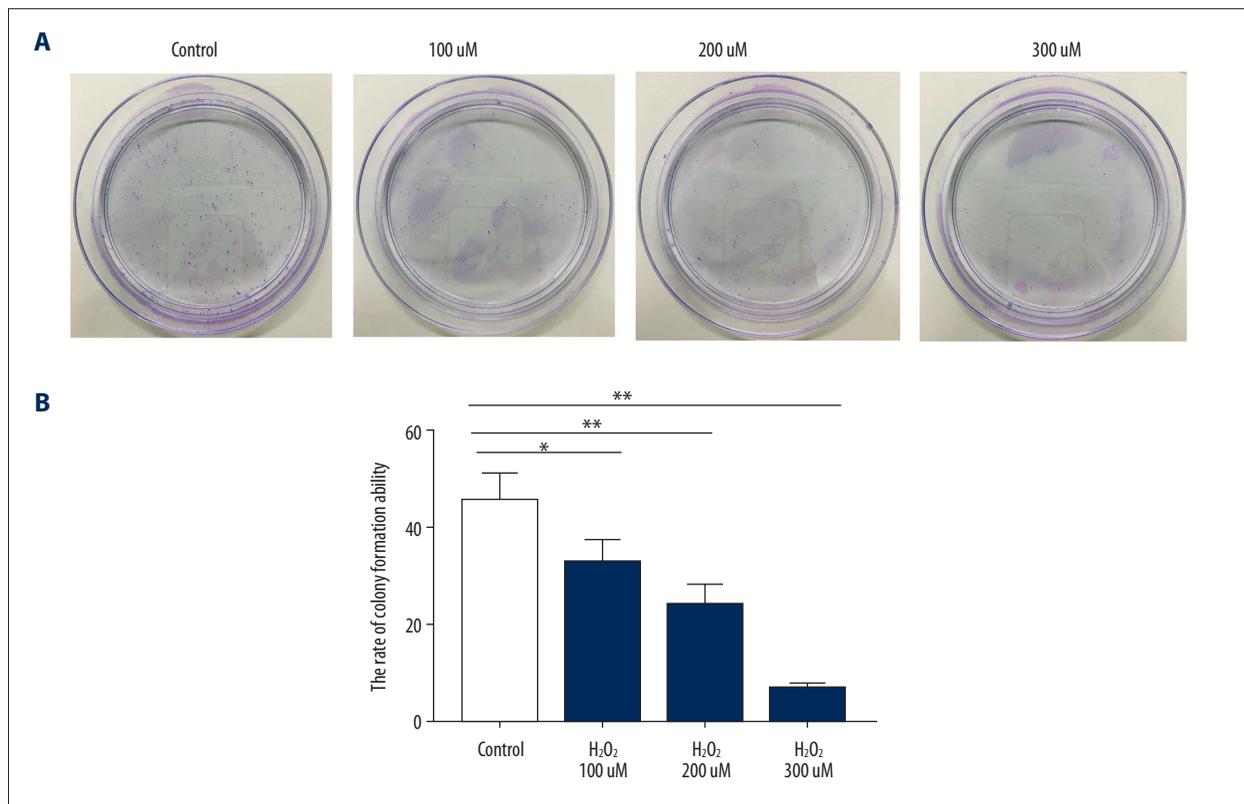
All experiments were performed at least 3 times. Results are presented as the mean ± standard deviation (SD). The *t* test was used for 2-group comparisons. One-way analysis of variance (ANOVA) was used to compare multiple sets of data. *P*<0.05 was considered statistically significant.

## Results

H<sub>2</sub>O<sub>2</sub> induced apoptosis at the concentration of 400–600 uM but did not significantly affect cell viability at concentrations below 400 uM. Our data revealed that H<sub>2</sub>O<sub>2</sub> kills BMSCs at concentrations above 400 uM; about 60% of cells died when H<sub>2</sub>O<sub>2</sub> reached 400 uM and about 90% cells died when H<sub>2</sub>O<sub>2</sub> reached 600 uM. However, when the concentration of H<sub>2</sub>O<sub>2</sub> fluctuated within the range of 100–300 uM, H<sub>2</sub>O<sub>2</sub> had no significant effect on BMSC survival as detected by survival assay (Figure 1).



**Figure 2.** Effect of H<sub>2</sub>O<sub>2</sub> on the differentiation of BMSCs. When BMSCs were exposed to different concentrations of H<sub>2</sub>O<sub>2</sub>, alizarin red staining, alcian blue staining, and oil red O staining were used for detection of osteogenic differentiation, chondrogenic differentiation, and adipogenic differentiation (A). The expression of 3-line differentiation relative genes were also detected by PCR (B).



**Figure 3.** Effect of H<sub>2</sub>O<sub>2</sub> on colony formation ability. The crystal violet staining of cell colony formation in a petri dish (A) and the counts of cell colony formation rate (B).

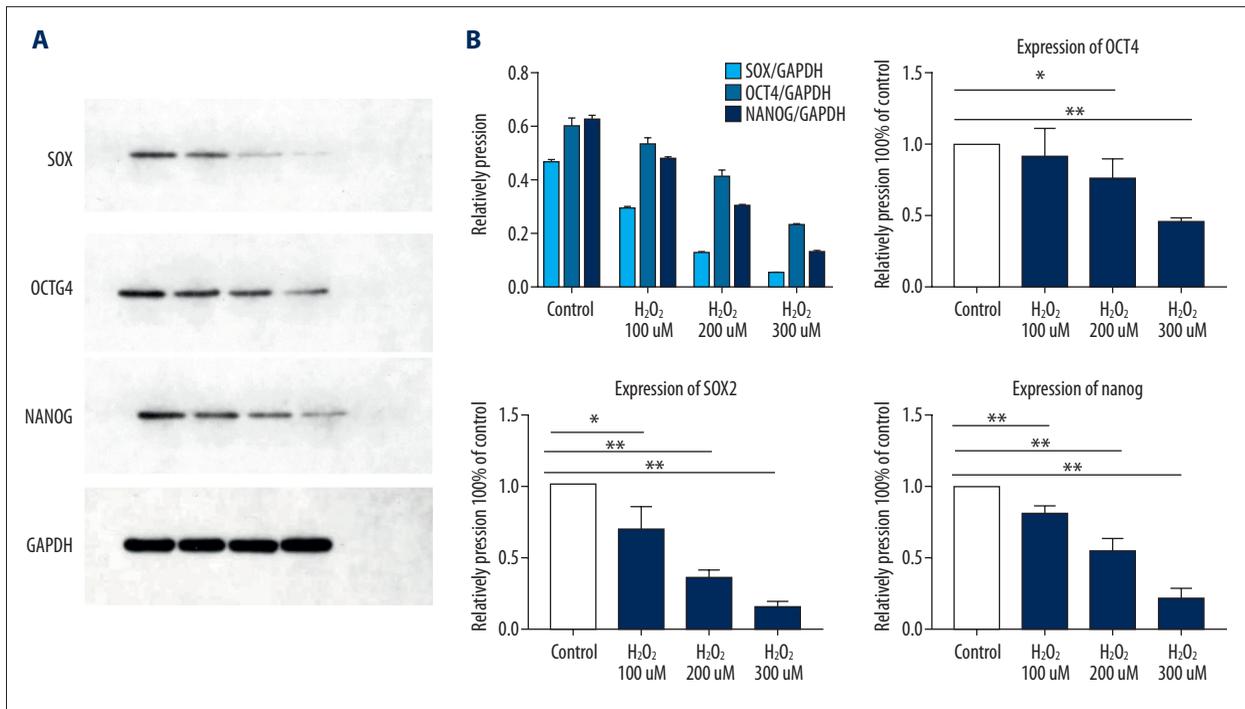
H<sub>2</sub>O<sub>2</sub> interferes with the ability of BMSC to differentiate into 3 cell lineages at concentrations of 200 uM to 300 uM. This was shown by staining with alizarin red, toluidine blue, and oil red O (Figure 2A), and by analyzing the expression of genes that are representative of the differentiation process (Figure 2B). At a concentration of 100 uM, calcium nodule formation by BMSCs was clearly affected (Figure 2A), whereas OC and RUNX2 expression showed no significant differences (Figure 2B). For adipogenesis, the effect of H<sub>2</sub>O<sub>2</sub> was not as significant as in osteogenesis and chondrogenesis. Another point worth mentioning is that the chondrogenic differentiation ability of BMSCs was affected by H<sub>2</sub>O<sub>2</sub>, as evidenced by changes in SOX9 and col II gene expression, but was not visible in the toluidine blue staining.

H<sub>2</sub>O<sub>2</sub> affects colony formation by BMSC at concentrations of 100-300uM (Figure 3). Not all stem cells proliferate and finally form colonies, but the adherent and proliferating cells must be stem cells. Colony formation reflects 2 important cellular traits – the population dependence ability and the ability to proliferate. The initial concentration of cells was 500/well and after 2 weeks of culture, colonies with more than 50 cells were counted. When cells were exposed to 100–300 uM H<sub>2</sub>O<sub>2</sub>, the colony formation ability decreased when compared with

the control group (Figure 3A). The colony formation rate is shown in the bar graph in Figure 2B.

H<sub>2</sub>O<sub>2</sub> decreases the expression of stemness-related proteins in BMSCs at concentrations of 100–300 uM (Figure 4). Different genes play important roles in various biological processes of stem cells, such as proliferation, differentiation, stress response, and apoptosis [21,22]. Oct4, Sox2, and nanog are 3 stemness-related proteins that are highly expressed in stem cells, but whose levels of expression decreases with cellular senescence or after exposure to adverse environmental conditions, such as H<sub>2</sub>O<sub>2</sub>. Changes in the levels of expression of these stemness-related proteins were detected by Western blot analysis (Figure 4A). The bar graphs in Figure 3B show the relative levels of expression of these proteins.

H<sub>2</sub>O<sub>2</sub> affects BMSC migration capacity at concentrations of 100–300 uM (Figure 5). Migration is a characteristic of tumor cells and stem cells, and the scratch test can measure this property clearly. We found that when there was no H<sub>2</sub>O<sub>2</sub> present, BMSCs had the ability to migrate by about 60%. When the concentration of H<sub>2</sub>O<sub>2</sub> increased, the cell migration area decreased. When the concentration of H<sub>2</sub>O<sub>2</sub> reached 300 uM, BMSCs lost their ability to migrate.



**Figure 4.** Effect of H<sub>2</sub>O<sub>2</sub> on the expression of stemness-related proteins. The expression of Sox2, Oct4 and Nanog, 3 stemness-related proteins, were detected by Western blot analysis (A). The relative expression of target proteins was compared with GAPDH, and the statistical results are shown (B).

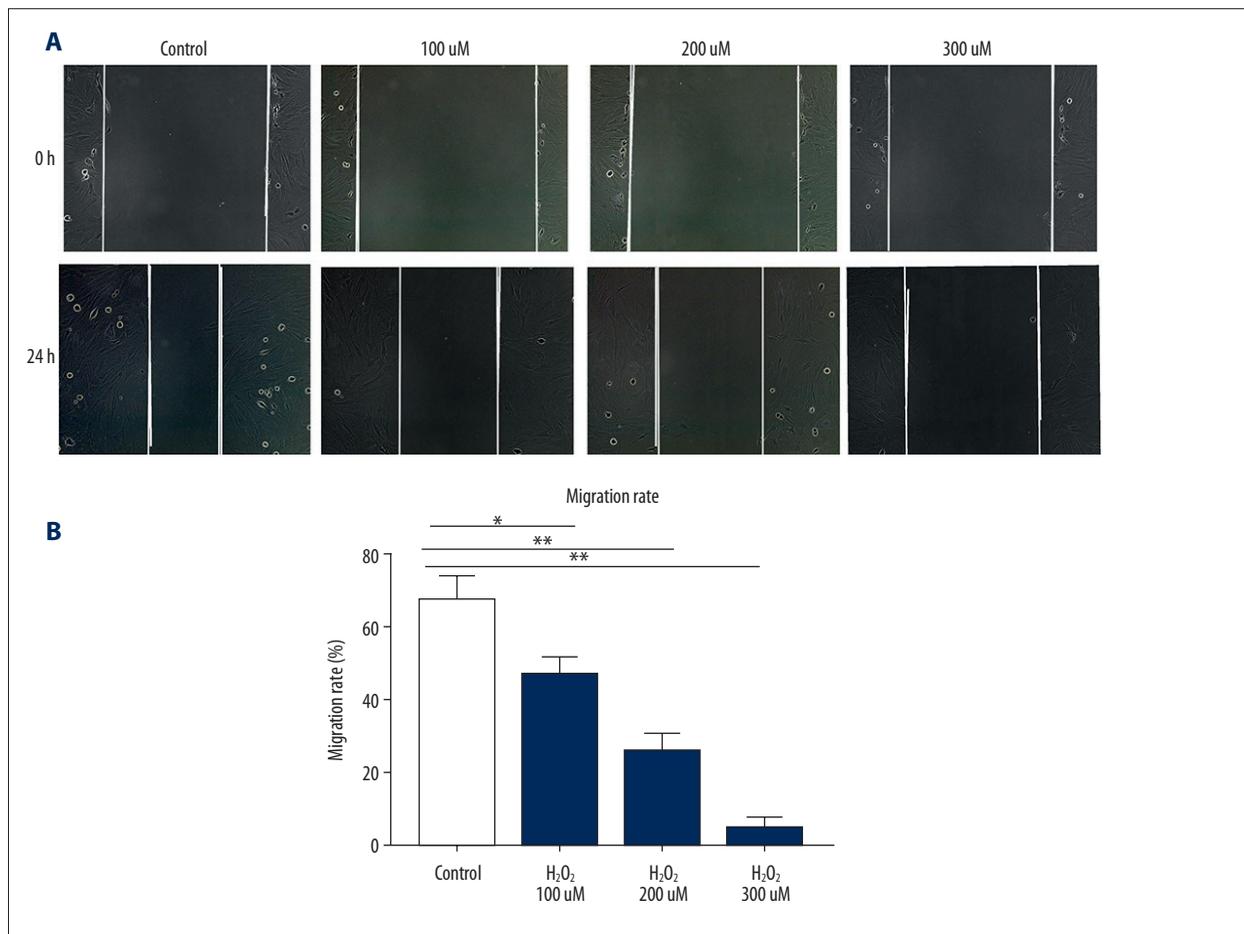
## Discussion

BMSCs have a wide range of applications in multiple research fields due to their ability to differentiate into many cell types, such as cardiomyocytes [23], tenocytes [24], neural cells [25,26], and, the most commonly used type, osteocytes. H<sub>2</sub>O<sub>2</sub> is an important factor to consider when BMSCs are going to be transplanted. In the present study, we found that H<sub>2</sub>O<sub>2</sub> exerted a negative effect when its concentration reached 100 uM to 300 uM. As previous publications have reported [20], low oxidative stress (10 uM of H<sub>2</sub>O<sub>2</sub>) promotes the proliferation of adult stem cells and is beneficial for wound tissue regeneration by acting through the JNK-FOXO3a-catalase signaling pathway. However, in other cases, like in spinal cord injury [27,28], H<sub>2</sub>O<sub>2</sub> can reach a relatively high local concentration, which is why BMSCs do not survive and are difficult to transplant. When patients have to be transplanted, the level of H<sub>2</sub>O<sub>2</sub> *in vivo* is different and depends on factors such as the patient's basic health level, age, sex, and comorbidities. We can give clinic guidance and a possible explanation about why some patients failed in transplantation by testing the effect of H<sub>2</sub>O<sub>2</sub> on stem cells. When we need to transplant in some situation, we have to test the level of H<sub>2</sub>O<sub>2</sub> in bone marrow. Only the appropriate level of H<sub>2</sub>O<sub>2</sub> can achieve success in transplantation. Thus, it is important to define which H<sub>2</sub>O<sub>2</sub> concentration most benefits BMSCs.

We found that at a concentration of 100 uM, H<sub>2</sub>O<sub>2</sub> affected the calcium nodule formation ability of BMSCs, but the expression of OC and RUNX2 showed no significant differences, perhaps because OC and RUNX2 expression and calcium nodule formation ability were measured on different days. In contrast, we found evidence of inhibited chondrogenic and adipogenic differentiation based on gene expression analysis, but not by cell staining. We hypothesize that the different induction and conditioning environments may be responsible for this phenomenon, as well as differences in detection times. Additional *in vivo* experiments are needed to determine the damaging effects of H<sub>2</sub>O<sub>2</sub> on the differentiation ability of BMSCs.

Colony formation, stemness protein expression, and migration capacity are 3 important characteristics of BMSCs. We found that these 3 characteristics were negatively affected to a different degree when cells were exposed to H<sub>2</sub>O<sub>2</sub>. In our study, we focused on the loss of stemness, a condition of cellular dysfunction (not apoptosis) induced when stem cells are exposed to a concentration of H<sub>2</sub>O<sub>2</sub> below 600 uM [18]. Stemness plays an important role in the ability of stem cells to self-renew [29], to synthesize extracellular matrix components [30,31], and to differentiate [32]. Therefore, we need to ensure that the stemness of transplanted BMSCs is normal.

To conclude, this is the first study to determine the range of H<sub>2</sub>O<sub>2</sub> concentrations that have a damaging effect on the



**Figure 5. (A, B)** Effect of H<sub>2</sub>O<sub>2</sub> on the migration ability of BMSCs as detected by scratch test.

stemness of BMSCs. We also developed a model of “stemness loss” for BMSCs, which can be used in other research areas.

## Conclusions

We report for the first time that a range of H<sub>2</sub>O<sub>2</sub> concentrations can have a damaging effect on the stemness of BMSCs, an effect that has to be taken into consideration when BMSCs are transplanted. We also developed a model of BMSC “stemness loss” that can be applied in different areas of research.

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## Conflicts of interest

None.

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