

The involvement of senescence induced by the telomere shortness in the decline of osteogenic differentiation in BMSCs

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Abstract. – **OBJECTIVE:** BMSC (Bone marrow mesenchymal stem cells) is an important seed cell for the repair of bone and cartilage defect in the tissue engineering. The proliferation rate and differentiation capacity of BMSCs from the old donors were less than that from young donors; however, the related mechanism remained unclear.

MATERIALS AND METHODS: Sprague Dawley (SD) rats BMSCs were cultured and treated. Hydrogen peroxide (H₂O₂) and continual passage of BMSCs were performed to induce senescence. Senescence was detected by the SA-β-Gal staining and the telomere length analysis. Cell proliferation and osteogenic differentiation were also observed. Finally, Olaparib was used to maintain the telomere length and investigate the role of telomere length and senescence on the cell proliferation and differentiation.

RESULTS: H₂O₂ could increase the positive rate of SA-β-Gal staining in BMSCs and shorten the length of the telomere. The proliferation rate and ALP activity were also decreased by the H₂O₂. The senescence and decline of osteogenic differentiation could also be observed after prolonged passage of BMSCs. Inhibition of telomere length decline could attenuate the increased positive rate of SA-β-Gal staining induced by the H₂O₂, promote the cell proliferation, and enhance the capacity of osteogenic differentiation.

CONCLUSIONS: Senescence induced by the decline of telomere length could reduce the capacity of osteogenic differentiation and inhibit cell proliferation in BMSCs.

Key Words:

Bone marrow mesenchymal stem cells, Senescence, Telomere, Osteogenic differentiation, Olaparib.

Introduction

Bone mesenchymal stem cells (BMSCs) are usually served as a major source of cells for tis-

sue repair in the histological engineering. BMSCs are easily obtained from the bone marrow of adults; additionally, BMSCs are characterized with the pluripotent, such as differentiation into the chondrocytes, osteoblast, and myoblast¹. However, recent studies found that BMSCs, just like other kinds of somatic cells, are also susceptible to the senescence, which can affect its potent in differentiation. Besides, the impacts of oxidative stress and the passage numbers on the senescence of BMSCs remain unknown. In this study, we observed the variations in senescence, proliferation and osteogenic differentiation using the hydrogen peroxide solution and multiple passages. Cellular senescence refers to an irreversible stage, in which the state of cell metabolism can be changed from active to stagnant. Senescence in replicative form is always accompanied by the shortening of the telomere. Telomere, the ribonucleoprotein complex located at the end of the linear chromosome in eukaryote, consists of the non-code repetitive sequence of DNA and relevant proteins. In human beings, the telomere is composed of tandem repeat sequences of TTAGGG in 2000-3000 bps, and sequences in the 3' end of telomere will fold back to protect the double-stranded DNA by forming T or D-loop. This can avoid the false infusion of non-homologous ends and homologous recombination in DNA repair to guarantee the intact replication of encoding DNA². If cells are under the oxidative stress or the number of cell passage is increased, the deficiency of telomere at the end of the chromosome will easily cause the degradation or infusion of DNA. Thus, the checkpoint of DNA for damage can be activated. Telosome, also known as telomere-associated proteins that are a complex composed of 6 proteins, can form the T-loop and maintain the stability to avoid the abbreviation of telomere³.

In recent years, researches have found that ATM kinase signal pathway is involved in the responses of cells to the stress. Furthermore, some studies revealed that ATM kinase signal pathway is correlated with the length of telomere⁴. In the yeast and human cells, the shortage of ATM kinase might lead to the abbreviation of telomere⁵. Poly (ADP-ribose) polymerase 1 (PARP1) inhibitor can activate the ATM kinase. Lee et al⁴ found that olaparib, a kind of strong inhibitor of PARP1, can promote the activation of ATM kinase, and the extension of telomere in skin fibroblast for about 20-26%⁶. In this study, we observed the impact of olaparib on the length of telomere in BMSCs, and the influence of variations in length of telomere on the proliferation and differentiation of BMSCs.

Materials and Methods

Reagent and Antibody

In this study, all animal experiment procedures were approved by the Animal Ethics Committee. β -galactosidase kit (Beyotime Biotech Ltd., Co, Shanghai, China); reagents used in cell culture, including DMEM (Dulbecco's modified Eagle medium), fetal bovine serum (FBS), and 0.25% trypsin (Gibco, Grand Island, NY, USA); CCK-8 (Cell Counting Kit-8, Dojindo, Japan); alkaline phosphatase kit (Nanjing Jiancheng Technology Co., Ltd, Nanjing, Jiangsu, China).

Primary Culture of Nucleus Pulposus Cells

First, we selected 30 male SD rats (Slac Laboratory Animal, Shanghai, China) aged 3 months and weighed about 250-300 g, which were executed via the excessive aesthesia using 3.5 ml/kg chloral hydrate. On the septic operation table, after the bilateral femurs were isolated, the pulp cavity was exposed and rinsed using DMEM/F12 culture solution containing 15% FBS and 1% penicillin-streptomycin. Cells that were flushed out were collected in an incubator containing 5% CO₂ for incubation at 37°C for 3 days followed by replacement of culture solutions. Later, the solutions were exchanged every 3 days, and the changes in cell growth were observed using inverted phase contrast microscope. Cells were then digested using 0.25% trypsin for passaging.

Design of Experiment

To detect the effect of hydrogen peroxide solution on the senescence, proliferation and

differentiation of BMSCs, we divided the cells into 3 groups, i.e. the control group, 100 μ M H₂O₂-2H group (cells were treated using 100 μ M hydrogen peroxide for 2 h), and 100 μ M H₂O₂-4H group (cells were treated using 100 μ M hydrogen peroxide for 4 h). After the treatment of cells, the cells were cultured for 24 h for morphological observation, β -galactosidase staining, detection of cell proliferation, measurement of telomere length and the activity of alkaline phosphatase. To detect the influence of numbers of passage on the senescence, proliferation and differentiation of BMSCs, cells were divided into 4 groups, i.e. the P2 group, P4 group, P8 group and P16 group. When the cells were passaged to a specified generation, we observed the morphology of cells, performed β -galactosidase staining, detected the cell proliferation, and measured telomere length and the activity of alkaline phosphatase. While in detecting the effect of telomere length on the senescence, proliferation and differentiation of BMSCs, we divided the cells into 4 groups, i.e. the Control group, 100 μ M H₂O₂-4H group, 3 μ M olaparib-24H group (Cells were treated using 3 μ M olaparib for 24 h), and 100 μ M H₂O₂-4H + 3 μ M olaparib group (olaparib was added into the BMSCs at 2 h before adding the H₂O₂, and after 4 h interaction with H₂O₂, olaparib was then used to treat the cells for 24 h).

Cell Morphology Observation

After the BMSCs were treated using 100 μ M hydrogen peroxide solution for 2 h and 4 h, they were cultured for 24 h followed by the observation under the inverted microscope, through which we specifically detected the quantity of flat mast cells and its ratio to the whole quantity of cells.

Cell Counting Kit-8 Analysis

2000 cells were inoculated onto a well of 96-well plate for incubation overnight. Cells were treated according to the procedures in above experiments, and then the original medium was replaced by the fresh DMEM-F12 medium followed by 2-week incubation, during which the medium was replaced every 3 days. Thereafter, the medium in each well of the 96-well plate was replaced by 100 μ L DMEM/F12 medium, and then 10 μ L of reaction agent was added to each well. Cells were then incubated in 37°C incubator for 1 h. Microplate reader was used to detecting the OD₄₅₀ value, which was about the value of the control group.

β-galactosidase Staining

BMSCs were cultured in a 6-well plate, and treated according to the procedures in the experiment design. Then, they were fixed for 15 min at room temperature using the stationary liquid of β-galactosidase in the kit followed by washing for 3 times using PBS. BMSCs were then stained for 12 h using staining solution of β-galactosidase. β-galactosidase contained 1 mg/mL 5-Br-4-Cl-3-indoxyl-galactoside (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride and 2 mM magnesium chloride. After staining, cells were placed under the inverted microscope to observe the ratio of positive cells in the total cells in the vision that was amplified by 200 times.

Analysis of Telomere Length

BMSCs that were cultured in the 6-well plate, after being treated according to the procedures in the above experiment design, were cultured for 2 weeks to analyze the length of telomere. In accordance with the instruction of manufacture, we used the High Pure PCR Template Preparation Kit (Roche, Berlin, Germany) to isolate the genomic DNA of BMSCs, and detected the concentration as well as the purification of DNA using spectrophotometry. To analyze the length of telomere, the extracted 20 μg DNA was digested using restriction enzyme I (New England Biolabs, Ipswich, MA, USA), and then 0.8% agarose gel was used for electrophoresis for 16 h at a constant voltage of 60 V to isolate the DNA above. DNA in the gel was firstly transferred to the capillary filled by standard sodium citrate-hydrochloric acid buffer (3 M sodium chloride/0.3 M sodium citrate), and then to the cation nylon membrane (Osmonics, Minnetonka, MN, USA), followed by incubation for 14 h. After the nylon membrane was washed using sodium citrate buffer for 3 times, the nylon membrane was heated at 120°C for 30 min. Then, the restriction fragment at the end of membrane was hybridized to the digoxin-marked oligonucleotide probe of telomere (Roche, Berlin, Germany). Buffer was used to eluting the extra free digoxin-marked probes, and then the membrane was blocked with skimmed milk to cover the sites spread by non-specific antibodies. The specific antibody of digoxin coupled with alkaline phosphatase and CDP-Star (Roche, Berlin, Germany), the chemiluminescent substrate was used to detect the strength of digoxin probe. The antibody solution with the resistance to digoxin (Roche, Berlin, Germany) was used for incubation for 30 min. After the membrane was washed

using buffer, CDP-Star was added onto the surface containing DNA facing upward and evenly spread over the whole surface followed by incubation for 5 min without light. Then, the color development was carried out in a dark room, and the images obtained were processed by ImageQuant™ RT ECL™, and the length of telomere was calculated using Telometric 1.2 software.

Activity Analysis of Alkaline Phosphatase

Then, cells that were cultured in the 6-well plate (1×10^5 /well) were intervened according to the procedures in the above experimental design, and osteogenic induction culture was conducted using the osteogenic induction medium, containing 10% v/v FBS, 0.1 μmol/L dexamethasone, 50 mg/L ascorbic acid, and 10 mmol/L sodium β-glycerophosphate. After 14 days of culture, we analyzed the activity of alkaline phosphatase in BMSCs. The experiment principle was as follow: alkaline could be disintegrated into disodium phenyl phosphate, further producing phosphate and free phenol, in which the latter could interact with the 4-aminoantipyrine in the alkaline environment, and oxidized by potassium ferricyanide to generate the red perylenequinonoid derivatives, so we could determine the activity of alkaline phosphatase through the shade of red.

After BMSCs in the above procedure were collected, cell lysis was carried out using 0.05% of triton and ultrasonic technique (ice bath, 150 W, 3 s interval). Then, the detection of alkaline phosphatase was performed at the wavelength of 405 nm using a microplate reader (BMG, Labtech, Ortenberg, Germany) according to the instruction of kit (Nanjing Jiancheng Technology Co., Ltd, Nanjing, Jiangsu, China).

Statistical Analysis

ANOVA was used for comparison among different groups, and the statistics were carried out using SPSS 15 software (SPSS Inc., Chicago, IL, USA). Bonferroni method was applied in the pairwise comparison if the difference in ANOVA was statistically significant. $p < 0.05$ suggested that the difference was statistically significant.

Results***Impact of Oxidative Stress on the Morphology of BMSCs***

In this study, to verify whether oxidative stress could lead to the senescence of BMSCs, we used

100 μ M hydrogen peroxide solution to treat the cells. After incubations for 2 h and 4 h, respectively, cells were then cultured for 24 h followed by morphological observation under the inverted microscope. The results showed that BMSCs in the control group were manifested as the regular spindle shape (Figure 1A), while among the BMSCs treated using hydrogen peroxide solution, especially those treated with hydrogen peroxide solution for 4 h, the quantity of typical senile cells in flat and loose shape was increased remarkably (Figure 1B-C, $p < 0.05$). Compared to the control group, the ratio of BMSCs in flat and loose shape to all BMSCs could be increased to 57.85% in 2 h after they were treated using hydrogen peroxide solution, indicating that oxidative stress could accelerate the senescence of BMSCs.

Oxidative Stress Leading to the Accelerated Senescence, Decreased Proliferation, Shortened Length of Telomere and Reduced Activity of Alkaline Phosphatase of BMSCs

β -galactosidase staining was a classical method for detecting the cell senescence. After BMSCs had been treated using hydrogen peroxide solution, the positive rate of BMSCs in β -galactosidase staining was significantly increased. There were azure particles surrounding the cell nucleus (Figure 2A-B; $p < 0.05$). This represented the positive result in β -galactosidase staining. CCK-8 analysis revealed that after the treatment of hydrogen peroxide solution, the proliferation of BMSCs was decreased and OD value was lower than that in the control group (Figure 2C), in

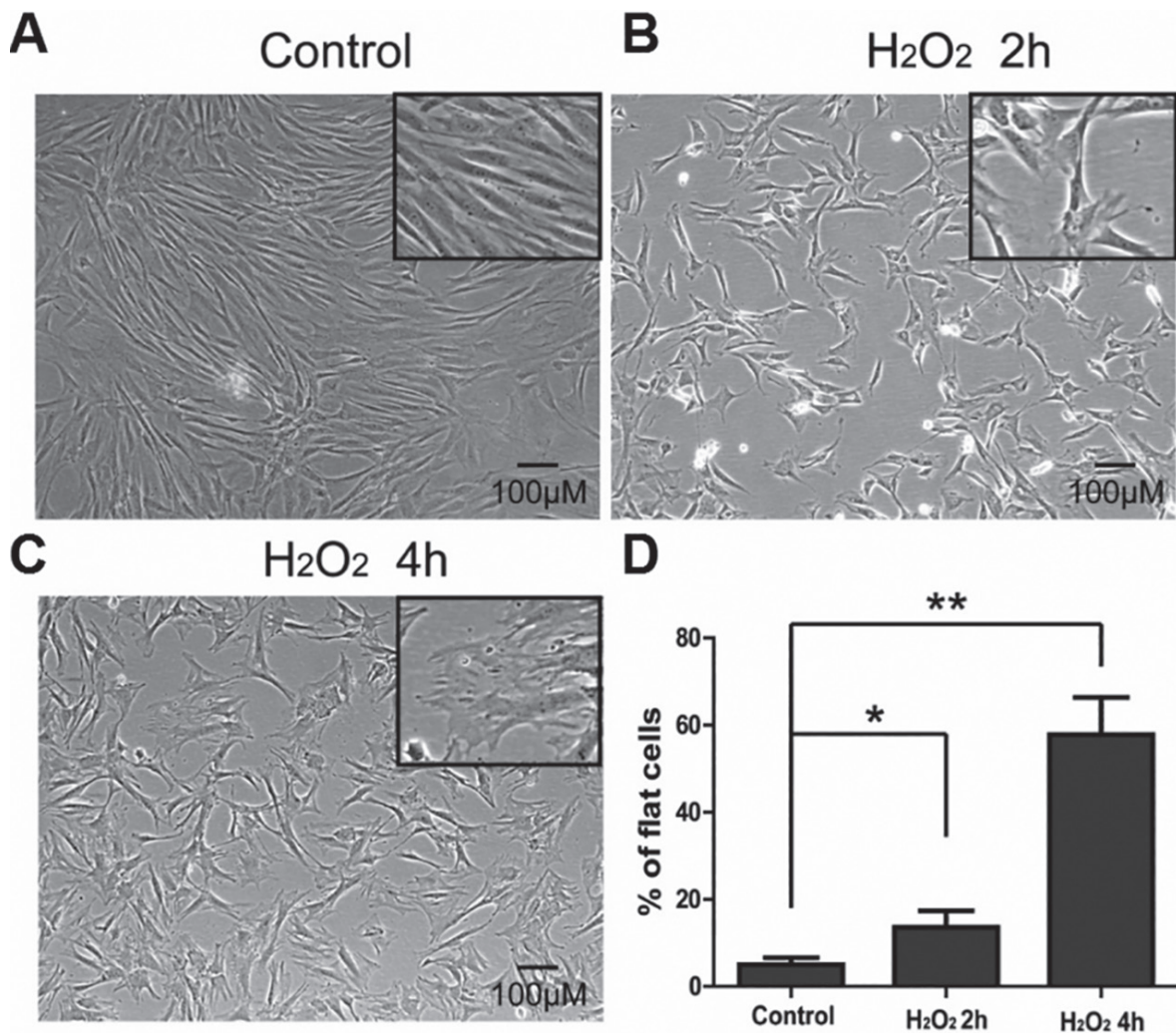


Figure 1. The cell morphology of BMSCs under the stimulation of hydrogen peroxide solution. **A**, Control group. **B**, 100 μ M H₂O₂-2H group. **C**, 100 μ M H₂O₂-4H group, the cells under the inverted phase contrast microscope. **D**, Percentage of senile cells in flat and loose shape. * $p < 0.05$; ** $p < 0.01$, $n = 6$.

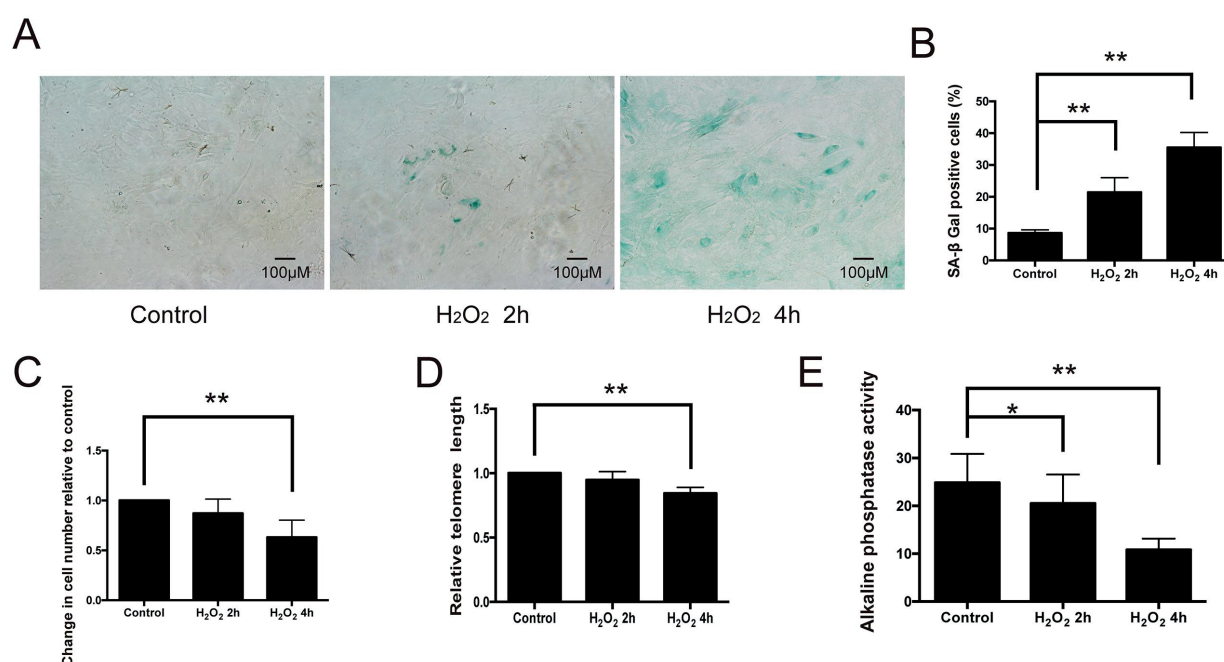


Figure 2. The senescence, proliferation, telomere length and the activity of alkaline phosphatase of BMSCs under the effect of hydrogen peroxide solution. **A**, β -galactosidase staining. **B**, Percentage of positive cells in β -galactosidase staining. **C**, Cell proliferation detected by CCK-8. **D**, Detection of telomere length. **E**, Activity of alkaline phosphatase in cells. * $p < 0.05$; ** $p < 0.01$, $n = 6$.

which the OD values in 100 μ M H₂O₂-2H group and 100 μ M H₂O₂-4H group were respectively 87.17% and 63.17% of those in the control group. In the 100 μ M H₂O₂-4H group, the length of telomere was shortened by about 16% compared to that in the control group (Figure 2D, $p < 0.05$); in terms of the cell differentiation, the activity of alkaline phosphatase in BMSCs was significantly decreased (Figure 2E, $p < 0.05$), indicating that oxidative stress not only accelerated the senescence of cells, but also decreased the osteogenic differentiation capability.

Excessive Passages Leading to the Accelerated Senescence, Decreased Proliferation, Shortened Length of Telomere and Reduced Activity of Alkaline Phosphatase of BMSCs

Positive ratio of BMSCs in β -galactosidase staining was increased in a passage number-dependent pattern with the augmentation of numbers of passage. The positive ratios of BMSCs in P8 group and P16 group were increased to 21.2% and 35% compared to that in the P2 group (Figure 3A-B, $p < 0.05$). More passages suggested a weaker proliferation capability of cell. A remarkable decline was identified in the proliferation capability of BMSCs in P8 group and P16 group

(Figure 3C). The telomere length of BMSCs in P16 group was shortened by about 9% compared to that in the P2 group (Figure 3D, $p < 0.05$), but no significant abbreviations were observed in comparisons of telomere lengths among P8 group, P4 group and P2 group. Similar to those cells that were treated using hydrogen peroxide solution, the activity of alkaline phosphatase of BMSCs in P16 group was also significantly decreased (Figure 3E, $p < 0.05$), indicating that the differentiation capability of cells was weakened with an increase of the number of passage.

Inhibiting the Abbreviation of Telomere Length Could Alleviate the Cell Senescence and Decreased Differentiation Capability Caused by Oxidative Stress

To confirm the effect of telomere length on cell senescence and cell differentiation capability, we applied olaparib, the PARP1 inhibitor, to activate the ATM kinase to inhibit the abbreviation in telomere length. Compared with the single treatment using hydrogen peroxide solution for 4 h, olaparib + hydrogen peroxide solution could significantly inhibit the abbreviation in telomere length (Figure 4C, $p < 0.05$), while the single application of hydrogen peroxide solution would

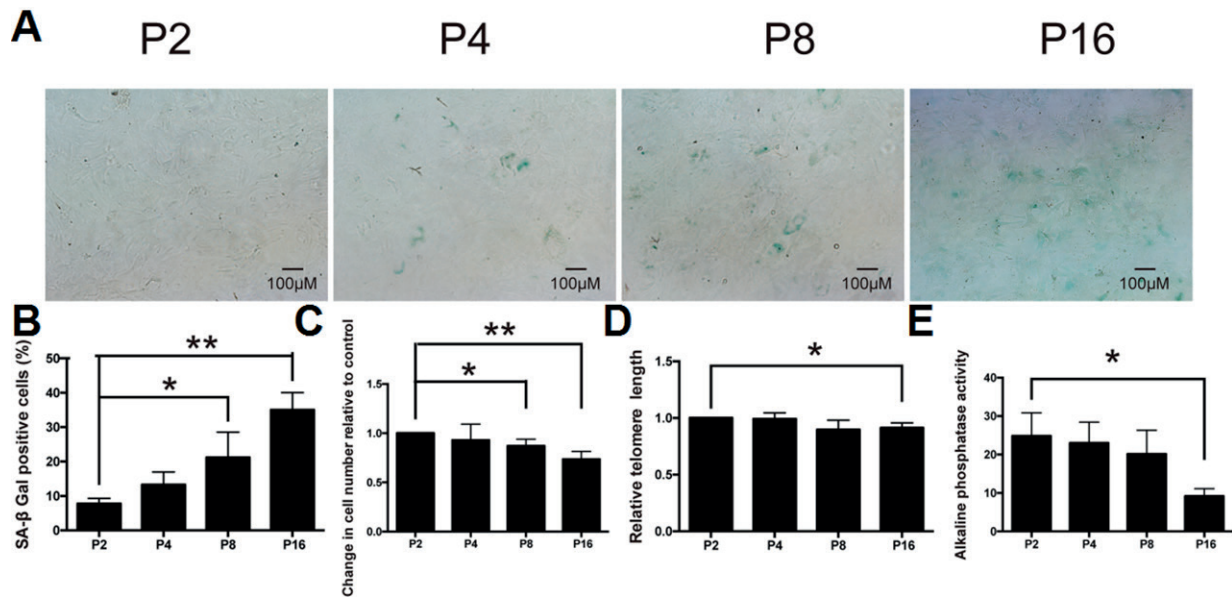


Figure 3. The senescence, proliferation, telomere length and the activity of alkaline phosphatase of BMSCs in different passages. **A**, β -galactosidase staining. **B**, Percentage of positive cells in β -galactosidase staining. **C**, Cell proliferation detected by CCK-8. **D**, Detection of telomere length. **E**, Activity of alkaline phosphatase in cells. * $p < 0.05$; ** $p < 0.01$, $n = 6$.

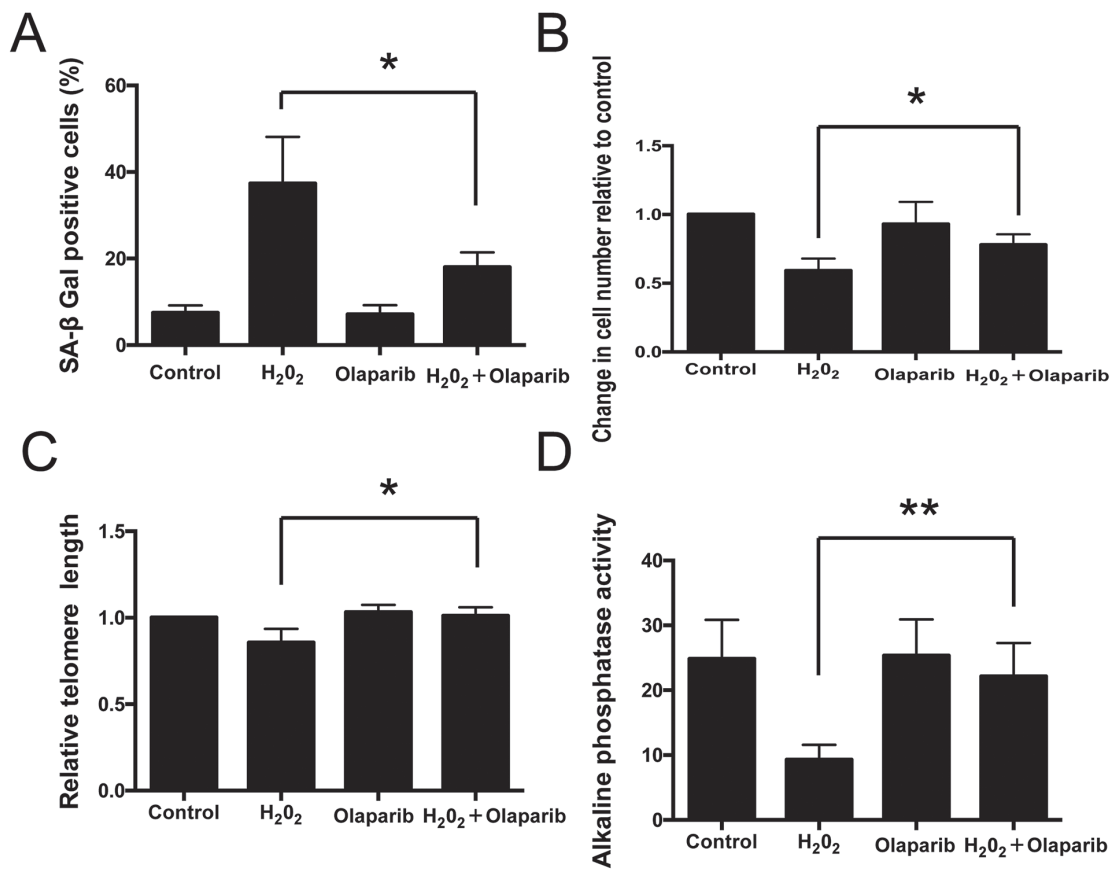


Figure 4. The senescence, proliferation, telomere length and the activity of alkaline phosphatase of BMSCs after the abbreviations in telomere length were inhibited. **A**, Percentage of positive cells in β -galactosidase staining. **B**, Cell proliferation detected by CCK-8. **C**, Detection of telomere length. **D**, Activity of alkaline phosphatase in cells. * $p < 0.05$; ** $p < 0.01$, $n = 6$.

remarkably shorten the length of telomere. Furthermore, we found that olaparib could offset the increase in positive ratio in β -galactosidase staining that was caused by the hydrogen peroxide solution (Figure 4A, $p < 0.05$), suggesting that olaparib could relieve the senescence of BMSCs, and augment the proliferation capability (Figure 4B, $p < 0.05$). Finally, we needed to figure out the influence of variations in length of telomere on the cell differentiation. The activity analysis of alkaline phosphatase revealed that olaparib could increase the activity of alkaline phosphatase that was decreased by hydrogen peroxide solution, indicating that the decrease in the cell differentiation was alleviated (Figure 4D, $p < 0.05$).

Discussion

In this study, we observed that the oxidative stress caused by hydrogen peroxide solution could accelerate the senescence of BMSCs through following perspectives: cell morphology, β -galactosidase staining and cell proliferation. Oxidative stress has been a common model inducing the cell senescence, and it has been validated that senescence could be induced in chondrocytes and fibroblast cell line that are treated by hydrogen peroxide solution⁷, indicating that the senescence of BMSCs, just like the somatic cells, could be triggered by the oxidative stress. However, BMSCs, after the treatment of hydrogen peroxide, could still proliferate, which is different from the stagnancy in proliferation of chondrocytes. Moreover, the senescence induction of BMSCs needed 4 h of treatment using 100 μ M hydrogen peroxide, but only 2 h treatment could realize the significant senescence in chondrocytes, suggesting that regarding the tolerance to the oxidative stress, BMSCs shows a higher tolerance capability compared to the somatic cells.

On the other hand, we found that an increase in the passages could give rise to the senescence, decrease in the proliferation capability, abbreviation in telomere length and decline in the osteogenic differentiation of BMSCs. During the *in vitro* culture, a high concentration of O_2 in the incubator can cause the accumulation of reactive oxygen species (ROS) in cells, activate the signal pathway of stress, and thus, lead to the cell senescence through the oxidative stress, which is similar to the action mechanism of hydrogen peroxide⁸. Therefore, to inhibit or delay the cell senescence in *in vitro* culture for a long

time, we should maintain a low concentration of O_2 in the incubator⁹ or add the antioxidant¹⁰. The length of telomere can affect the cell senescence, and oxidative stress can adjust the consumption of telomere end. Similar to chondrocytes and fibroblast⁷, oxidative stress can also facilitate the abbreviation of telomere in BMSCs. However, some studies reported that the short-term stimulation of hydrogen peroxide could not cause the abbreviation in the length of telomere of fibroblast¹¹, indicating that the abbreviation in length of telomere is gradually induced in the replication of chromosome. Hence, to eliminate the influence of an excessively short period of culture, we arranged the analysis of telomere length after two weeks of culture following the cell stimulation using hydrogen peroxide. DNA rupture caused by oxidative stress is considered as the action mechanism mediating the abbreviation of telomere, in which the repeated fragments in telomere are more susceptible to the attack of oxidative stress compared to the non-repeated fragments. For example, repeated sequence of GGG is frequently ruptured due to the attack of ROS¹². The rupture of DNA double-strand could simultaneously prevent the cell proliferation and facilitate the self-repairing system. Therefore, proliferation capability was weakened with the proceeding of cell senescence in this study. The rupture of a single strand in DNA could facilitate the abbreviation of telomere due to a relatively low efficiency in repairing mechanism of single-strand rupture¹³. In this research, we selected the olaparib to alleviate the abbreviation in length of telomere in BMSCs. Olaparib cannot only inhibit the senescence of cell, but also enhance cell proliferation and osteogenic differentiation, which can be served as the sufficient evidence for the effect of telomere in senescence and cell function of BMSCs. Olaparib can maintain the length of telomere mainly through increasing the activity of ATM kinase. In the cells of mammals, the active ATM kinase could activate the function of telosome through promoting the phosphorylation of telomere-associated protein TRF1, thus maintaining the length of telomere¹⁴.

The senescence of BMSCs could cause the decrease in osteogenic differentiation. In this work, we found that cell senescence caused by hydrogen peroxide or excessive passages could result in the reduction of activity of alkaline phosphatase, the marker of osteogenic differentiation, and partially reverse the decrease in the alkaline phosphatase through inhibiting the

senescence by alleviating the abbreviation in telomere length. In our previous experiments, we also found that with an increase in the age of donors, a decline is seen in the osteogenic differentiation capability of BMSCs, which is coincident with the results of this study¹⁵. With an increase in age, we also identified decreases in the quality and quantity of BMSCs¹⁶. Saeed et al¹⁷ discovered that the deficiency of telomerase gene could give rise to the dysfunction of osteogenic differentiation of BMSCs, and telomerase is a key factor in maintaining the telomere length and the depletion of telomerase could lead to the cell senescence.

Conclusions

In this study, we employed the stimulation of hydrogen peroxide and multiple passages, and found that the oxidative stress and an increase in the number of passages can cause the hypertrophy of BMSCs, and further give rise to the senescence, abbreviation in telomere and the decrease in osteogenic differentiation. Thus, inhibiting the abbreviation in telomere could not only reduce the senescence and facilitate cell proliferation, but also increase the osteogenic differentiation capability of BMSCs.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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