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# Decreased proliferation ability and differentiation potential of mesenchymal stem cells of osteoporosis rat

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ABSTRACT

**Objective:** To explore decreased proliferation ability and differentiation potential of mesenchymal stem cells (MSCs) of osteoporosis rat. **Methods:** MSCs were obtained from osteoporosis rat, and proliferation potency and impaired osteogenic differentiation potential were determined. **Results:** The result showed a significant downregulation of MSCs pluripotency related gene (Oct 4) and osteogenic genes (BSP, OCN) expression in OVX MSCs compared with Sham MSCs ($P<0.05$). **Conclusions:** These data suggest that MSCs are aging in osteoporosis body, and autologous OVX MSCs transplantation is not appropriate to treat osteoporosis if necessary. There will be a possibility in establishing a new clinical application of MSCs autologous transplantation to treat osteoporosis, if OVX MSCs have stronger proliferation and differentiation.

## 1. Introduction

Osteoporosis is a systemic disease meaning low bone mass, microstructure damage, skeleton disorder, and more fragile bone, leading to an increasing of the bone fracture risk. The imbalance of the bone remodeling, about bone resorption and formation, seems to be the cause of osteoporosis, and the osteoblasts and osteoclasts come to be disproportionate[1-3]. Although the RANK/RANKL/OPG system has been confirmed[4,5], the detailed pathologic mechanism, especially the molecular cause, keeps more mystery. With the population life extending and the aging society, osteoporosis because of aging bone loss is very common in the elderly, threatening the health seriously. By now, the treatment of primary osteoporosis generally is medicines for correcting the disorder of bone formation and resorption. The medicine therapy is a long-term treatment, which needs strict adherence[6,7].

Mesenchymal stem cells (MSCs) from bone marrow have the ability for self-renewal, and the potential to differentiate into multilineage cells, such as osteoblasts, chondrocytes, myocytes, adipocytes, neurons, cardiomyocytes[8-11]. So MSCs seem to be the one of greatest therapeutic resources for gene therapy, cell therapy and tissue engineering[12-14]. MSCs must have life span *in vivo* and *in vitro*, the potential of multilineage differentiation and self-renewal seems to loss with aging. Some studies suggest that there is an age-related decline of MSC frequency and physiology[15]. It has been reported that if MSCs come aging, the balance of osteoblastic and adipocytic differentiation is broken, and the population of osteoblasts seems to declines[8,16,17].

In the present study, we hypothesized that osteoporosis is the result of the aging MSCs. In our study, we established an ovariectomized (OVX) osteoporosis rat model, isolated MSCs from OVX and normal rats, and characterized MSCs in vitro. We report that the MSCs from osteoporosis rat have less proliferation potency and impaired osteogenic

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differentiation potential. These data suggest that MSCs are aging in osteoporosis body, and autologous OVX MSCs transplantation is not appropriate to treat osteoporosis if necessary.

## 2. Materials and methods

### 2.1. Development of osteoporosis rat model

Female Sprague Dawley rats (6-9 months old) were used for establishing an OVX osteoporosis rat model. Chloral hydrate (100 mg/mL) 350 mg/kg was injected into abdominal cavity to induce and maintain anesthesia. Rats were randomly divided into two groups: OVX group ($n$=7) and Sham group ($n$=7). OVX operation had two longitudinal incisions along both sides of the spine. After having ligated the ovarian vessels and fallopian tubes, the bilateral ovaries were excised. The Sham group was performed the same operation, but only the periovarian fat pads were removed. The mass which was removed had a histopathological examination to confirm ovary excision. Bone mineral density (BMD) of the femurs of two groups was measured by dual energy X-ray absorptiometry (Hologic Corporation, Discovery Wi) 3 months postsurgery to confirm the osteoporosis status. The uterus weight was measured at the same time, and histopathologic examination of the femurs was done as well.

### 2.2. Isolation and culture of bone marrow MSCs

MSCs of the Sham group and OVX group were isolated from bone marrow of femur and tibia. Separation of muscles and tendons from femurs and tibias was done before MSCs isolation. MSCs culture medium (Cyagen Bio, China) was used to flush the marrow cavity of femurs and tibias. Until the bone seemed white, the bone marrow was washed out completely. The culture medium used to flush containing MSCs was collected, eliminating the thrombus, and seeded into 25 $cm^2$ flasks. MSCs were adherent to the bottom of the flask by their adherence characteristic. Culture medium was changed at 24, 48, and 72 h to isolate MSCs from other types of nonadherent cells. The nonadherent cells were washed out from the flask by changing culture medium. MSCs were cultured in an incubator at 37 ℃ with 95% air and 5% $CO_2$. The medium was replaced every 3 days. After 7-10 days culture, when the cells reached 70% confluency, they were trypsinized and passaged.

### 2.3. Flow cytometry analysis

Cells were trypsinized at the time of 90% confluency, and centrifuged at 244 $g$ for 4 min. The supernatant was discarded. $3.0\times10^4$ cells were resuspended in 100 μL buffer which was phosphate-buffered saline (PBS) solution with 1% bovine serum albumin. Cells were incubated in the dark with antibody for PE anti-rat CD45, PE CD34, Mouse anti-rat CD44 PE, PE anti-mouse/rat CD29 (all from Biolegend) at 4 ℃ for 30 min. Then cells were washed and resuspended with buffer , analyzed with flow cytometry (FACSCalibur, BD, USA).

### 2.4. Cells morphology and growth curve

Cells of two groups were examined with an inverted phase contrast microscope (Olympus IX50, Olympus, Japan). A growth curve was described with a counting chamber. Cells were trypsinized at the time of reaching 70% confluency, and centrifuged at 244 $g$ for 4 min. Cells were resuspended in the culture medium, and seeded into the 6-pore plate at the initial concentration of $2.1\times10^3/cm^2$. At 1st, 2nd, 3rd, 4th, 5th, and 6th day of culture, the cells in one pore were trypsinized and counted.

### 2.5. Osteogenic differentiation of MSCs

Cells were plated at a concentration of $3.1\times10^3/cm^2$ on 6-pore plate with MSCs culture medium (Cyagen Bio, China), incubating at 37 ℃ with 95% air and 5% $CO_2$. When cells were 50%-70% confluency 24 h later, the medium was changed by 2 mL Osteogenic differentiation medium (Cyagen Bio, China) every pore. The osteogenic differentiation medium was replaced every 2 days. On the 10th day of osteogenic differentiation, cell samples were collected for reverse-transcription PCR. And on the 21st day of osteogenic differentiation, cells were stained with alizarin red S.

### 2.6. Alizarin red S staining

On the 21st day of osteogenic differentiation, medium was discarded. And cells were washed by PBS solution twice. Then cells were fixed with 4% neutral buffered formalin for 30 min. Then cells were washed with PBS and stained with 1ml alizarin red S each pore for 3-5 min. The pores were washed by PBS, and calcium nodules were stained and

visible under microscope.

### 2.7. Reverse-transcription and RT-PCR

When cells were normal culture for 14 days or osteogenic differentiation culture in 10 days, total RNA was extracted and isolated with RNAprep Pure Cell Kit (TIANGEN, Beijing, China) according to the manufacturer's instrucions. The total RNA was dissolved in DEPC H2O,and stored at -20℃. As reverse-transcription, cDNA was synthesized from 1 μg total RNA with Oligo-dT (50 pmoles) and random primer (50 pmoles) using AccuPower® RocketScriptTM RT PreMix (BIONEER, Korea) according to manual's protocol. The PCR reactions were performed by using 25 μL 2× Es *Taq* MasterMix (CWBIO, China ) in a 50 μL reaction mixture with cDNA 1 μL and 20 pmoles of forward and reverse primers (sequences are shown in Table 1). PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium-bromide staining with a gel documentation system of ChampGel 5500 (Sage Creation, Beijing). The band intensities of gel image were quantitated with Scion Image (IBM PC, USA).

**Table 1**
PCR primer sequences.

| Gene | Primer sequences | Size of product (bp) |
|---|---|---|
| Actin | F 5′-AGACCTTCAACACCCCAGC-3′ | 260 |
| | F 5′-GTCACGCACGATTTCCCT-3′ | |
| Oct 4 | F 5′-GGACACCTGGCTTCAGACTT-3′ | 197 |
| | R 5′-ATCCCTCCACAGAACTCGTATG-3′ | |
| OCN | F 5′-ACAAGTCCCACACAGCAACTC-3′ | 103 |
| | R 5′-CCAGGTCAGAGAGGCAGAAT-3′ | |
| BSP | F 5′-AAAGAGCAGCACGGTTGAGTAT-3′ | 175 |

### 2.8. Statistical analysis

Statistical analysis was performed using SPSS software for Windows (SPSS statistics 21, IBM, USA). All data are expressed as the mean±standard deviation (SD). Analysis of variance (ANOVA) was used for statistical comparisons between different groups. $P<0.05$ was taken to indicate statistical significance.

## 3. Results

### 3.1. Establishment of osteoporosis rat model

All rats both the OVX group ($n=7$) and the Sham group ($n=7$) survivd as there were no postoperative complications and infection. OVX caused the changes to the level of ostrogen and bone metabolism, leading to the decrease of weight, uterus weight and BMD. The average weight of the OVX rats was (417.29±33.20) g at 3 months postoperatively, which of the Sham group was (430.42±36.23) g ($P<0.05$). There was the significant difference in the OVX group between preoperative and postoperative weight [(443.14±40.27) g] ($P<0.05$), while the difference was not significant in Sham group [(434.29±45.42) g]. BMD of the OVX group was (0.199 ±0.006) g/cm$^2$ and (0.238±0.015) g/cm$^2$ for the Sham rats ($P<0.05$). The difference of uterus weight/body weight ratio between two groups was significant as well, (0.039±0.010)% for the OVX and (0.058±0.006)% for the Sham. The results of histopathologic examination showed that a significant bone loss in the OVX group, and there were full of erode-like cavities in bone tissues (Figure 1).

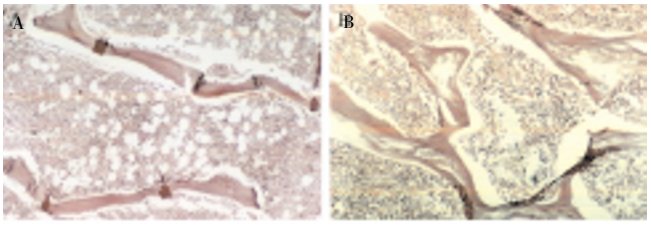


**Figure 1.** Bone histopathologic examination of the femurs of OVX and Sham rats, ×40. (A) OVX group; (B) Sham group.

### 3.2. Flow cytometry analysis

After initial cell culture, the results of flow cytometry analysis showed that both OVX and Sham MSCs from the bone marrow showed positive responses to MSCs markers like CD29, CD44. Also they showed cells were negative for the hematopoietic markers such as CD34, CD45. Positive CD29 and CD44 were 99.86% and 88.04%, respectively in OVX group, 99.86% and 99.47% in Sham group. Positive CD34 and CD45 were 0.10% and 0.14%, respectively in OVX group, 0.64% and 0.35% in Sham group. All of these results showed those cells conformed to MSCs.

### 3.3. Cells morphology and growth curve

Primary MSCs from Sham rats and OVX rats were examined after 14 days culture. The results showed that compared to Sham MSCs, OVX MSCs were slenderer, more nuclear concentrated, and more intercellular spaced (Figure 2). In the same time of culture, the OVX MSCs also showed lower colony-forming rate and growth rate. According the growth

curve (Figure 3), OVX MSCs showed a lower proliferation rate than the Sham ($P$<0.05). And there was no peak of population for the OVX in 7 days culture, while the Sham MSCs had a top of ( 6.75±0.31)×105 at the 4th day.

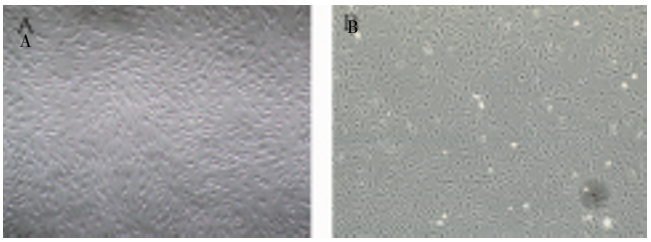


**Figure 2.** Cells morphology of OVX and Sham primary MSCs after 14 days culture, ×40. (A) Sham MSCs; (B) OVX MSCs.

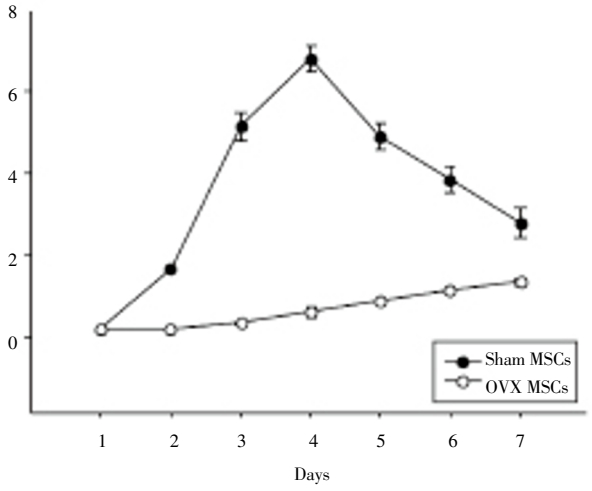


**Figure 3.** Grouth curve of OVX MSCs and normal MSCs.

### *3.4. Charaterization and Osteogenic differentiation of MSCs*

To examine the MSCs pluripotency, we made RT-PCR analysis of Oct4 when cells were normal culture by 14 days. RT-PCR revealed the downregulation of Oct 4 in OVX MSCs compared with Sham (Figure 5). And these were associated with differentiation of MSCs. We also chose RT-PCR analysis of bone sialoprotein (BSP) and osteocalcin (OCN) gene with the 10 days osteogenic differentiation cultured MSCs. The result showed a significant downregulation of osteogenic genes (BSP, OCN) expression in OVX MSCs compared with Sham MSCs ($P$<0.05).

With 21 days of osteogenic differentiation, nodule formation was examed by alizarin red S stain (Figure 4). The results showed that OVX MSCs formed less calcium nodules compared with Sham MSCs in the same area, and OVX MSCs had decreased osteogenic differentiation potential.

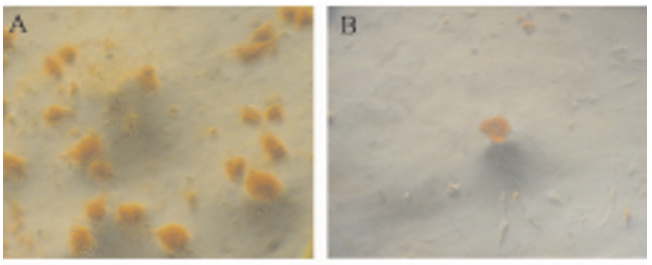


**Figure 4.** OVX and Sham MSCs stained by Alizarin red S after 21 days osteogenic differentiation culture, ×200. (A) Sham MSCs; (B) OVX MSCs.

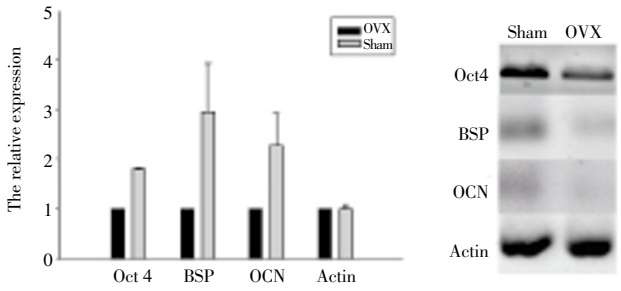


**Figure 5.** Differental gene expression between OVX and Sham MSCs. RT-PCR analysis of MSCs pluripotency related gene (Oct 4) and osteogenic differential genes(BSP, OCN), $P$<0.05.

## 4. Discussion

With the population life extending and the aging society, the number of the patients with osteoporosis is increasing. Autologous MSCs transplantation may be a potential strategy to increase bone formation in osteoporosis patients, if their MSCs have nice ability of proliferation and differentiation[18-20]. To make sure the probablity, in the present study, we have demonstrated osteoporosis rat model establishment, MSCs isolation, and characterized MSCs *in vitro*. Rat model has been used as a substitute for human patients in the study of bone defects[21], cerebral infarction[22], myocardial ischemia[23]. OVX models appear to undergo the same bone remodeling as estrogen-deficiency osteoporosis in postmenopausal women. Porcine and canine models are inappropriate for study of osteoporosis because they have little bone loss after OVX[24,25]. Rat models need less time to achieve osteoporosis compared with 6 months of rabbits[26]. In our study, we established rat osteoporosis model after OVX 3 months. Initial cell culture had difficulties such as lower proliferation rates. We successfully cultured MSCs from the OVX rats to examine the proliferation and differentiation potentials.

Currently, the mainly accepted mechanism of osteoporosis

is that the number and activity of osteoblasts reducing, the number and activity of osteoclasts increasing result in decreased bone formation and increased bone resorption. Osteoblasts derive from MSCs, indicating MSCs are one of the keys to osteoporosis. MSCs have the ability to self-renewal and differentiation in multiple lineages. Since MSCs have pluripotency of multiple tissues in vitro and vivo, they are usually attached to cell and gene therapy.

As a result, it is necessary to evaluate the proliferative capacity and differential ability to maintain the treatment after MSCs transplant. In our study, OVX MSCs had a lower proliferation rate than Sham MSCs, in the 7 days culture. The key point may be telomerase reverse transcriptase (TERT). TERT can improve the activity of telomerase to extend telomere lengths and maintain telomere stable when cell division making telomere short. That makes cell proliferation enhanced and cell life span extended. However it reported that in MSCs the expression of TERT was rarelly[27-29], causing telomerase activity limited leading to MSCs aging[30]. And they also had a weaker osteogenic differentiation than the normal. Oct4 as a embryonic transcription factor is normally expressed in ESCs to enhance self-renewal and pluripotency, which can be examined to evaluate stem-like properties loss in MSCs[31]. And an increase of Oct4 expression was reported in MSCs during differentiation[32]. Therefore we checked the semi-quantative RT-PCR analysis of Oct4 in OVX and Sham MSCs. Results revealed that lower mRNA levels of Oct4 in OVX than Shan, suggesting loss of stem-like and differential properties in the OVX MSCs. In our study, we also examined the expression of osteogenic differential marker genes such as BSP, OCN[33,34] after 10 days osteogenic differentiation culture. We noted a significant downregulation of expression for these genes in OVX MSCs compared with Sham, suggesting that OVX MSCs had a lower osteogenic differential ability.

Although autologous MSCs transplantation was reported to treat osteoporosis, there are many difficulties remaining in future clinical applications. In our study, we revealed that OVX MSCs had lower proliferative capacity and weaker osteogenic differentiation than normal MSCs, which made it inappropriate for autologous transplantation treatment. There will be a possibility in establishing a new clinical application of MSCs autologous transplantation to treat osteoporosis, if OVX MSCs have stronger proliferation and differentiation.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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