Proliferation ability of bone marrow mesenchymal stem cells in corticosteroid-induced osteonecrosis of femoral head

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Abstract

BACKGROUND: Corticosteroid-induced osteonecrosis of femoral head is one of the major causes of the loss of hip joint function. More and more studies have shown that corticosteroid-induced osteonecrosis of femoral head may be associated with proliferation ability of bone marrow mesenchymal stem cells.

OBJECTIVE: To detect the proliferation and differentiation ability of bone marrow mesenchymal stem cells isolated from patients with steroid-induced osteonecrosis of femoral head, providing rational evidences for treatment of corticosteroid-induced osteonecrosis of femoral head with the transplantation of autologous bone marrow containing bone marrow mesenchymal stem cells into the necrotic area of femoral head.

METHODS: Bone marrow mesenchymal stem cells from the femoral heads were collected from patients with corticosteroid-induced osteonecrosis of femoral head, and new femoral neck fractures without osteonecrosis who were scheduled for total hip arthroplasty. In another group, bone marrow mesenchymal stem cells were collected from ilium bone marrow of the same steroid-induced osteonecrosis of femoral head patients. The femoral neck fracture was defined as fracture without preceding trauma or in response to minimal trauma. Cases with corticoid treatment were excluded from the femur neck fracture patients. All bone marrow mesenchymal stem cells were divided three groups: femoral neck fracture group; femoral head group of corticosteroid-induced osteonecrosis of femoral head; ilium group of corticosteroid-induced osteonecrosis of femoral head. The bone marrow mesenchymal stem cells were isolated by enzyme digestion or density gradient centrifugation from bone marrow blood of the three detecting area, and then selected by the adhesive method. Passage 3 bone marrow mesenchymal stem cells were selected for experiments.

RESULTS AND CONCLUSION: The results of methyl-thiazolyl-tetrazolium assay indicated that the bone marrow mesenchymal stem cells obtained from the femoral head group showed reduced proliferation ability compared with those obtained from the other two groups. The percentage of bone marrow mesenchymal stem cells was increased at G_0/G_1, but decreased significantly at $S+G_2/M$ in the femoral head group ($P < 0.05$). The bone marrow mesenchymal stem cells obtained from the ilium group were proliferated best. The decreased proliferation ability of bone marrow mesenchymal stem cells may play a role in the low repair capacity of corticosteroid-induced osteonecrosis of femoral head, and bone marrow mesenchymal stem cells from the ilium of patients with corticosteroid-induced osteonecrosis of femoral head have a better proliferative ability.

Subject headings: femur head necrosis; femur head; transplantation, mesenchymal stem cell; bone marrow; femur

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INTRODUCTION

Reflecting the widespread use of steroids, corticosteroid-induced osteonecrosis of femoral head (ONFH) ranks the first among the etiologies for non-traumatic ONFH in China. More and more studies have shown the association between bone marrow mesenchymal stem cells (BMSCs) and non-traumatic ONFH, which may result from the decreased number and lessened reproductive activity of BMSCs containing osteoprogenitor cells in the femoral head[1-3]. Glucocorticoid has been found to exert powerful regulatory effects on proliferation and differentiation of BMSCs. In some conditions, glucocorticoid may induce abnormal proliferation and/or differentiation of BMSCs, even osteonecrosis[4-6].

There are few reports regarding proliferative activity of BMSCs of femoral head and ilium area in patients with corticosteroid -induced ONFH in comparison with normal population. We postulate that proliferative activity of BMSCs could be altered in patients with corticosteroid-induced ONFH.

To examine this hypothesis, we investigated the proliferative activity ability of the BMSCs isolated from the femoral head and ilium area with corticosteroid-induced ONFH and compared it with the proliferative ability in

patients with femoral neck fractures. It will provide supportive evidence for implantation of autologous bone marrow containing BMSCs in treatment of ONFN.

MATERIALS AND METHODS

Design

Cytology *in vitro* and comparative observation.

Time and setting

The experiment was performed at Orthopaedic Laboratory of Institute of Clinical Medical Science of China-Japan Friendship Hospital in China between March and December 2011.

Materials

BMSCs from femoral heads which were collected from two clinical groups (corticosteroid-induced ONFH and new femoral neck fracture without osteonecrosis) were isolated and cultured *in vitro*. The femoral heads were obtained from 20 corticosteroid-induced ONFH patients (ARCO stage, III–IV; 10 males and 10 females; average age of 43 years, range 35–50 years), and 20 new femoral neck fractures without osteonecrosis patients (10 males and 10 females; average age of 59 years, range 55–75 years) who underwent total hip arthroplasty.

In another group, BMSCs were collected from the ilium bone marrow of the same corticosteroid-induced ONFH group. The femoral neck fracture was defined as fracture without preceding trauma or in response to minimal trauma. Cases with corticoid treatment were excluded from the femur neck fracture patients. This study was approved by the Administrative Regulations on Medical Institution[7].

Before the study, enrolled patients were informed of the program and risk of study, and the written informed consent was obtained for all patients for research.

Reagents and equipments:	
Reagent and equipment	Source
LG-DMEM culture median	Gibco, USA
Fetal bovine serum culture median, Methyl-thiazolyl-tetrazolium (MTT), Trypsin, dimethyl sulfoxide (DMSO)	Sigma, USA
Microplate reader (Athnos 2010)	Salzburg, Australia
Mouse anti-human CD29-FITC, rat anti-human CD44-PE, CD133, CD34, CD14, rabbit anti-mouse IgG-FITC	eBioscience, USA
Phase contrast microscope (Nikon Eclipse Ts 100), Fluorescence microscopy	Nikon, Japan
Flow cytometry	Beckman Counter Company, Germany

Methods

Isolation, culture and identification of BMSCs

All BMSCs were divided into three groups: femoral neck fracture group, corticosteroid-induced ONFH femoral head group,and corticosteroid-induced ONFH ilium group. All denied exposure to alcohol or other predisposing factors for ONFH. All patients showed no evidence of concurrent illness and were not receiving any medications that could affect the bone metabolism. The BMSCs were isolated by density gradient centrifugation from bone marrow blood (5–10 mL)of the three detecting area, and then selected by the adhesive method[3]. The BMSCs were identified by immunofluorescent staining method (CD29-FITC, CD44-PE, CD133, CD34, CD14, rabbit anti- mouse IgG-FITC).

Proliferative activity of BMSCs

The cells of second generation were digested, resuspended and seeded in 96-well plates. For each plate, one well containing 20% FBS-DMEM free of cells was taken as baseline well. Each plate was picked out at 1, 3, 5, 7 or 9 days respectively and added with 20 μL of 5 mg/L MTT solution. Then, the plates were placed, avoiding light, in a humidified incubator saturated with 5% CO_2 at 37 ℃ for 4 hours. After supernatant was discarded, each well was washed thrice with 0.1 mol/L PBS and added with 150 μL of DSMO. After the plates were shaken for 10 minutes, the precipitation was resolved completely. The absorbance value of each well was measured using an ELISA reader at 490 nm and then recorded. The curve of cell growth was drawn with time as X-axis and absorbance values as Y-axis. The results obtained were recorded respectively.

Cell growth period of BMSCs

When the cells of second generation covered 90% of bottom of culture flask, they were digested and resuspended at the density of 1×10^6/mL as above-mentioned. Then the cells were fixed in 80% pre-cooled alcohol at 4 ℃ overnight. After washed with PBS and centrifuged for 5 minutes, the supernatant was discarded. The cells were added with 10 μL of PI (100 mg/L), 10 μL of RNase (100 mg/L) and 40 μL of PBS and kept in the dark for 30 minutes. Then the cell cycles of BMSCs were measured with a FCAS instrument. Proliferation index (PI) was used to assess the levels of BMSCs proliferation and calculated with the following formula: $PI=[(S+G_2/M)/(G_0/G_1+S+G_2/M)]\times100\%$. In the same time, DNA ploidy of BMSCs was also analyzed. The results obtained were recorded respectively.

Main outcome measures

Proliferative activity of BMSCs from corticosteroid-induced ONFH patients.

Statistical analysis

All statistical analyses were completed using SPSS Statistical Software 10.0 (SPSS, Chicago, IL, USA). Mean±SD and frequencies were calculated for general demographic and routine clinical data. The Mann-Whitney *U* test was used to compare the non-parametric data between two independent samples. $P < 0.05$ was

considered to be statistically significant.

RESULTS

Identification of BMSCs from corticosteroid-induced ONFH patients

There was marked difference in growth state of BMSCs between corticosteroid-induced ONFH femoral head group and the other two groups under an inverted microscope 24 hours after primary culture, and BMSCs from all groups adhered and extended in spindle or polygonal shape. Forty-eight hours later, the clone formed and adherent cells increased. However, the number of clones in the corticosteroid-induced ONFH femoral head group was much less than that in the other two groups. BMSCs resembled typical features of fibroblasts 3 days later in the other two groups and 5 days later in the corticosteroid-induced ONFH femoral head group. Clones enlarged and fused into monolayer 7 days later in the femoral neck fracture group and the corticosteroid-induced ONFH ilium group and 10 days later in corticosteroid-induced ONFH femoral head group. In the femoral neck fracture group and the corticosteroid-induced ONFH ilium group, BMSCs proliferated and distributed evenly after passage and adhered partially 2 hours later and completely in funicular shape 24 hours later. In contrast, BMSCs from corticosteroid-induced ONFH femoral head group proliferated very slowly and overgrew the bottle till 10–12 days later. The immunofluorescent staining showed that the cultured cells expressed typical BMSCs markers such as CD29 and CD44, but not typical haematopoietic cell markers including CD34, CD14 and CD133 (**Figures 1 A–C**).

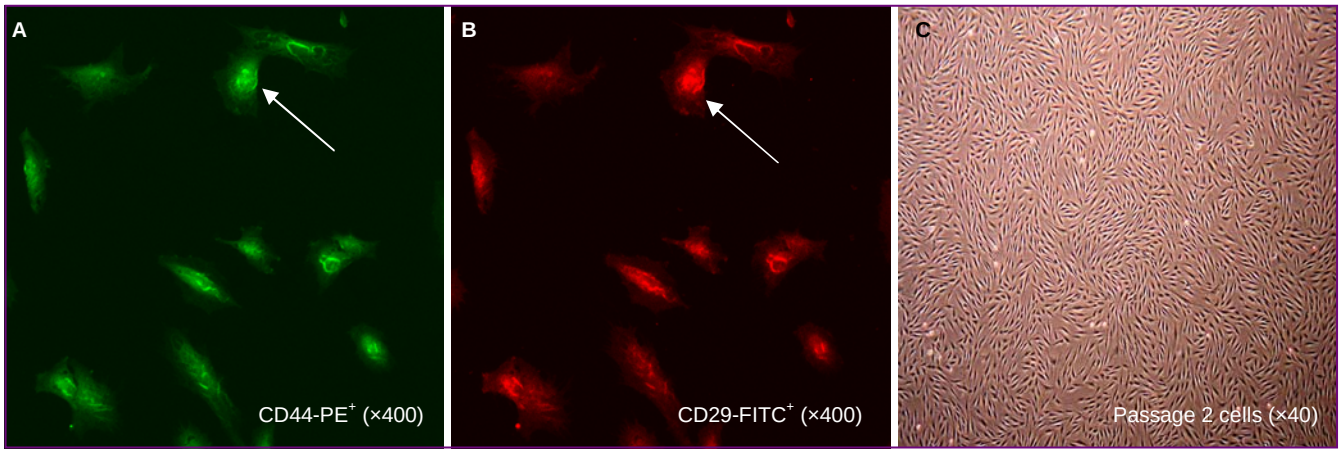

Figure 1 Morphology of bone marrow mesenchymal stem cells (BMSCs) from corticosteroid-induced osteonecrosis of femoral head patients (immunofluorescent staining)

Notes: The BMSCs are identified by immunofluorescent staining method and selected by the adhesive method and cultured, and positive staining is then observed in the cells localized in a perinuclear pattern (arrow).

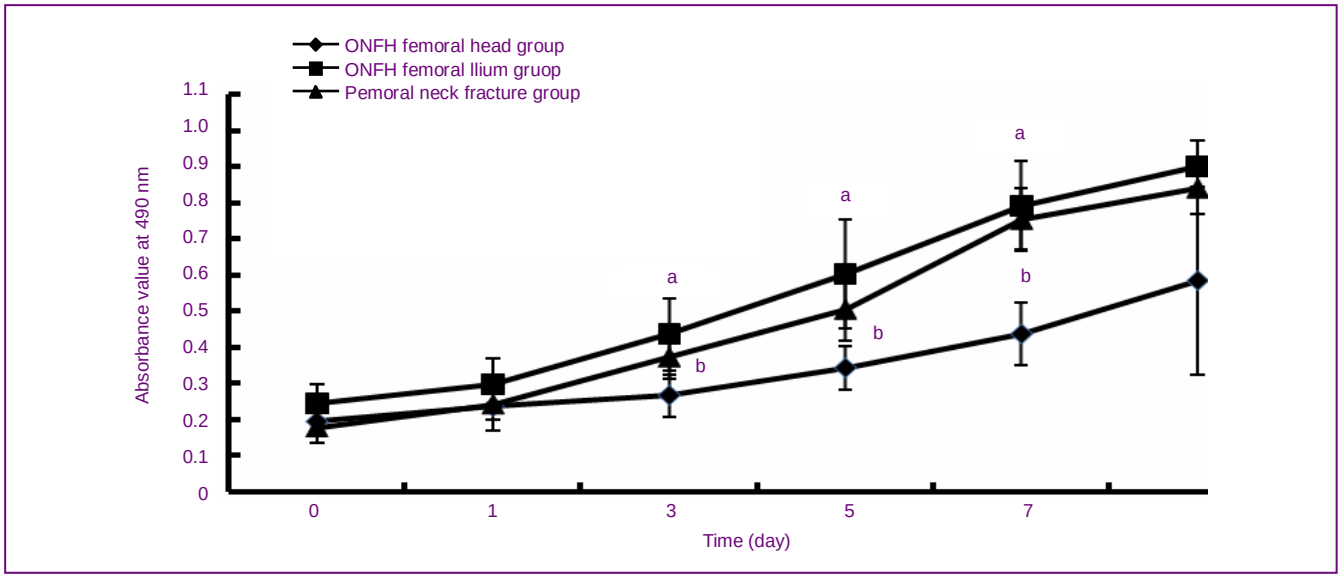

Figure 2 The growth curves of bone marrow mesenchymal stem cells from corticosteroid-induced osteonecrosis of femoral head (ONFH) patients reflecting the proliferative activities by MTT detecting methods

Notes: Data are represented as the optical absorption values at 490 nm. [a]*P* < 0.05, *vs*. ONFH femoral head group; [b]*P* < 0.05, *vs.* femoral neck fracture group.

Proliferative activity of BMSCs from corticosteroid-induced ONFH patients

The proliferative activities of BMSCs using MTT methods were obtained from the three groups and drawn into the growth curves. As shown in the figures, the proliferative activity of BMSCs in the femoral neck fracture group and the corticosteroid-induced ONFH ilium group were remarkably stronger than that in the corticosteroid-induced ONFH femoral head group. For the corticosteroid-induced ONFH femoral head group, BMSCs growth fell into the stagnant stage within 1–7 days after seeding, turned into the logarithmic stage at 8 days, and came to the plateau stage thereafter. However, the growth curve in the femoral neck fracture group and the corticosteroid-induced ONFH ilium group moved left obviously and the peak increased (**Figure 2**).

Growth period of BMSCs from corticosteroid-induced ONFH patients

For FCAS, the percentage of cells in S stage or G_2/M+S stages was usually taken as PI to indicate the proliferation of cells. Compared with the other two groups, the percentage of cells in G_0/G_1 stages in the corticosteroid-induced ONFH femoral head group was increased significantly while the percentage in G_2/M+S stages (PI) was decreased significantly ($P < 0.05$). The percentage of cells in G_2 stage in the corticosteroid-induced ONFH ilium group was the highest in the three groups. These were in consistent with results revealed by MTT method (**Table 1**).

Table 1 Growth period of BMSCs from corticosteroid-induced ONFH patients ($\bar{x}\pm s$, n=20, %)

Growth period	Femoral neck fracture group	ONFH femoral head group	ONFH ilium group
G_0/G_1	9.5±0.3[a]	88.5±0.4	77.4±0.6[a]
S	12.6±0.5[a]	3.6±0.5	13.9±0.5[a]
G_2/M	8.0±0.4[a]	7.9±0.5	9.3±0.43[a]
PI	20.5±0.5[a]	11.6±0.6	23.2±0.5[a]

[a]$P < 0.05$, *vs*. ONFH femoral head group. ONFH: osteonecrosis of femoral head; BMSCs: bone marrow mesenchymal stem cells.

DISCUSSION

ONFH is one of the major causes of disability in young patients for it usually leads to the loss of hip joint function in early stages of the disease[8]. Reflecting the widespread use of steroids, corticosteroid-induced ONFH ranks the first among the etiologies for non-traumatic ONFH in China. Although there are many theories on the disease, the underlying mechanism remains unclear. Currently the only effective treatment for collapse of femoral head is total hip replacement or rotational transtrochanteric osteotomy[1-3, 10-13]. It has been suggested recently that ONFH may be a disease of BMSCs, due to abnormal proliferation and/or differentiation of BMSCs[5-7]. It is a kind of multipotent stem cells that can differentiate into multiple types of cells including osteoblasts, adipose cells, fibroblasts, chondrocytes and so on. These kinds of cells play an important role in the stability of bone and cartilage tissue and in post-damage tissue repair, such as bony fracture[4]. In some conditions, glucocorticoid can induce differentiation of BMSCs into adipose cells and suppress differentiation into osteoblasts and the proliferative capacity of BMSCs[14-17]. By far, the detection of the proliferative ability of BMSCs in patients with glucocorticoid-induced ONFH has not yet been reported internationally. Therefore, the proliferative ability of BMSCs was measured in these patients, and the relationship was investigated between the alterations of the proliferative ability of BMSCs and the pathogenesis of these diseases in the present study. It is another purpose for providing supportive evidence for autologous bone marrow transplantation containing BMSCs in treatment of ONFH.

Our results showed that the proliferation of BMSCs from the corticosteroid-induced ONFH femoral head group was obviously worse than that from the other two groups. There was remarkable difference in the growth state of BMSCs between the three groups under an inverted microscope. In details, BMSCs in the corticosteroid-induced ONFH femoral head group usually became coarse or slim in shape and light-colored in the cytoplasm, and proliferated slowly in an uneven manner. In contrast, BMSCs in the femoral neck fracture group and the corticosteroid-induced ONFH ilium group usually became funicular in shape or formed clones and proliferated quickly in an even manner within dark-colored cytoplasm. Twenty-four hours after primary culture, BMSCs adhered and extended in spindle or polygonal shape. Forty-eight hours later, the clones were formed and adherent cells were increased. However, the number of clones in the corticosteroid-induced ONFH femoral head group was much less than that in the other two groups. BMSCs resembled typical features of fibroblasts 3 days later in the femoral neck fracture group and the corticosteroid-induced ONFH ilium group, and 5 days later in the corticosteroid-induced ONFH femoral head group. Clones were enlarged and fused into monolayer 7 days later in the femoral neck fracture group and the corticosteroid-induced ONFH ilium group, and 10 days later in the corticosteroid-induced ONFH femoral head group. In the femoral neck fracture group and the corticosteroid-induced ONFH ilium group, BMSCs proliferated and distributed evenly after passages and adhered partially 2 hours later, formed clones and adhered completely 24 hours later, and covered the bottle 6-7 days later. In contrast, BMSCs in the corticosteroid-induced ONFH femoral head group proliferated very slowly and covered the bottle 10–12 days later. As demonstrated in the growth curve, BMSCs in the corticosteroid-induced ONFH femoral head group proliferated extremely slowly within 1–7 days after seeding and then gradually faster since 8 days and then came into the platform stage. However, the growth curve of BMSCs in the femoral neck fracture group and the corticosteroid-induced ONFH ilium group advanced obviously at the logarithmic stage proceeding for at least 2 days. Moreover, as an indicator of cell numbers, the peak of the growth curve increased significantly. The cells of the corticosteroid-induced ONFH ilium group grew better than the femoral neck fracture group; the cell cycle of multiplication was shorter.

Cell cycle is composed mainly of G_1, S and G_2/M stages. S stage and G_2/M stage are usually stable and the time of a cell cycle is dominantly dependent on the time of G_1. For FCAS, the percentage of cells in S stage or G_2/M+S stages is usually taken as PI to indicate proliferation of cells. Our results showed that the percentage of cells in G_0/G_1 stages increased significantly in the corticosteroid-induced ONFH femoral head group and the percentage in G_2/M+S stages (PI) decreased significantly in comparison with the other two groups.

In the present study, all controls were the patients with fracture of femoral neck. Fracture of femoral neck is common in the elder patients. And the majority of controls (the femoral neck fracture group) in the present study were thus elder patients. In contrast, all the patients with ONFH were much younger than the controls. It has been well demonstrated that the proliferative capacity of BMSCs decreases with age[18-20]. At least we did not find out any reference implying that the proliferative capacity of BMSCs increases with age[21-22]. In comparison with the femoral neck fracture group, the proliferative capacity of BMSCs decreased obviously in the patients with ONFH in the corticosteroid-induced ONFH femoral head group, though they were much younger than the controls.

The decreased proliferative capacity of BMSCs obtained from the corticosteroid-induced ONFH femoral head group seemed to participate in the pathogenesis of glucocorticoid-induced osteonecrosis. However, the mechanism underlying the influence of corticosterroid BMSCs remains very unclear. Kuen Tak in Korea found that, through measuring their differential ability, the proliferative capacity of BMSCs at the proximal femur decreased remarkably in the patients with alcohol-induced osteonecrosis. In these patients, it was also found that the capacity of BMSCs to differentiate into osteoblasts decreased and the capacity to differentiate into fat cells increased. In addition, the research conducted the correlation analysis between the activity of BMSCs with age and sex. These studies on alcohol-induced osteonecrosis provided a theoretic basis for corticoid-induced osteonecrosis since it has been well-established that the pathogenic mechanism of corticoid-induced osteonecrosis is similar to that of alcohol-induced osteonecrosis[23-25]. It has been widely known that glucocorticoid inhibits proliferation of various types of cells, including BMSCs and osteoblastic cells. Although these effect seem to be more related to osteoporosis than osteonecrosis, several studies recently demonstrated that these effect had occurred in osteonecrosis[26-28]. Weinstein *et al* [29-30] first proposed that corticoid could promote apoptosis of osteoblasts and osteocytes and suppress production of osteoblasts, leading to decrease of osteocytes. By comparison with other kinds of osteonecrosis, Hernigou *et al* [5] also found that the number of BMSCs at the proximal femoral was significantly decreased in the case of corticoid-induced osteonecrosis and thus speculated that corticoid induces damage to osteoblasts at femoral head and exerts deleterious effect on bone tissue. Wang *et al* [6] believed that the differentiation of BMSCs into fat cells induced by corticoid accounted for osteonecrosis. Li *et al* [14] confirmed that corticoid did induce the differentiation of BMSCs into fat cells and inhibit the differentiation BMSCs into osteoblasts at the level of gene expression regulation. Moreover, Hofbauer *et al* [31] found that corticoid could activate osteoclasts by which transcription and expression of osteoprotegerin/ osteoclastogenesis inhibitory factor was inhibited in osteoblasts/BMSCs and expression of osteoclast differentiation factor/osteoprotegerin ligand was promoted in bone matrix cells, and then resulted in decreased osteocytes and bone mass, which might be involved in corticoid-induced osteonecrosis. Our results revealed that the proliferation activity of the cells from the corticosteroid-induced ONFH ilium group was better than that in the corticosteroid-induced ONFH femoral head group. We assumed that it may be concerned with the environment where the cells live. But we did not know whether the cells in the corticosteroid-induced ONFH ilium group were suppressed at some degree or not, for we did not compare it to the cells from non-osteonecrosis patients. So it might be beneficial to treat non-traumatic ONFH with the transplantation of autologous bone marrow which contain BMSCs from the ilium into the necrotic area of femoral head.

We found that the proliferative capacity of cultured BMSCs that obtained from the patients with osteonecrosis decreased, even in absence of corticoid, suggesting that the persisting alterations in function of BMSCs may play a role in pathogenesis of corticoid-induced osteonecrosis. It is reported that ONFH consists of necrotic, repair, and normal regions. In the earlier stage of osteonecrosis, sufficient repair capacity can reverse the process of disease[32-35]. Insufficient repair capacity due to decreased ossification may be one of major reasons for further development of osteonecrosis, since bone formation rate is impaired, to a large extent, by decreased proliferation of progenitors[36-38]. Our results confirm that the pathogenesis of glucocorticoid-induced osteonecrosis is associated with decreased proliferative capacity of BMSCs at the proximal femur. Glucocorticoid induces osteonecrosis through lowering the proliferative activity or differential capacity as well as altering the differentiation direction of BMSCs, even the decreased repair capacity due to decreased proliferative capacity of BMSCs in normal regions surrounding necrotic regions of ONFH may be one of major reasons for progression of osteonecrosis. The limited bone remodeling and repair may be a result of the decreased number and lessened reproductive activity of BMSCs containing osteoprogenitor cells; therefore, it might be beneficial to treat non-traumatic ONFH with the implantation of autologous bone marrow which contain BMSCs into the necrotic area of femoral head.

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骨髓间充质干细胞增殖能力与皮质类固醇性骨坏死

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文章亮点：

皮质类固醇性股骨头坏死的自行修复能力有限可能与此类患者股骨头内骨髓间充质干细胞增殖活性下降有关。实验通过检测股骨头坏死患者股骨头内和髂骨处来源的的骨髓间充质干细胞的增殖活性，并与股骨颈骨折的患者做对照，发现股骨头坏死患者股骨头内的骨髓间充质干细胞增殖活性明显下降，明显低于股骨颈骨折患者和其髂骨来源的骨髓间充质干细胞，而其髂骨来源的骨髓间充质干细胞活性增殖活性最高，这就为移植含有骨髓间充质干细胞的浓缩骨髓血治疗股骨头坏死提供了理论依据。

关键词：

干细胞；骨髓干细胞；股骨头坏死；皮质类固醇；髂骨；细胞增殖；细胞周期；髋关节

主题词：

股骨头坏死；股骨头；干细胞移植间充质；骨髓；股骨

摘要

背景：皮质类固醇激素性骨坏死是造成髋关节功能丧失的主要病因之一。近年研究表明，激素性股骨头坏死可能与激素引起的骨髓间充质干细胞增殖能力有关。

目的：检测皮质类固醇性骨坏死患者骨髓间充质干细胞的增殖活性，为建立自体骨髓干细胞移植治疗股骨头坏死的合理性寻求证据。

方法：选取皮质类固醇性股骨头坏死病例设为股骨头坏死组，按取材部位不同再分为股骨头坏死股骨头组、股骨头坏死髂骨组，同时选取无股骨头坏死、无激素应用的拟行人工关节置换的股骨颈骨折患者设为对照组。用密度梯度离心法分离各组骨髓间充质干细胞，再经贴壁筛选法筛选，选取第 3 代细胞进行实验。

结果与结论：MTT 结果显示，股骨头坏死股骨头组增殖能力明显弱于其他 2 组，其骨髓间充质干细胞在培养后 1-7 d 为生长滞留期，第 8 天达到对数生长期，以后进入到平台期，而其他 2 组较病例组生长曲线明显前移，并且峰值增高。流式细胞仪测定的细胞周期结果显示，股骨头坏死股骨头组中 G_0/G_1 细胞比例明显增高，而 $S+G_2/M$ 期细胞比例降低，细胞增殖指数较其他 2 组降低($P < 0.05$)，而股骨头坏死髂骨组的细胞增殖活性最强。结果证实，皮质类固醇性骨坏死患者股骨头来源的骨髓间充质干细胞增殖活性较低，髂骨来源的骨髓间充质干细胞活性增殖活性较高。

作者贡献：全部作者均参与了实验的设计，实施和评估，均受过正规培训。王佰亮进行实验设计，王佰亮和李铁军进行实验实施，孙伟进行实验评估，资料收集为岳德波、孙伟，王佰亮审校并对文章负责。

利益冲突：文章及内容不涉及相关利益冲突。

伦理要求：参与实验的患病个体及其家属自愿参加，所有参与者均对实验过程完全知情同意，在充分了解本治疗方案的前提下签署“知情同意书”。实验干预及治疗方案获中日友好医院伦理委员会批准(伦理审批号：20110103)。王佰亮和李铁军均具有细胞培养技术，岳德波和孙伟均具有股骨头坏死在治疗经验。所有实验实施者均是临床医师，经过相关培训，具有从事实验所要求的资质。

学术术语：股骨头坏死-是股骨头血供中断或受损，引起骨细胞及骨髓成分死亡及随后的修复，继而导致股骨头结构改变、股骨头塌陷和关节功能障碍的疾病，可分为创伤性和非创伤性 2 类。

作者声明：文章为原创作品，无抄袭剽窃，无泄密及署名和专利争议，内容及数据真实，文责自负。

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