Correlation between the efficacy of stem cell therapy to osteonecrosis of the femoral head and cell viability

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Abstract

Background: Osteonecrosis of the femoral head (ONFH) is a common disease that greatly affects the quality of life of patients. Repair of necrotic area is the key to treatment.At present, the combination of stem cell transplantation and decompression is used clinically to promote the repair of necrotic areas through the characteristics of stem cells. However, a considerable number of patients cannot achieve a satisfactory outcome in repairing the femoral head necrotic area. It is very important to find out the reasons for the poor curative effect. The aim of this study was to investigate the correlation between stem cell viability and the repair efficacy of stem cell therapy combined with core decompression to early-stage of ONFH.

Methods: A total of 30 patients with idiopathic ONFH were performed core decompression combined with autologous stem cell transplantation. The Harris score (HHS) and necrosis area change of patients before and after operation were observed. The mean value of repair ratio was set as a threshold dividing the patients into group A (ratios greater than the mean value) and group B (ratios less than the mean value). The ultrastructure, proliferative capacity and multidirectional differentiation ability were compared between the groups.

Results: At 9 months after surgery, HHS and magnetic resonance imaging (MRI) findings had improved by varying degrees. Based on the repair ratio, i.e., (62.2 ± 27.0) %, 62.2% was set as a threshold dividing the patients into group A and group B. Better repair(Group A) showed faster proliferation efficiency and healthier ultrastructure. The cells of Group A also showed stronger specific staining after osteogenesis and chondrogenesis induced differentiation. The activity of alkaline phosphatase (ALP) was also higher in group A (OD 2.39 ± 0.44 vs 1.85 ± 0.52; $P < 0.05$) after osteogenic differentiation.

Conclusions: The quality of implanted stem cells is closely related to the efficacy of this procedure and determines whether the defects of self-repair in the necrotic areas can be corrected to enhance the repair capacity of necrotic tissue and to promote the repair of necrotic areas to achieve the desired therapeutic outcome.

Background

Osteonecrosis of the femoral head (ONFH) is a common disease that greatly affects the quality of life of patients [1]. The course of this disease is progressive, and the severity of arthritis gradually increases [2], with late-stage patients needing to undergo total hip arthroplasty (THA) [3]. Determining how to prevent progression of early-stage ONFH or even to completely repair the necrotic areas to avoid undergoing THA is a very challenging issue in clinical practice. Core decompression is one of the early treatments available [4] and can significantly relieve the early symptoms of pain in patients with ONFH [5]. However, a considerable number of patients cannot achieve a satisfactory outcome in repairing the femoral head necrotic area [5]. Femoral head necrosis is actually a disease involving the activity of local stem cells in the femoral head [6]. In ONFH patients, number and viability of Mesenchymal stem cells (MSCs) in the

femoral head was found to be decreased [7]. Therefore, the implantation of new stem cells may be one of the solutions. MSCs are capable of self-renewal and divergence into multiple lineages, including bone, cartilage, adipose tissue, muscle and tendon [8]. Theoretically, implanted stem cells with renewable and multi-differentiational ability could drive and lead the repair of the necrotic area, compensating the repair difficulty resulted from functional defect of local stem cells [9]. Both clinical doctors and patients have great hope for it. However, in practical clinical application process, stem cell therapy combined with core decompression has slight advantages over core decompression procedure only[10], but it still does not achieve anticipated therapeutic effects, with no apparent repair in the necrotic area for some postoperative patients [11]. For those patients, they not only paid for the expensive hospitalization fee but also endured psychological and physical pain. Hence it is of great importance to find out the cause of this problem. In our opinion, the differences in stem cell proliferation and differentiation capacity may be important factors affecting the efficacy of the combined therapy. Therefore, this study aimed to investigate the mechanism of how the quality and number of stem cells affect the correlation between stem cell viability and the repair efficacy of ONFH.

Methods

General data

The study was reviewed and approved by the University Ethics Committee. Written informed consent was obtained from all subjects.

This study included 19 men and 11 women with idiopathic ONFH (Arco stage II), with a mean age of 30.6 years. All patients received the following evaluations before surgery: The Harris score (HHS), the visual analogue scale (VAS) of pain, routine blood test, liver and kidney function, comprehensive coagulation tests, erythrocyte sedimentation rate (ESR), C-reactive protein level, electrocardiogram (ECG), chest anteroposterior radiography, bilateral hip anteroposterior radiography, and bilateral hip plain MRI scan.

Harvest and isolation of autologous stem cells

Prior to surgery, patients received recombinant granulocyte colony-stimulating factor (GCSF, 30 IU IM Qd ×5 days) for stem cell mobilization in the bone marrow [12]. Under effective anesthesia, the patient was placed in the supine position. Bone marrow aspiration from the iliac crest was performed, and 100 ml of bone marrow was harvested. Additionally, 100 ml of peripheral blood was also collected. The bone marrow and blood were placed in heparin-coated centrifuge tubes and centrifuged twice at 4000 r/min for 10 min. A 30 ml cell suspension was obtained. The 15 ml of suspension was added into a collagen sponge to build a gel-like cell-material composite for surgery. The remaining 15 ml was used for assessing the number and vitality of cells.

MNCs count

The Mononuclear cells (MNCs) were isolated by density gradient centrifugation with Percoll. Cell suspension was performed with gradient centrifugation in the speed of 3000 rpm for 30 min by cell separator. The centrifugal liquid was divided into three layers, middle layer was the MNCs, MNCs suspension were obtained from the middle layer after separation to performed the MNCs count under microscope eyepiece grid.

Isolation and culture of hBMSCs

The cells were re-suspended according to a 1:1 proportion with the culture medium (i.e., Dulbeco's modifild of eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin) and then placed in cell culture flasks at a density of 3×10^6 cells/mL in an incubator at 37°C and 5% CO_2. The cells were passaged by treatment with a 0.2% trypsin solution when the cell confluence reached 80%. The third-passage cells were used for the subsequent experiments.

Immunophenotypic characterization of hBMSCs

The third-passage cells were collected after digestion.A 100 µl volume of suspension containing 1×10^6 cells were immunostained for cell surface markers using an Aria SE flow cytometer and Cell Quest Pro software. The hBMSCs were identified as a population of cells expressing CD105, CD73, CD44 and CD90, with an absence of CD34, CD45 and HLA-DR. Cells were incubated with CD44-APC, CD90-FITC, CD105-CY5.5, CD73-PE, CD34-PE, CD45-PE and HLA-DR-PE. Antibodies were used at the suppliers recommended dilutions. Sample was incubated with antibody for 45 minutes at room temperature protected from light. After 2 times of PBS washing, flow cytometry was performed.

Ultrastructural observation

The ultrastructural observation of cells was conducted using a transmission electron microscopy (TEM, Tecnai 10, FEI, Hillsboro, OR, USA). The specimen preparation of TEM was the following the third-passage cells concentrated by centrifugation at low speed (2000 rpm). The small cell pellets were pre-fixed in 2.5% glutaraldehyde, and then rinsed three times with phosphate buffer solution (PBS). Subsequently, the small cell pellets were post-fixed with 1% osmium tetroxide, rinsed three time with PBS and dehydrated by a series of mixture of acetone and distilled water (30%, 50%, 70%, 90%, 95%, and 100%), respectively. Finally, the dehydrated cell pellets were embedded and then were sectioned with diamond knives. The ultra-thin sections of specimens were stained with uranyl acetate and lead citrate each for 30 min and observed under TEM.

Multilineage differentiation and quantification of differentiation

The third-passage cell were trypsinized and replated onto 100 mm^2 tissue culture plates at 10^5 cells per plate. After incubation in the culture medium for 1 day, the medium was replaced with either osteogenic medium, containing 10 mM b-glycerophosphate, 0.1 M dexamethasone, 50 g L-ascorbic acid 2-phosphate/mL and 10 g insulin/mL; or chondrogenic medium, containing DMEM, 1% (v/v) FBS, 10 ng rh-TGFb1/ml, 50 mg ascorbic acid/l, 6.25 mg insulin/ ml, 10–7 M dexamethasone, 100 units penicillin/ml, 100 mg streptomycin/ml and 12 lM L-glutamine. Culture media were replaced every 3 days. Cells were assessed at 14 days after differentiation. Quantitative analysis of osteogenic differentiation was performed by measurement of alkaline phosphatase (ALP) activity, by an Alkaline Phosphatase Assay Kit (Abcam, Cambridge, MA) per the manufacturer protocol, and quantification of Alizarin Red S adhered to calcified tissues, following the protocol. The staining of toluidine blue (D8857, NobleRyder, China) were performed to ascertain chondrogenic differentiation.

Image analysis

Alizarin Red S staining after a 14-day osteogenic induction of hBMSCs and Toluidine Blue staining after a 14-day chondrogenic induction of hBMSCs were evaluated. The area percentage of color density of each specific staining was determined individually by Image J image analyzing system version # 1.50d. Images were first converted into grey scale via selecting RGB stalk type of image, then thresholding is activated and adjusted to select region of interest as compared to the original colored image. The region of interest becomes then highlighted in red within the grey scale, and then area percentages measurements were made.

Core decompression and stem cell-material composite implantation

Upon successful anesthesia, a 3 cm incision was made below the greater trochanter. Under C-arm fluoroscopy guidance, a 2.5 mm Kirschner wire (K-wire) was drilled into the lesion site of the femoral head (2–3 mm beyond the subchondral level) via the femoral neck. A 6.5 mm drill was drilled into the same site over the K-wire. A customized long-handled curette was used to completely remove the lesion tissue beneath the cartilage. Fluoroscopy was performed to ensure the completion of the lesion removal. Next, the collagen sponge-cell composites were injected to fill this site. A piece of muscle membrane was used to cover the filling site to prevent cell leakage. All procedures were performed by the same surgical team (Fig. 1a-f).

Postoperative management and follow-up

The patients were instructed to walk with a supportive device for 9 months after surgery. HHS, and imaging studies of the treated hip were used to assess the clinical efficacy during the follow-up. Patients' assessments of pain were marked on a VAS from 0 cm (no pain) to 100 cm (severe pain).

Assessment criteria

HHS of the hip: The delta of HHS was calculated as the difference between the HHS before the surgery and the HHS at 9 months after surgery. A greater difference was believed to represent more significant functional improvement.

Necrotic area evaluation by MRI: A GE Signa 1.5T superconducting MR (USA) was used for the hip examination. The coronal T1-weighted images were selected to measure the necrotic area angle α and the central angle β of the femoral head (Fig. 2a, b). According to FengChao Zhao's method for necrotic area ratio calculation, the necrotic area ratio (before vs. after treatment) of each hip was determined [13]. The repair ratio is calculated according to the following formula. A greater repair ratio represents more significant lesion repair.

[Due to technical limitations, the formula could not be displayed here. Please see the supplementary files section to access the formula.]

The mean value of repair ratio was set as a threshold dividing the patients into group A (ratios greater than the mean value) and group B (ratios less than the mean value). The ultrastructure, proliferative capacity and multidirectional differentiation ability were compared between the groups.

Statistical analysis

The SPSS version 21.0 was used for statistical analysis. Paired t test, nonpaired t tests and Spearman correlation analysis were used. All tests were two-tailed at the 5% level of significance.

Results

MRI-determined necrotic area ratio

This ratio significantly decreased from (35.51 ± 9.57) % preoperative to (13.74 ± 10.70) % at 9 months after surgery. At 24 months after operation, the necrotic area ratio was (13.24 ± 9.49) %, which had no significant change compared with 9 months after operation ($p > 0.05$) (Fig. 3a-l, Fig. 4a).

HHS and VAS of the hip

The HHS significantly increased from (71.63 ± 8.05) preoperative to (84.66 ± 6.97) at 9 months after surgery ($p < 0.05$). At 24 months after operation, the HHS was (85.27 ± 7.97), which was slightly higher than that of 9 months after operation ($p > 0.05$). The delta of HHS (13.04 ± 5.86) was obtained by subtracting the score at 9 months after surgery from the preoperative score (Fig. 4b). The VAS significantly decreased from (3.33 ± 0.77) preoperative to (1.91 ± 0.53) at 9 months after surgery ($p < 0.05$). At 24 months after operation, the VAS was (1.87 ± 0.60), which was slightly lower than that of 9 months after operation ($p > 0.05$). (Fig. 4c).

Correlation between the repair ratio and the delta of the HHS

An increased repair ratio was associated with a greater HHS (Table 1). This result suggests that the extent of the lesion repair is correlated with the extent of functional improvement. Based on the repair ratio, i.e., (62.2 ± 27.0) %, 62.2% was set as a threshold dividing the patients into group A and group B (Table 2). There were no significant differences in the baseline characteristics (Table 3).

Table 1 Correlations among repair ratio, delta of the HHS, and age

Parameter	Repair ratio	
	r value	*p* value
Delta of the HHS	0.850	< 0.05
Age (years)	0.084	> 0.05

$p < 0.05$ was considered to be statistically significant.

Table 2 Repair ratios (%) between groups A and B

N	Group A	Group B
1	62.5	8.7
2	62.8	12.4
3	67.2	18.7
4	69.1	23.7
5	75.3	35.9
6	76.4	38.1
7	84.2	38.5
8	85.7	40.7
9	87.3	42.3
10	87.8	49.7
11	88.5	53.1
12	91.9	55.7
13	94.2	57.3
14	96.5	60.4
15	100	
16	100	

Table 3 Baseline patient characteristics

Variable	Group A (N = 16)	Group B (N = 14)	*p* value
Side of treated hip			
Left (%)	10 (62.5%)	6 (42.9%)	> 0.05
Right (%)	6 (37.5%)	8 (57.1%)	> 0.05
Age (mean ± SD)	30.69 ± 5.87	30.43 ± 4.45	> 0.05
Gender (male/female)	10/6	9/5	> 0.05
Preoperative HHS (mean ±SD)	68.67 ± 8.38	75.01 ± 6.37	> 0.05
Preoperative necrotic area ratio (%)	34.17 ± 9.01	37.05 ± 10.29	> 0.05
Concentration of MNCs (mean±SD×10^9/L)	9.94 ± 1.46	10.04 ± 1.47	> 0.05
Hospitalization expenses ($)	3203.31 ± 115.23	3190.14 ± 134.37	> 0.05

No variables were significantly different at baseline between groups A and B.

Ultrastructural characteristics of hBMSCs and the times of cell passages

The hBMSCs from group A exhibited large nuclei that were irregular round or oval in shape, intact nuclear membranes, and large and obvious nucleoli with even heterochromatic distribution. The cells were rich in cytoplasm, showing a medium electron density. The organelles, such as the rough endoplasmic reticulum, Golgi apparatus, and mitochondria, were simple but abundant, with clear structure. Group B featured the hBMSCs with decreased electron density in the cytoplasm and plentiful vacuoles and autophagosomes in varying sizes. The autophagosomes contained incompletely digested residual organelles, cytoplasmic components, and ridge-dissolved mitochondria. (Fig. 5a-d) A group compared with B group, cell ultrastructure showed more characteristics of healthy cells. The times of cell passages 0 (P0) was 9.19 ± 0.98 days in group A and 10.21 ± 1.19 in group B ($p < 0.05$), and P2 reduced to 6.19 ± 1.72 in group A and 8.07 ± 1.94 in group B ($p < 0.05$). P3 was 5.63 ± 1.03 days in group A and 7.36 ± 3.13 in group B ($p < 0.05$). P0, P2, and P3 in group A were significantly shorter than those in group B ($p < 0.05$), but there was no significant difference in P1 between groups A and B (Fig. 5e).

Cell surface marker expression

Flow cytometry was used to detect the surface antigen molecules of third-passage hBMSCs in groups A and B. The cells highly expressed CD105, CD73, CD44 and CD90, but not the hematopoietic stem cell marker CD34, CD45 and HLA-DR (Fig. 6).

Multilineage differentiation test

After a 14-day induction, the hBMSCs of two groups were able to maintain different degrees of osteogenic and chondrogenic differentiation. The specific staining of Group A was stronger than Groups B (Fig. 7a-f). The hBMSCs of Group A demonstrated superior ALP activity after osteogenesis induction compared to Group B (OD 2.39 ± 0.44 vs 1.85 ± 0.52; $p < 0.05$) (Fig. 8).

Alizarin Red S staining was expressed as red calcium nodules staining.Toluidine Blue staining was expressed as blue granular cytoplasmic staining. Via using image J image analyzing software,the specific staining area percentage was evaluated. The area percentage range of Alizarin Red S staining was 16.44 ± 8.48 in Group A and was 6.52 ± 5.31 in Group B (Fig. 7c). The area percentage range of Toluidine Blue staining was 25.39 ±9.24 in Group A and was 12.99 ± 4.08 in Group B (Fig. 7f). Both of these specific stainings showed a signifificantly higher mean area percentage of specific staining when compared to Group B. ($p < 0.05$)

Discussion

Based on this study, we can conclude that the efficacy of autologous stem cell combined with core decompression for the treatment of early-stage ONFH is associated with autologous stem cell viability.

The higher proliferation and differentiation capacity of hBMSCs can improve the efficacy of therapy.

The traditional treatment of early ONFH is core decompression [14]. It can release the pressure in the femoral head, open the small vessels in the femoral head which are blocked by pressure, and relieve the pain symptoms [15]. However, the imaging data obtained from follow-up after operation showed that the necrosis area of femoral head did not shrink significantly in quite a number of patients [16], and even in some cases it was observed that the necrosis area would continue to expand [17], which eventually led to the collapse and deformation of femoral head [5]. Therefore, core decompression alone can not make the femoral head necrosis area reconstructed and repaired well[18]. But stem cells give us hope. Sugaya's team confirmed the efficacy of local transplantation of BMSCs in the treatment of femoral head necrosis through animal experiments, and confirmed that BMSCs can survive, proliferate, differentiate into bone directly in the necrotic area and promote the repair of necrotic area [19, 20]. But stem cell therapy for osteonecrosis has not achieved theoretical repair effect in clinical application [7]. Wojciech Pepke compared the efficacy of bone marrow cell implantation and core decompression alone in the treatment of early ONFH. Comparing the volume change of necrotic area after operation, there was no significant difference between the two groups [11]. The activity and quantity of stem cells should be the key factors influencing the therapeutic effect.

9 months after operation, the HHS was significantly lower than that before operation. From 9 months to 2 years later, some patients'function continued to improve, and some patients' hip function had a deteriorating trend, but the overall trend was stable. Valérie Gangji followed up 13 patients with similar results[21]. The VAS score of 9 months after operation decreased significantly, and the trend of change was similar to that of functional score. There was no significant change in VAS score at 2 years after operation. Tabatabaee RM also obtained similar results [22]. In this study, the diversity of regional repair of femoral head necrosis was also observed. The HHS of patients with better repair also changed significantly, which indicated that the repair of necrotic area was closely related to the improvement of function [12]. There was no significant difference in the necrotic area between 2 years and 9 months after operation, indicating that the repair and reconstruction of necrotic area mainly occurred within 9 months after operation, which was similar to the healing process of fracture [23]. Therefore, the repair of necrotic area at 9 months after operation could evaluate the therapeutic effect of stem cell implantation.

In 1999, Hernigou reported a decrease in the activity of stem cells in ONFH [24]. The decreased activity of stem cells may affect local tissue and vascular regeneration, the tissue's oxygen supply, and osteogenic function, eventually leading to ONFH [25]. Moreover, the decreased activity of the stem cells can hinder the repair of necrotic areas, forming a vicious circle [26]. Therefore, it is necessary to improve the growth and differentiation capacity of local stem cells in the necrotic area of the femoral head [27, 28]. Implanted hBMSCs can differentiate into various types of cells that can perform vascularization and osteogenesis functions to promote the repair of the necrotic areas [29]. The concept of combining stem cell transplantation with core decompression for the treatment of early-stage ONFH was first proposed by Hernigou in 2002 [30]. He observed that the number of transplanted cells was closely related to the prognosis of stem cell transplantation. In 2005, in a study evaluating stem cell transplantation for the

treatment of bone nonunion and osteonecrosis, he proposed that the key to effective stem cell transplantation is that the concentrations of the implanted stem cells should be greater than 2 million MNCs per ml [31]. However, the differentiation capacity of implanted stem cells was not discussed in either his study or those of others.

This study aimed to discuss the relationship between the efficacy of core decompression combined with stem cells transplantation and the proliferation and differentiation capacity of implanted stem cells in patients with idiopathic ONFH. Using TEM, we found that the implanted hBMSCs showing poor repair capacity had decreased electron density in the cytoplasm, with a large number of vacuoles and autophagosomes of varying sizes. The autophagosomes contained incompletely digested residual organelles and cytoplasm components. Based on the morphology of the vacuoles, we speculated that these vacuoles may be derived from mitochondrial vacuolization, which can affect aerobic respiration [32] and can further compromise the capacity for cell proliferation and differentiation [33]. Autophagosomes and digested myelin-like bodies seen in a large number of cells indicate cell aging and initiation of the self-protective response [34, 35]. We speculate that abnormal changes in organelles (e.g., mitochondria) may affect the synthesis of certain enzymes in the cell and cell metabolism [36], which is consistent with our finding that the time of cell passage of this group of cells was longer, indicating that metabolic abnormalities in cells affect their proliferative activity.

The osteogenic and chondrogenic-specific induction and staining also showed that the better outcome occurred with Group A. In especial, after osteogenic induction of hBMSCs, the alkaline phosphatase activity assay and Alizarin Red S staining showed that the osteogenic activity of the hBMSCs in patients with poor necrotic area repair was lower than in those with good repair. Because healing reactions of hBMSCs are essential [28], the osteosynthesis defects partially explain the poor repair of necrotic areas following stem cell implantation. This defect may also be one of the causative agents of femoral head necrosis in these patients [37].

In this study, the viability of hBMSCs of patients was observed in two groups based on the repair ratio. The reason for this grouping is that the greater the repair of the necrotic area is, the better the effect of the treatment is.There is not a clear value to define the curative effect. At the same time, there is no related research to tell us the extent to which the repair of necrotic area can achieve satisfactory clinical results.

The total costs of stem cell therapy combined with core decompression is about 3200 dollars, while the costs of stem cell viability detection is about 170 dollars. Detection of cell viability before operation has great predictive value of this combined therapy, provides theoretical basis for treatment decision, and avoids additional both economic burdens and psychological and physical pain caused by ineffective therapy.

In summary, core decompression combined with stem cells transplantation is currently a popular treatment option for early-stage ONFH. But the use of undifferentiated stem cell therapy and the lack of assessment of the quality of the cells to be implanted are likely to compromise the treatment and expected efficacy, which can increase the economic burden on patients and even delay disease diagnosis

and treatment. In future clinical work, initially examination of the quality and quantity of the peripheral blood and bone marrow needs to be done. In cases with low numbers of stem cells or poor stem cells osteogenesis activity, the regimen should be adjusted. Finding a more effective, noninvasive, simple, and inexpensive preoperative stem cell assessment method will be the priorities in the next steps.

Due to the small sample size and short follow-up period in this study, the best indications and long-term efficacy of this treatment need to be determined through further studies.

Conclusion

The efficacy of core decompression combined with autologous stem cell transplantation for the treatment of early-stage ONFH is closely related to stem cell viability in patients.

Abbreviations

ONFH: Osteonecrosis of the femoral head; BMSCs: Bone marrow mesenchymal stem cells; MNCs: Mononuclear cells; HHS: Harris hip score; MRI: Magnetic resonance imaging; ALP: Alkaline phosphatase; DMEM: Dulbeco's modifild of eagle medium; FBS: 10% Fetal bovine serum; GCSF: Granulocyte colony-stimulating factor; OD: Optical density

Declarations

Ethics approval and consent to participate

This study was registered in the Chinese Clinical Trial Registry（ChiCTR-ORC–17011698）.The study was reviewed and approved by Guizhou Medical University Ethics Committee. The patients and their family members were fully informed and signed the consent forms.

Consent for publication

Written informed consent was obtained from the patient for publication of this study and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Availability of data and material

The datasets generated and analyzed during the current study are not publicly available, but are available as de-identified data sheet from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Z.-Y. Wu, Q. Sun and Q. Zou completed the data measurement. M. Liu and Z.-X. He completed the statistical analysis. Z.-Y. Wu completed the article writing. At last B. Grottkau and C. Ye completed the article changes. All authors read and approved the final manuscript.

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Figures

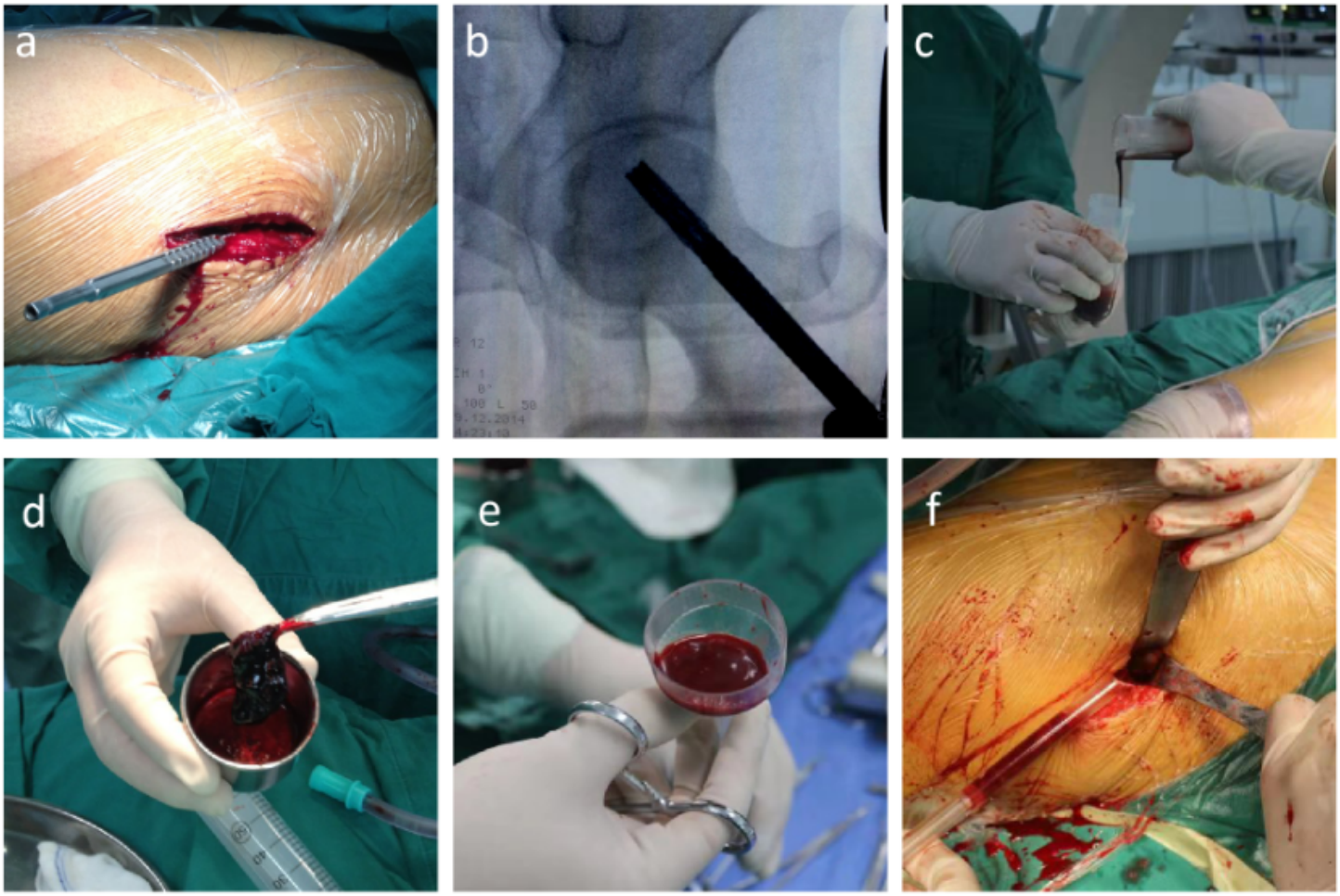

Figure 1

a Core decompression. b Intraoperative C arm fluoroscopy. c Stem cell suspension obtained by centrifugation. d Collagen sponge-cell composites. e Implantation device of Collagen sponge-cell composites. f Materials Implantation.

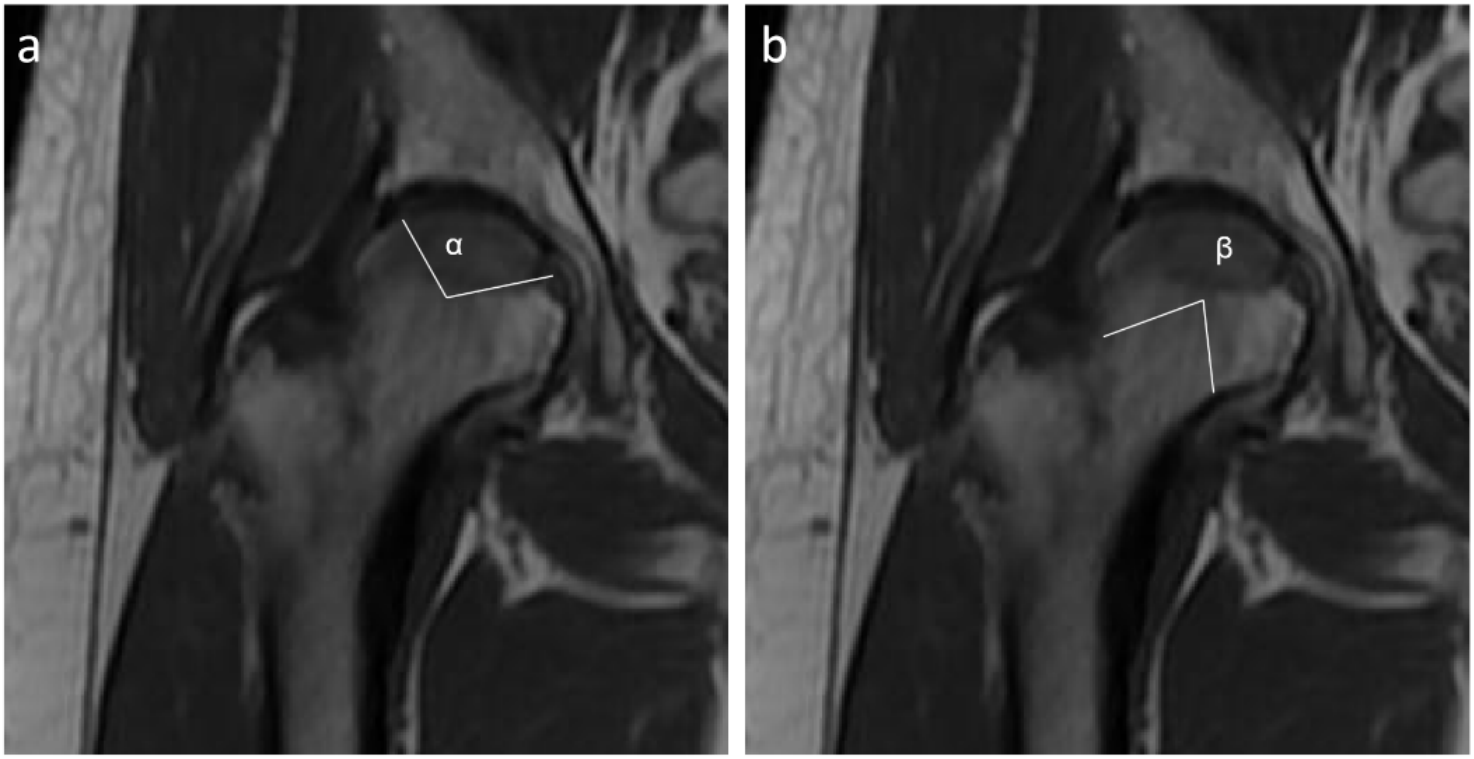

Figure 2

a The angle α was defined as the angle corresponding to the necrotic site on an MRI image. b The angle β was defined as the central angle corresponding to the femoral head in the same image (the angle between the connecting lines from the center of the femoral head to the femoral head-neck junctions).

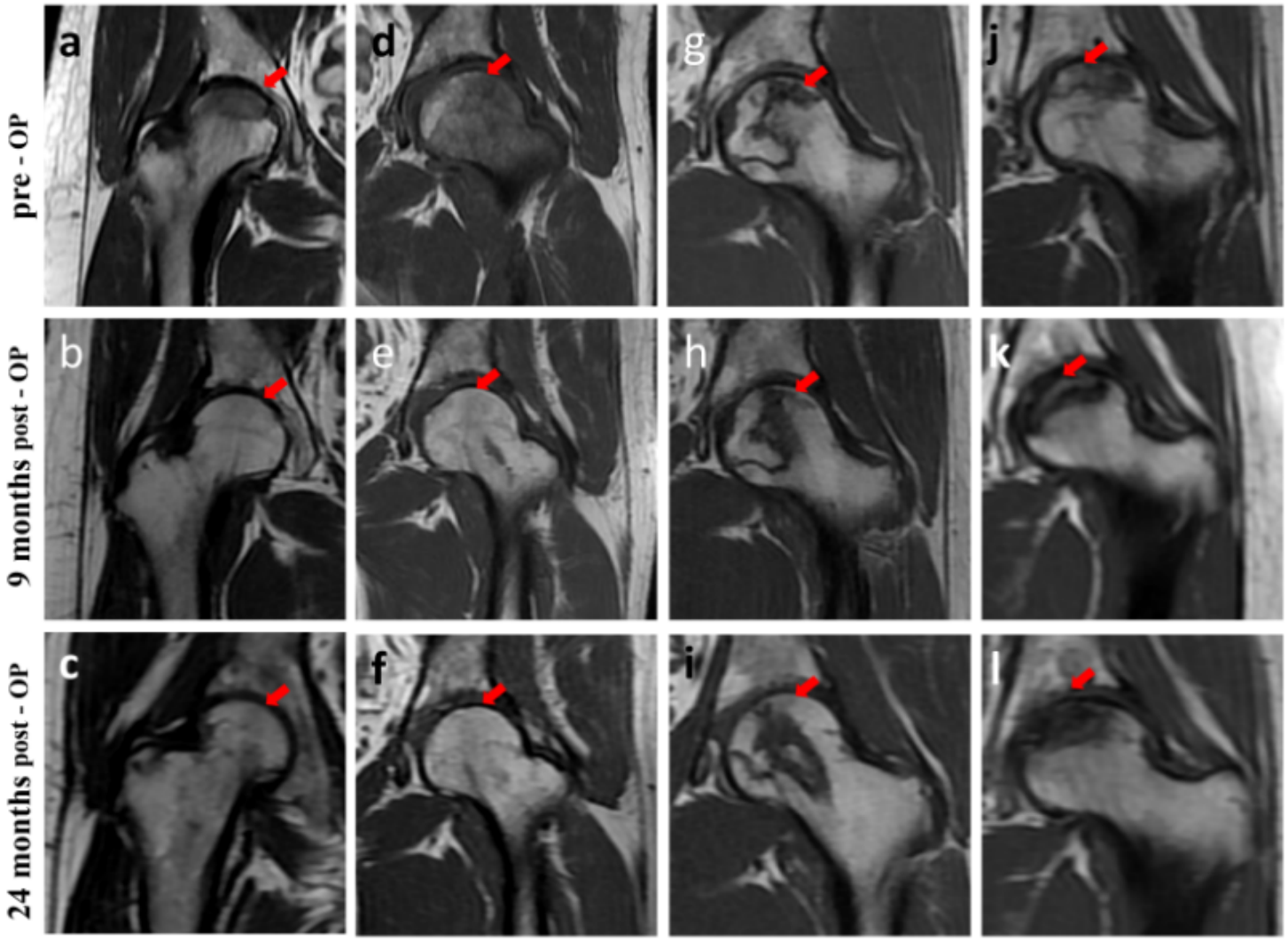

Figure 3

a-l The T1-weighted images on MRI of the necrotic area before and after operation. arrow The location of the necrotic area. a-f In a part of patients, the necrotic area ratio at 9 months after surgery was significantly lower than that before surgery. g-l In the other part of patients, the necrotic area ratio at 9 months after surgery was not significantly lower than that before surgery. In all patients, the necrotic area at 24 months after operation did not change much compared with that at 9 months after operation.

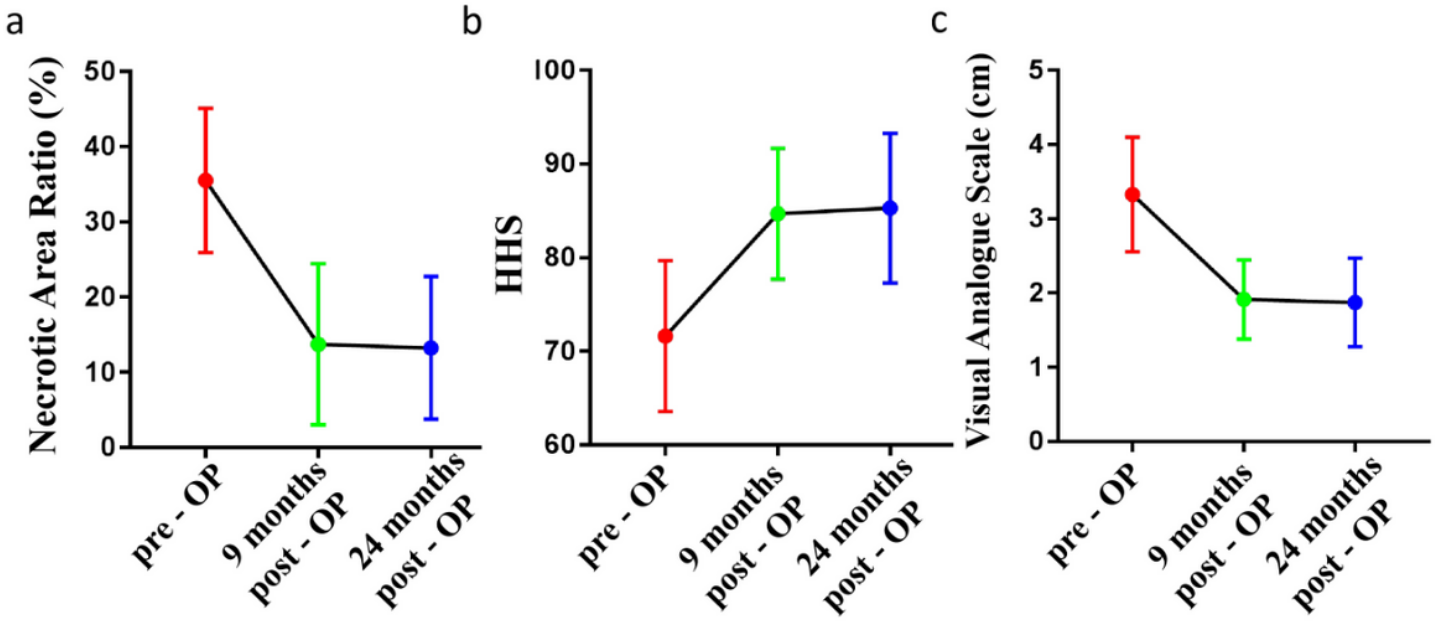

Figure 4

a The necrotic area ratio (%) at 9 months after surgery was significantly lower than that before surgery ($p < 0.05$). The necrotic area at 24 months after operation did not change much compared with that at 9 months after operation. b The HHS was significantly higher at 9 months after surgery than that before surgery ($p < 0.05$). c The VAS score of 9 months after operation was significantly lower than that before operation ($p < 0.05$). At 24 months after operation, the VAS was (1.87 ± 0.60), which was slightly lower than that of 9 months after operation ($p > 0.05$).

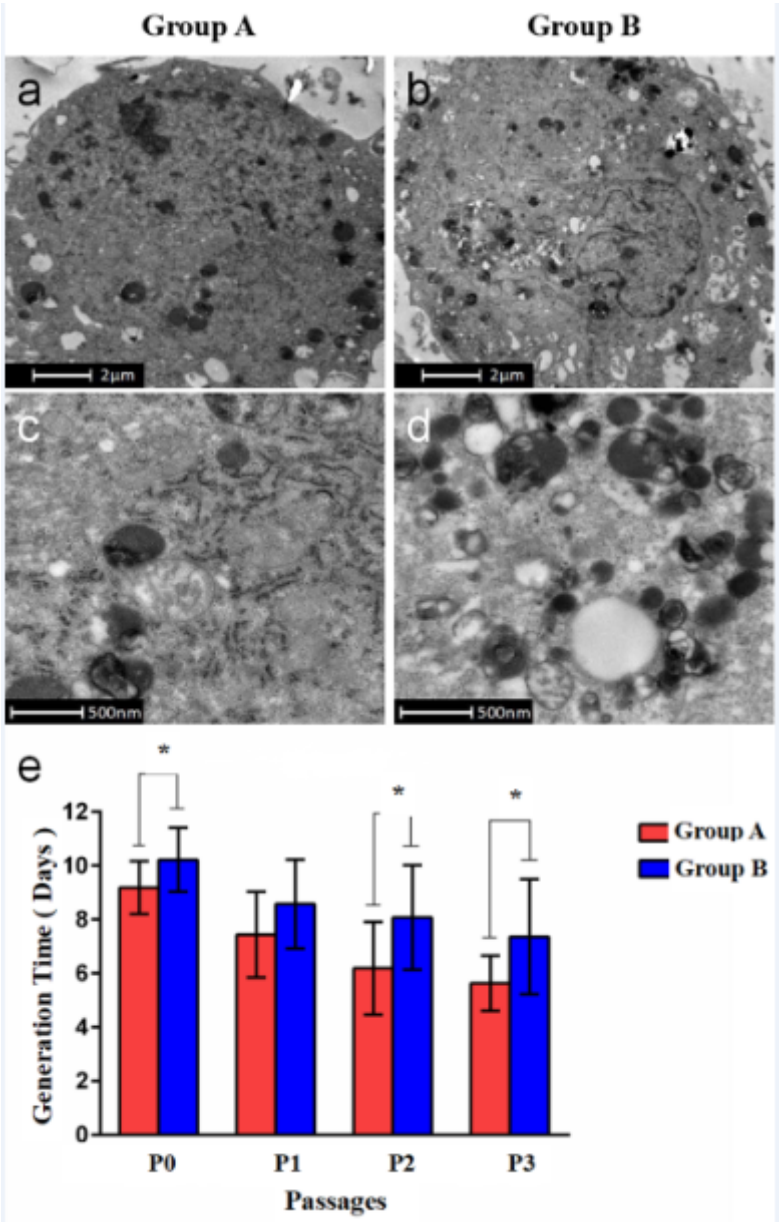

Figure 5

a-d The hBMSCs from group A have large nuclei and large and obvious nucleoli with even heterochromatic distribution, along with rich cytoplasm with a medium electron density. The hBMSCs from group B have decreased electron density of the cytoplasm and large numbers of vacuoles and autophagosomes in varying sizes. e Comparison of generation times of hBMSCs passage between groups A and B. * $p < 0.05$.

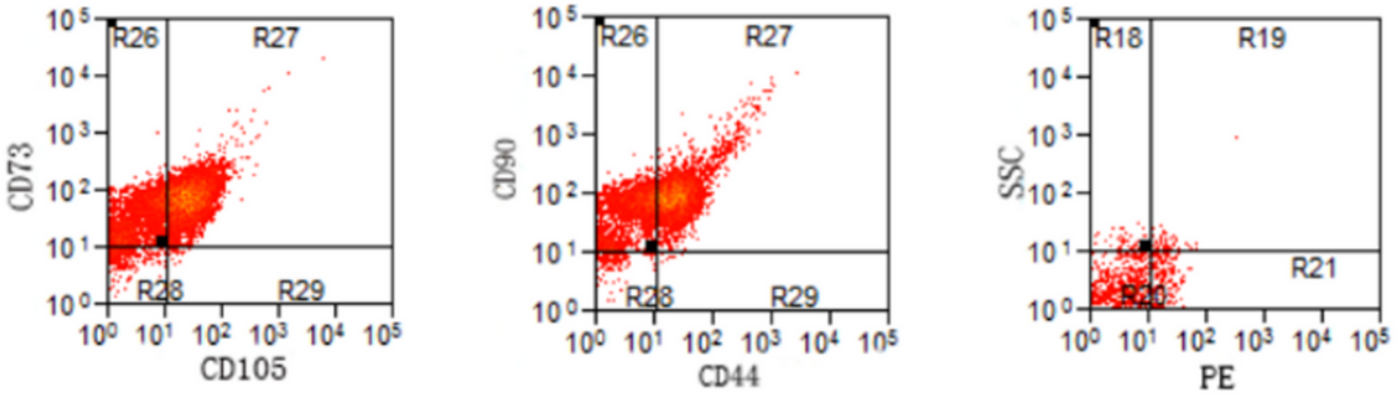

Figure 6

The results of flow cytometry. The cells highly expressed CD105, CD73, CD44 and CD90, but not CD34, CD45 and HLA-DR.

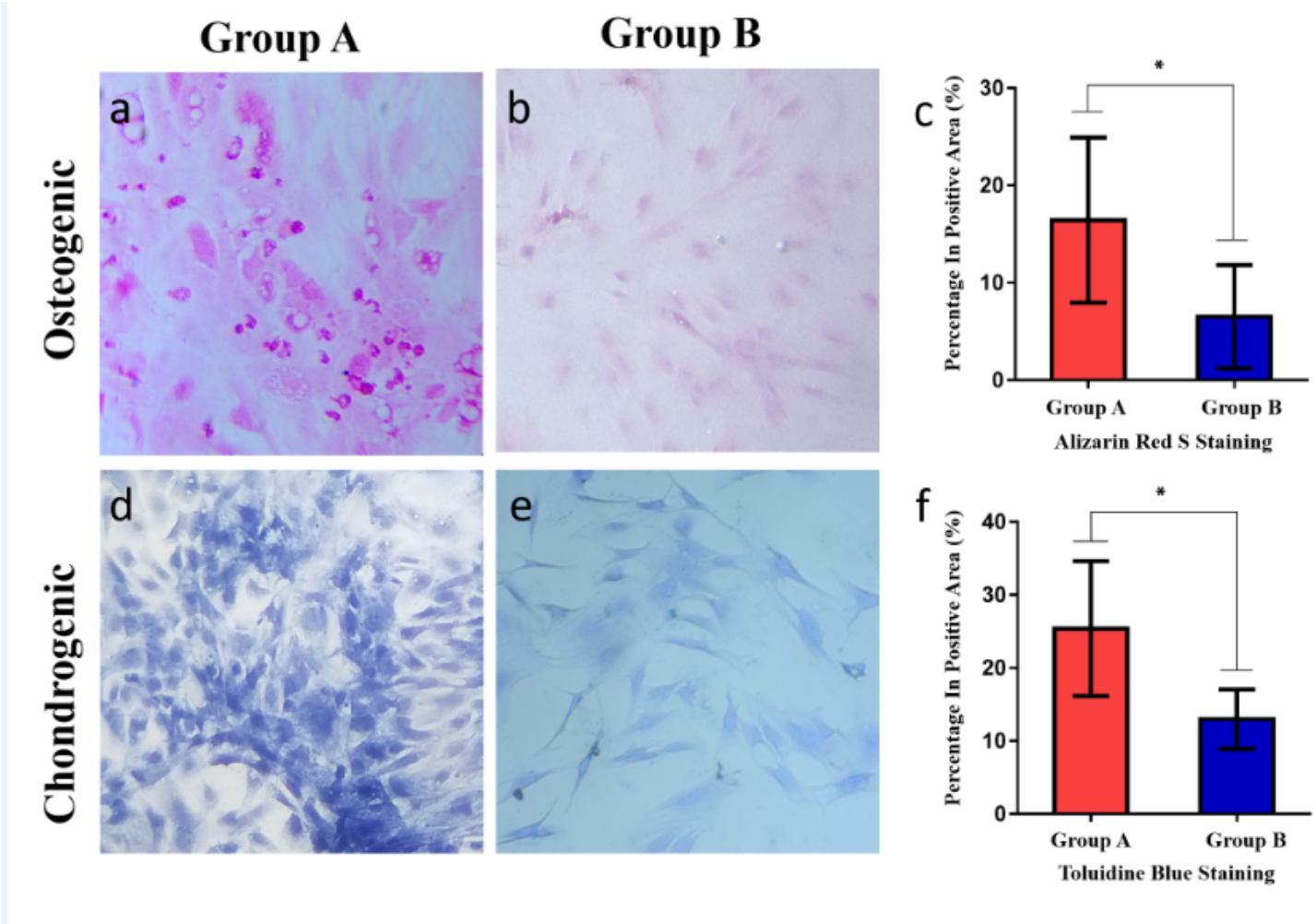

Figure 7

a-f Comparison of multilineage differentiation. (a, b) Alizarin Red S staining (×100) after a 14-day osteogenic induction of hBMSCs. (d, e) Toluidine Blue staining (×100) after a 14-day chondrogenic

induction of hBMSCs. (c, f) The specific stainings showed a signifificantly higher mean area percentage of specific staining when Group A compared to Group B.

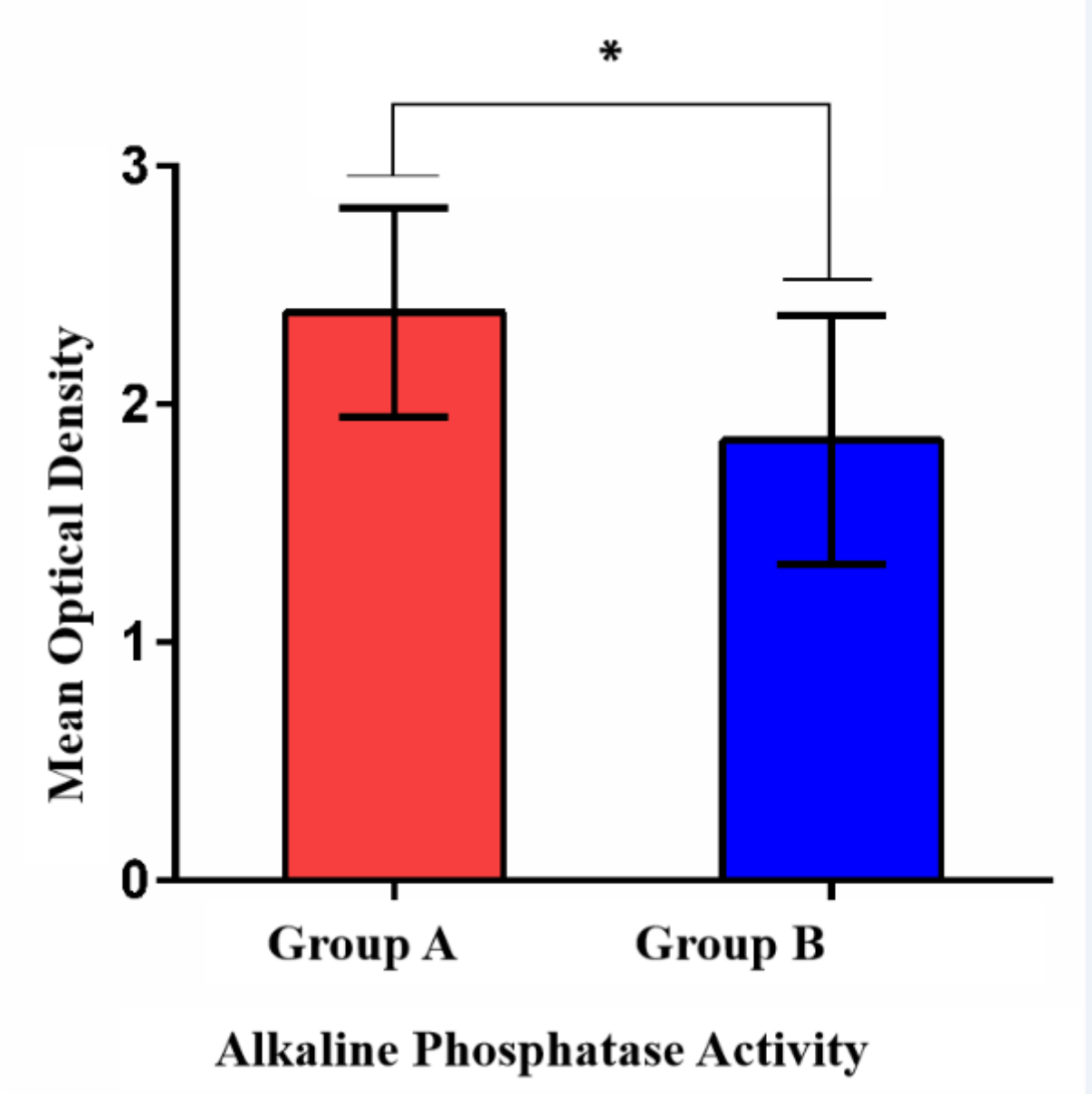

Figure 8

The determination of ALP activity after induced differentiation of the hBMSCs from groups A and B. * $p < 0.05$.

Supplementary Files

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- Methodsformula.docx