The effects of autologous bone marrow mesenchymal stem cell arterial perfusion on vascular repair and angiogenesis in osteonecrosis of the femoral head in dogs

Hongting Jin · Bingjiang Xia · Nanze Yu · Bangjian He · Yan Shen · Luwei Xiao · Peijian Tong

Received: 13 August 2012 / Accepted: 27 September 2012 / Published online: 13 October 2012
© Springer-Verlag Berlin Heidelberg 2012

Abstract

Purpose The purpose of this study was to observe the effects of marrow mesenchymal stem cell (MSCs) arterial perfusion on vascular repair and angiogenesis in osteonecrosis of the femoral head (ONFH).

Methods Twelve healthy male adult Beagle dogs were randomly divided into two groups: group A (the control group) and group B (the MSCs arterial perfusion group). ONFH animal models were established by hip dislocation and liquid nitrogen. At the same time, MSCs were obtained, cultured and proliferated. After three weeks, arterial perfusion was performed in all animals. Group B was given 1 ml MSCs (5×10^6 – 1×10^7 /ml), while 0.9 % normal saline was used in group A. After four weeks or eight weeks, the dogs were put to death. The changes of main arteries, the expression of vascular endothelial growth factor (VEGF), VEGF mRNA and microvessel density (MVD) of ONFH were observed. All the data were analysed by SPSS13.0.

Results In digital subtraction angiography (DSA), after four or eight weeks of treatment, the quantity and diameter of the main arteries of the femoral head in group B were improved, compared to group A ($P < 0.05$, $P < 0.01$). Concerning histology and immunohistochemistry, after four or eight weeks of

treatment, the expression of VEGF and MVD were significantly higher than that of group A ($P < 0.05$, $P < 0.01$). For real-time quantitative polymerase chain reaction (RT-PCR), after four or eight weeks of treatment, the expression of VEGF mRNA in group B was significantly higher than that of group A ($P < 0.05$, $P < 0.01$), and after eight weeks of treatment, the expression of VEGF mRNA were significantly higher than that of four-weeks treatment in group A ($P < 0.01$).

Conclusions MSCs arterial perfusion can promote vascular repair and angiogenesis and then improve blood supply and repair of femoral head.

Introduction

Osteonecrosis of femoral head (ONFH) is a difficult and complicated problem in the orthopaedic clinic, which commonly affects young adults between 30 and 50 years old. If untreated, it will lead to femoral head collapse requiring total hip replacement [1–3]. ONFH can be clinically divided into two types: traumatic and non-traumatic. The former usually results from a fracture of the femoral neck or dislocation of the hip joint [4, 5], while the latter is often the complication caused by overuse or abuse of hormones or alcohol [6, 7]. Although the pathogenesis and aetiology of both types has not yet been revealed completely, the decrease of blood perfusion to the femoral head seems to be the major factor [8, 9]. How to promote vascular repair and angiogenesis in ONFH becomes a crucial part of the treatment. Vascular growth consists of two mechanisms, namely, vasculogenesis and angiogenesis. The vasculogenesis is a process of the body forming the earliest blood vessels, including mesoderm formation, the establishment of the endothelial cell lineage and the organisation into cord-like vascular structures. However, the angiogenesis means

H. Jin · B. Xia · N. Yu · B. He · Y. Shen · L. Xiao · P. Tong
Zhejiang Chinese Medical University,
Hangzhou, Zhejiang 310053, China

L. Xiao · P. Tong (✉)
The First Affiliated Hospital of Zhejiang Chinese Medical University,
Youdian Road,
Hangzhou, Zhejiang 310006, China
e-mail: zhongxiyijieheguke@163.com

P. Tong
National Traditional Chinese Medicine Clinical Research Bases,
Hangzhou, Zhejiang 310006, China

forming new vessels by sprouting from or by splitting of pre-existing vascular structures, which occurs in response to metabolic stress within hypoxic tissues, enhancing tissue capillarisation [10]. Angiogenesis involves the interplay of endothelial progenitor cells, pericytes, growth factors, and cellular matrix components. Angiogenesis is providing crucial for the repair of the necrotic femoral head, the nutrients and removing waste products. Several methods aimed at promoting blood supply are offered to cure ONFH, e.g. physiotherapy, interventional therapy, core decompression [11, 12], however, the outcomes are unsatisfactory.

Marrow mesenchymal stem cells (MSCs), the hot topic of leading worldwide research, are a kind of cell with the function of self-renewal and proliferation. A number of favourable biological characteristics make MSCs an ideal therapeutic agent for regeneration. Firstly, MSCs are easily isolated and can be greatly expanded *in vivo* while maintaining genetic stability [13, 14]. Second, MSCs are immunologically inert and are transplantable between HLA-mismatched individuals [15, 16]. Third, the use of MSCs do not pose ethical concerns. Hernigou and Beaujean first used bone marrow grafting to treat osteonecrosis and the results are encouraging [17]. Recently, studies have indicated that the direct injection of autologous MSCs into femurs prevents corticosteroid-induced ONFH [18, 19]. In this study, we aimed at investigating the effects of MSCs arterial perfusion on vascular repair and angiogenesis in ONFH of dogs.

Materials and methods

Animal models

Twelve mature male beagle dogs weighing 10 ± 0.5 kg were used in the study. All the animals were kept and maintained according to the Guidelines for the Care and Use of Laboratory Animals required by the Animal Experiment Center of Zhejiang University of Traditional Chinese medicine. The animals were divided into two groups randomly, namely, group A (the control group) and group B (the MSCs-transplant group). The animal models of ONFH were established by hip dislocation and liquid nitrogen, which was similar to the method of Takaoka et al. [20]. After the induction of anaesthesia with an intramuscular injection of ketamine hydrochloride (25 mg/kg, Abbott, USA), sodium pentobarbital (5 mg/kg per hour, Abbott, USA) was administered intramuscularly to maintain general anaesthesia. A 5-cm-long incision was made at the lateral side of the right hip. Via the gap between gluteus medius and gluteus superficialis, the joint capsule was exposed and opened. The ligamentum teres was cut and the hip was dislocated. Dry gauze was used to fill the surroundings of the femoral head, and 100–150 ml liquid nitrogen was

applied to freeze it through a special funnel for three minutes. The wounds were then bandaged after rewarming with 37 °C saline. Antibacterial prophylaxis was obtained with penicillin 40,000 UI/kg for ten days.

Isolation and labelling of MSCs

Fifteen millilitres of bone marrow were collected from the iliac crest using 5-ml syringes and a 18G bone marrow biopsy needle (Biomedical, USA). The aspirated bone marrow of the dogs was filtered in a 100- μ m mesh and diluted in phosphate buffer solution (PBS) in a ratio of 1:3. Solutions were homogenised and added to Ficoll (Sigma, USA). After centrifugation at 2,000 RPM for 30 minutes at 20 °C, cells were plated in 25 cm² flasks at initial density of 2.0×10^5 /cm. The medium was supplemented with 10 % foetal bovine serum (Gibco, USA) and 1 % total solution of antibiotics (100 U/ml of penicillin and 100 μ g/ml of streptomycin). Cells were incubated in a Thermo Electron Corporation incubator (Forma Series II Water Jacketed CO₂ Incubator HEPA Class 100) under culturing conditions of 37 °C, relative humidity of approximately 100 %, and 5 % CO₂. After three days, non adherent cell-derived bone marrow was removed, and the medium was replaced. Images of incubated cells were visualised. BMSCs of the second passage were seeded in tissue culture plates of 60-mm diameter and density of 1×10^5 in 4 ml of 10 % foetal bovine serum in Dulbecco's modified eagle medium. At 50–60 % confluence, 10 μ mol/L bromodeoxyuridine (BrdU, Sigma, USA) was added into the medium to label cells. At 90 % confluence, cells were harvested with 0.25 % trypsin (Sigma, USA) and prepared in a density of 5×10^5 – 1×10^6 /ml with PBS. Trypan blue staining was used to test the vitality of cells. Cells were characterised using flow cytometry with CD34.

MSCs arterial perfusion

Arterial perfusion was performed on all animals three weeks after establishment of ischaemic femoral heads. The right femoral artery was punctured by the Seldinger technique using a 4.0F Cobra catheter (Terumo, Japan). Then medial and lateral femoral circumflex arteries were found and catheterised. Seven ml iohexol were injected at a speed of 2 ml/s for DSA angiography. Images were acquired at a rate of 0.5/s. One millilitre MSCs with a cell density of 5×10^6 – 1×10^7 /ml were then injected into the artery. At the same time 0.9 % normal saline was used in the control group, and a pressure bandage was applied at the site of arterial puncture for four hours.

Radiological analysis

DSA angiography was performed at four and eight weeks after arterial perfusion. Images were acquired at a rate of

0.5/s. The number of vessels were counted, and the diameter of vessels were measured.

Histology, immunohistochemistry and histomorphometry

Histological analysis was performed to observe the changes of vessels in the femoral head. The femoral heads of three dogs of each group were harvested at four and eight weeks after treatment. The specimens were fixed in 10 % buffered formalin for two days, decalcified in 14 % ethylenediamine tetra-acetic acid for two weeks, embedded in paraffin, and cut into 3- μ m-thick coronal sections. The sections were stained with haematoxylin and eosin (H&E) to evaluate the presence of vessels. Next, 3- μ m paraffin sections were heated at 95 °C in citrate buffer (pH 6.0) for two hours, and then treated with dual endogenous enzyme blocking reagent for ten minutes (Dako, Danish). After being blocked with 1/20 normal goat serum (Vector, USA) for 30 minutes, sections were treated with 1/150 rabbit anti-VEGF antibody (Abcam, USA) and 1/300 mouse anti-BrdU (Sigma, USA) overnight at 4 °C and incubated with 1/200 secondary biotinylated goat anti-rabbit antibody (Vector, USA) and goat anti-mouse antibody (Vector, USA) for 30 minutes, followed by 1/250 streptavidine-peroxidase (Pierce, USA) for 30 minutes at room temperature. Peroxidase activity was revealed by Romulin AEC Chromogen (Biocare Medical, USA) staining. We quantified VEGF positive cells and BrdU-positive cells in four randomly-selected fields (one field=25 $\times$ 10⁻⁸) using ImagePro 4.5 (Leeds Precision Instruments, Minneapolis, MN, USA).

Total RNA extraction and real-time quantitative polymerase chain reaction

The samples were pulverised with a mortar and pestle under liquid nitrogen in a RNase-free condition. Total RNA was extracted using Trizol reagent (Invitrogen, USA) and precipitated in ethanol. The concentration of RNA was quantified by measuring the absorbance at 260 nm (A260) in a spectrophotometer, and the purity of RNA was assessed by the ratio of the readings at 260 nm and 280 nm (A260/A280). Then the integrity and size distribution of RNA was checked by formaldehyde-agarose gel electrophoresis and ethidium bromide staining. Reverse transcription followed by quantitative real-time polymerase chain reaction (qPCR) was performed. The primer sequences were: VEGF forward, GCTGAATTCGCTGCGCCTATGGCAGG; reverse, ATGATGGTCGACTCATCACCGCC; β -actin as an internal control forward, ATGCCATCCTGCGTCTG; reverse, ACTCCTGCTTGCTGATCCACAT. Real-time qPCR was done in triplicate on the cDNA with an iQTM5 real-

time qPCR (Bio-Rad, USA) following the recommended protocols. All data were analysed by $2^{-\Delta\Delta CT}$ (Livak) technique.

Quantification of microvessel density

Microvessel density (MVD), a measure of angiogenesis, was determined by light microscopy after immunostaining sections with anti-CD31 antibodies. Microvessels were counted on a $\times$ 200 field. Any single endothelial cell or cluster of endothelial cells clearly separated from adjacent microvessels was considered as one countable microvessel. Five representative areas of the section were counted. The evaluation was performed by three persons, without knowledge of the group.

Statistical analysis

All the data were presented as mean $\pm$ standard deviation ($\bar{x} \pm s$). Student's *t*-test or one-way ANOVA test was used to compare the means (SD) among the two groups at different time points. The statistical tests were performed by using the software SPSS 13.0. $P < 0.05$ was considered to be statistically significant.

Results

General condition of the animal models

In the following three days after the operation, all of the dogs suffered depression, poor appetite, hypo-activity, lameness, wounds of toes (bite by the dogs themselves for pain) and in the right posterior limbs. Three days later, they regained normal appearance, while lameness of the right posterior limbs persisted and they fell when jumping. After a four-week treatment, there was no significant improvement in the control group, while the MSCs-transplant group looked better both walking and jumping and the wounds of toes were almost cured. After an eight-week treatment, the control group seemed better in walking, and the MSCs-transplant group had improved activity, no visible lameness and normal toes.

After a four-week treatment, the femoral heads of group A had flat shape, smooth outline, unimpaired cartilage surface and visible subchondral trabeculae. The shape of the femoral heads of group B was almost normal. After an eight-week treatment, the femoral heads of group A were flat and mushroom-shaped, and that of group B had normal shape, smooth surface, unimpaired cartilage surface and clearly visible subchondral trabeculae. The animal models of ONFH have all been established successfully through the final pathological examination.

Isolation, culture and labelling of MSCs

It was observed by microscope that, 24 hours after inoculation, part of the round mononuclear cells were adherent, and 72 hours after inoculation, most of the round mononuclear cells were adherent and fibroblast-shaped (fusiform-shaped with small convexities and a large nucleus). On the seventh day of cultivation, the cells were fusiform-shaped and unilayered. Later, multilayers were also observed.

DSA observation

The photographs were taken by digital subtraction radiography. Before the treatment, the branches of medial and lateral femoral circumflex arteries were decreased and thinned, and superior and inferior head branches, which were thinned and shortened, did not reach the femoral head region, and blockage and interruption could be seen instead of collateral circulations (Fig. 1a, b). After four and eight weeks of treatments, it was observed from the MSCs-transplant group that the branches of medial and

lateral femoral circumflex arteries were increased and thickened, and superior and inferior head branches were developing sufficiently to reach the femoral head region, new collateral circulations were established and formerly blocked vessels were recanalised (Fig. 1c, d). The vascular numbers and diameters in group B four-week treatment were significantly higher than that of group A, and group B eight-week treatments were significantly higher than that of group B 4-week treatment (Figs. 2 and 3).

Histopathological and immunohistochemical measurement

Observation by light microscope

After a four-week treatment, incomplete periosteum, partly ablated and thinned cartilage layer, disorderly chondrocytes, thinned and disordered trabeculae, empty bone lacunae, karyopyknosed bone cells, blocked vessels, decreased haemopoietic tissue in bone marrow cavity and large amounts of fat cells were observed in group A. While in group B we found developing cartilage and trabeculae structure, incom-

Fig. 1 **a** Angiostenosis before DSA treatment. **b** Angiempfraxis before DSA treatment. **c** An enlarged vessel after DSA treatment. **d** A recanalised vessel after DSA treatment

Fig. 2 Comparison of the vascular counts between groups A and B. Group B four-week treatment was significantly higher than that of group A ($*P<0.05$); Group B eight-week treatment was significantly higher than that of group A ($#P<0.01$); Group B eight-week treatment was significantly higher than that of group B four-week treatment ($*P<0.01$)

plete trabeculae, some empty bone lacunae and decreased number of fat cells in bone marrow with a distinct border. After an eight-week treatment, outcomes of group A were severely destroyed cartilage surface with chaotic structure, ablated and broken cartilage layer, disorderly chondrocytes, large amounts of empty bone lacunae, thinned and incomplete trabeculae and tiny fractures. In group B, there were partly developed cartilage and trabeculae structure, presence of bone cells on trabeculae and decreased number of fat cells in bone marrow with distinct border (Fig. 4).

Observation by immunohistochemical measurement

Immunofluorescence staining showed that VEGF-positive stained cells and Brdu-positive stained cells were found in

Fig. 3 Comparison of the vascular diameters between groups A and B. Group B four-week treatment was significantly higher than that of group A ($*P<0.05$); Group B eight-week treatment was significantly higher than that of group A ($#P<0.01$); Group B eight-week treatment was significantly higher than that of group B four-week treatment ($*P<0.05$)

the transplant part in the MSCs-transplant group; and HE staining indicated that vessel density of the ischemic region in the experimental group was higher than that in the control group (Figs. 5 and 6).

The expression of VEGF mRNA

Quantitative analysis was made according to the $2^{-\Delta\Delta CT}$ (Livak) technique, and the results are shown in Fig. 7.

Quantification of microvessel density

The MVD in femoral head gradually increased in group B after four or eight weeks of treatment, there were significant statistical differences between the two groups (Fig. 8).

Discussion

The model of ONFH

Setting up an ideal animal model is very important in research and studies. A successful animal model should be similar to the prototype, and through analysis of models we can obtain partial information about prototypes. In our study, we established animal models of ONFH using a combination of dislocation and liquid nitrogen refrigeration [20]. Liquid nitrogen refrigeration can induce vasospasm, intravascular coagulation, injure vascular endothelial cells, increase vascular permeability, and then ischemia-reperfusion injury occurs after haemorrhage and rewarming. The hip dislocation would further injure the vessels around femoral heads of dogs, as in the human being's process of ONFH. Through our studies, we found the method of combining dislocation and liquid nitrogen refrigeration had two main advantages. First, it leaves no residual chemical substances, and also it does not affect the MSCs arterial perfusion during the treatment, which is different from other chemical methods. Second, it has short time model establishing, high success rate, low mortality of animals and good reproducibility. As fluid nitrogen and dislocation cause complete necrosis of the femoral head transiently, and at the same time allows a gradual repair process, it is suitable for our research on vascular repair and angiogenesis in osteonecrosis of femoral heads. However, the dog model as an animal model for ONFH has the inherent disadvantages of different biomechanical features from human beings. Because the dog is a type of quadruped, it could change the load mechanism of limbs and then change the mechanical environment during the process of bone repair.

Fig. 4 **a** H&E staining shows disordered trabeculae, incomplete, significant degeneration and necrosis with slight fractures (*arrows*) ($\times 40$). **b** H&E staining shows complete trabeculae structure, considerable amounts of bone cells with nucleus in the centre ($\times 40$)

The use of DSA

Digital subtraction angiography (DSA) is a technique that is used to clearly visualise blood vessels by subtracting a digitised tissue background image from an image of tissue injected with an intravascular contrast material, such as iodine. However, it is invasive. DSA is considered the gold standard for diagnosing arterial stenosis and blockage by imaging findings demonstrating narrowing or interruption of vessels [21, 22]. Some scholars have adopted DSA to observe the changes of blood supply after femoral neck fracture [23]. Zhao et al. [24] make use of the method of DSA to evaluate the haemodynamic changes after treatment of ONFH with vascularised bone flaps. They found that selective DSA is effective in evaluation of the haemodynamic changes in the necrotic femoral head. Through DSA performed on the patients with ONFH before operation, Yu et al. [25] point out that the DSA can show arterial stenosis and blockage precisely in the necrotic femoral head. In our studies, the branches of medial and lateral femoral circumflex arteries, superior and inferior head branches, and blockage and interruption could be seen clearly. Although an invasive method, it provides a clear image,

high resolution to the observation vascular lesions, and positioning of stenosis or blockage. So we consider it to be significant in evaluating the blood supply of femoral heads.

MSCs and angiogenesis in ONFH

With the development of cytotherapy, angiogenesis for ischaemic regions based on MSCs have become a hot topic. Considerable research has been made on this issue. Kinnaird et al. found that MSCs can enhance new blood vessel growth both in in vitro and in vivo models [26]. Qian et al. [27] transplanted autologous bone marrow mesenchymal stem cells into rabbit ischaemic hind limbs two weeks after transplantation; the inner diameter of the femoral artery and the peak velocity of blood flow in the experimental group were higher than the control group. At the same time, higher vessel density of the ischaemic region could be found in the experimental group. According to this research, MSCs have a positive effect on angiogenesis. In our research, we found similar results. From the images of DSA, we found that the branches of medial and lateral femoral circumflex arteries were increased and

Fig. 5 **a** Immunohistochemical staining shows brown particles in cytoplasm and VEGF positive stained cells ($\times 200$). **b** Immunohistochemical staining shows brown particles in the cytoplasm and BrdU-positive stained cells ($\times 200$)

Fig. 6 Comparison of positive VEGF expression among the groups. Those in group B, four-week treatment, were significantly higher than that of group A ($\ast\ast P<0.05$); Group B, eight-week treatment were significantly higher than that of group A ($\# P<0.01$); Group B, eight-week treatment were significantly higher than that of group B, four-week treatment: ($\ast P<0.01$)

thickened, superior and inferior head branches were developing sufficient length to reach the femoral head region, new collateral circulations were established, and blocked vessels were recanalised in femoral heads of dogs with MSCs transplant compared to the control group. At the same time, the MSCs-transplant group has a significantly higher MVD than that of the control group. Through our research, we have shown that arterial perfusion with MSCs has a promotive effect on regenerating vessels and improving blood supply in the ischemic femoral head. However, how MSCs promote angiogenesis in osteonecrotic femoral heads is unclear. Asahara et al. [28] established murine models of bone marrow implants that received bone marrow from transgenic mice expressing β -galactosidase under

Fig. 7 Comparison of VEGF mRNA expression among the groups. Those in group B with four-week treatment were significantly higher than that of group A ($\ast\ast P<0.05$); Group B, eight-week treatment were significantly higher than that of group A ($\# P<0.01$); Group B, eight-week treatment were significantly higher than that of group B, four-week treatment ($\ast P<0.01$)

Fig. 8 Comparison of MVD among groups A and B. Those in group B with four-week treatment were significantly higher than that of group A ($\ast\ast P<0.05$); Group B with eight-week treatment were significantly higher than that of group A ($\# P<0.01$); Group B, eight-week treatment, were significantly higher than that of group B, four-week treatment ($\ast P<0.01$)

the transcriptional control of an endothelial cell-specific promoter. They demonstrated that lacZ-positive endothelial progenitor cells derived from the bone marrow were incorporated into sites of neovascularisation where there was complete differentiation into endothelial cells. That means the MSCs may promote angiogenesis by differentiating into endothelial progenitor cells. In our study, we also found Brdu-positive cells in the newborn vessels. In recent years, some scholars [29] have suggested that transplantation of MSCs enhances vascular regeneration mainly through a paracrine action of VEGF, one of the most potent mediators of angiogenesis [30]. Its inhibition by soluble VEGF receptor treatment decreases angiogenesis [31]. Results of this research showed that the MSCs-transplant group had higher expression of VEGF, which indicated that MSCs can increase the expression of VEGF and then enhance angiogenesis. A possible mechanism may be that local hypoxia after vascular injury stimulates the expression of VEGF which can be promoted by MSCs, thus, more local vessels are generated to form a relatively complete vascular net. During ONFH, the decreased blood flow produces local hypoxia, thus stimulating VEGF expression. The decreased VEGF expression in our model also suggests a poor potential for angiogenesis.

Conclusion

This preliminary research shows that there is good therapeutic effect of MSCs on vascular repair and angiogenesis in femoral head osteonecrosis. The mechanism may promote the expression of VEGF and differentiate into endothelial progenitor cells. However, some problems remain unsolved, such as the best amount and time of MSCs transplantation, that need further studies.

Acknowledgments This publication was made possible by the National Natural Science Foundation of China (Grant No. 81202710), State Administration of Tradition Chinese Medicine of Zhejiang province (Grant No. 2009YA001), Zhejiang Provincial Natural Science Foundation of China (Grant No. Y2111184), and the Science Technology Department of Zhejiang province (Grant No. 2012R10064).

References

1. Lee MS, Hsieh PH, Shih CH et al (2010) Non-traumatic osteonecrosis of the femoral head—from clinical to bench. *Chang Gung Med J* 33(4):351–360
2. Aaron RK, Ciombor DM (2001) Coagulopathies and osteonecrosis. *Curr Opin Orthop* 12:378–383
3. Wang BL, Sun W, Shi ZC et al (2010) Treatment of nontraumatic osteonecrosis of the femoral head using bone impaction grafting through a femoral neck window. *Int Orthop* 34:635–639
4. Ehlinger M, Moser T, Adam P et al (2011) Early prediction of femoral head avascular necrosis following neck fracture. *Orthop Traumatol Surg Res* 97:79–88
5. Bastian JD, Turina M, Siebenrock KA et al (2011) Long-term outcome after traumatic anterior dislocation of the hip. *Arch Orthop Trauma Surg* 131:1273–1278
6. Mont MA, Hungerford DS (1995) Non-traumatic avascular necrosis of the femoral head. *J Bone Joint Surg Am* 77:459–474
7. Urbaniak JR, Harvey EJ (1998) Revascularization of the femoral head in osteonecrosis. *J Am Acad Orthop Surg* 6:44–54
8. Hong JM, Kim TH, Kim HJ et al (2010) Genetic association of angiogenesis- and hypoxia-related gene polymorphisms with osteonecrosis of the femoral head. *Exp Mol Med* 42:376–385
9. Hamilton TW, Goodman SM, Figgie M (2009) SAS weekly rounds: avascular necrosis. *HSS J* 5:99–113
10. Poole TJ, Coffin JD (1989) Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. *J Exp Zool* 251(2):224–231
11. Drescher W, Pufe T, Smeets R et al (2011) Avascular necrosis of the hip—diagnosis and treatment. *Z Orthop Unfall* 149:231–240
12. Li Q (2009) Interventional therapy in the treatment of avascular necrosis of femoral head and short-term efficacy. *Zhong Guo Gu Shang* 22:789–790
13. Bernardo ME, Zaffaroni N, Novara F et al (2007) Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 1:9142–9149
14. Sekiya I, Larson BL, Smith JR et al (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 20:530–541
15. Devine SM, Cobbs C, Jennings M et al (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* 101:2999–3001
16. Le Blanc K (2003) Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy* 5:485–489
17. Hernigou P, Beaujean F (2002) Treatment of osteonecrosis with autologous bone marrow grafting. *Clin Orthop Relat Res* 405:14–23
18. Gangji V, Hauzeur JP, Matos C et al (2004) Treatment of osteonecrosis of the femoral head with implantation of autologous bone marrow cells. A pilot study. *J Bone Joint Surg Am* 86A:1153–1160
19. Sun Y, Feng Y, Zhang CQ (2009) The effect of bone marrow mononuclear cells on vascularization and bone regeneration in steroid-induced osteonecrosis of the femoral head. *Joint Bone Spine* 76:685–690
20. Takaoka K, Yoshioka T, Hosoya T et al (1981) The repair process in experimentally induced avascular necrosis of the femoral head in dogs. *Arch Orthop Trauma Surg* 99:109–115
21. Barnett HJ, Taylor DW, Eliasziw M, Fox AJ et al (1998) Benefit of carotid endarterectomy in patients with symptomatic moderate or severe stenosis. *N Engl J Med* 339:1415–1425
22. Asymptomatic Carotid Surgery Trial (ACST) Collaborative Group (2004) Prevention of disabling and fatal strokes by successful carotid endarterectomy in patients without recent neurological symptoms: randomised controlled trial. *Lancet* 363:1491–1502
23. Ly TV, Swiontkowski MF (2008) Management of femoral neck fractures in young adults. *Indian J Orthop* 42:3–12
24. Zhao DW, Wang BJ, Wang WM et al (2006) Evaluation of hemodynamic changes by digital subtraction angiography after treatment of avascular osteonecrosis of femoral head with vascularized bone flaps. *Zhonghua Yi Xue Za Zhi* 86:438–441
25. Yu J, Xu TG (2008) Evaluation on therapeutic efficacy regarding the necrosis of femoral head by digital subtraction arteriography. *Anat Clin* 13:241–243
26. Kinnaird T, Stabile E, Burnett MS et al (2004) Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 94:678–685
27. Qian HG, Zhu DX, Zhu JZ et al (2008) Promotive effect of autologous bone marrow mesenchymal stem cell transplantation on angiogenesis in rabbit ischemic limbs. *J Clin Rehabil Tissue Eng Res* 12:10095–10097
28. Asahara T, Masuda H, Takahashi T et al (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 85:221–228
29. Schutmann P, Tavassol F, Lindhorst D et al (2009) Consequences of seeded cell type on vascularization of tissue engineering constructs in vivo. *Microvasc Res* 78:180–190
30. Gerber HP, Vu TH, Ryan AM et al (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 5:623–628
31. Street J, Bao M, deGuzman L et al (2002) Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci USA* 99:9656–9661