Vascular endothelial growth factor and nano-hydroxyapatite/collagen composite in the repair of femoral defect in rats*★

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Abstract

BACKGROUND: Previous studies have confirmed that nano-hydroxyapatite/collagen (nHAC) and mesenchymal stem cells for repair of bone defect have the ability of bone formation *in vivo*.

OBJECTIVE: To observe the effects of vascular endothelial growth factor (VEGF) and bone marrow mesenchymal stem cells (BMSCs), nHAC composite in the repair of femoral defect in rats.

METHODS: Sprague-Dawley rat models of middle part of the femur defect were established and randomly assigned to two groups. Control group was implanted with BMSCs/nHAC composite. Experimental group was implanted with VEGF/BMSCs/nHAC composite. At 2, 4 and 8 weeks postoperation, imaging and histology observation of femoral samples were performed. At 8 weeks postoperation, scanning electron microscopy was performed in new bony callus environment.

RESULTS AND CONCLUSION: nHAC composite implantation in the rats did not show rejection or inflammatory reaction. Moreover, bone formed rapidly using VEGF and BMSCs, nHAC composite, which exhibited better bone regeneration capacity compared with BMSCs/nHAC composite. The way of ossification mainly was endochondral ossification. It is presumed that VEGF promoted the formation of local microvessels, differentiation and proliferation of osteoblasts, speeded up the speed of endochondral ossification, shortened bone repair time, and elevated the quality and velocity of osteanagenesis.

INTRODUCTION

The development of tissue engineering successfully constructed tissue engineered bone, and provided experimental basis for treatment of bone defects. Bone marrow mesenchymal stem cells (BMSCs) are major seed cells in bone tissue engineering. A previous study has confirmed that rabbit autologous BMSCs combined with allogenic dried bone could repair rabbit radius 12-mm segment bone-periosteum defects, and verified that BMSCs combined with tissue engineered bone had a strong ability of repairing big-segment bone defects[1]. Nano-hydroxyapatite/collagen (nHAC) has been used in clinic as one of artificial bone in treatment of bone defects. A previous study has demonstrated that nHAC combined with BMSCs have osteogenic ability *in vivo*[2]. The present study utilized BMSCs as seed cells to culture with nHAC and to compound with vascular endothelial growth factor (VEGF), constructed VEGF/BMSCs/nHAC complex to repair rat femur defect, and observed VEGF effects on bone repair.

MATERIALS AND METHODS

Design

Completely randomized grouping design, contrast observational experiment.

Time and setting

Experiments were performed at the Laboratory of Clinical Medical Institute of Jiangsu University from December 2007 to August 2008.

Materials

A total of 20 SPF Sprague-Dawley rats aged 5–6 weeks and weighing 140–160 g, of both genders, as well as 4 Sprague-Dawley rats aged 4 weeks and weighing 60–100 g (for collecting BMSCs) were obtained from the Animal Experimental Center of Jiangsu University, animal production license No. SCXK(Su)2002-0009, and use license No. SCXK(Su)2002-0045.

Main reagent and equipment: nHAC (Department of Material, Tsinghua University), L-DMEM, fetal bovine serum and trypsin (Gbico), 40-μm pore diameter cell sieve (Amersham Biosciences, United Kingdom), 5810R refrigerated centrifuge (Eppendorf, Germany), optical microscope (OLYMPUS, Japan) and VEGF (PeproTech) were employed in this study.

Experimental methods

Collection and passage of rat BMSCs

Healthy juvenile Sprague-Dawley rats weighing about 80 g were intraperitoneally anesthetized with 0.3% sodium pentobarbital 35 mg/kg. The neck was cut off. Under sterile condition, bilateral femur was collected to remove soft tissue on the surface, and then washed in L-DMEM containing 10% fetal bovine serum. The medullary canal was exposed by removing bilateral osteoepiphysis, and washed using L-DMEM to obtain bone marrow. The bone marrow was triturated, made into single cell suspension, and centrifuged at 1 000 r/min for 20 minutes. Supernatant was removed. The sample was re-suspended in complete culture solution (L-DMEM), supplemented with 10% fetal bovine serum, 10 nmol/L dexamethasone, 50 mg/L vitamin C, and 10 mmol/L β-sodium glycerophosphate. Cell suspension was incubated in a 25 mL plastic culture flask, at 37 ℃, 5% CO_2 at saturated humidity. The medium was replaced at day 3. From then on, the medium was replaced every 3 days. Non-adhered cells were not removed. When 80% cells were

confluent, these cells were digested using 2.5 g/L trypsin and 0.4 g/L ethylenediamine tetraacetic acid, and subcultured at 1: 2. Obtained cells were stem cells by observation *in vivo* and immunofluorescence staining[1].

Preparation of VEGF/BMSCs/nHAC complex

nHAC after sterilization and package was cut into 2 mm× 2 mm×2 mm using operating knife blade in the super clean bench. The specimens were infiltrated in L-DMEM containing 15% fetal bovine serum in 24-well plates for 24 hours. L-DMEM in 24-well plates was absorbed. 100 μL L-DMEM containing 15% fetal bovine serum was added in each well. Rat BMSCs at the third passage were digested in 0.25% trypsin and made into cell suspension. Cell concentration was adjusted to 5×10^9/L using a cell counting plate. Following cells were triturated, 100 μL was dropped on the surface of nHAC using a micropipette at 37 ℃ in an incubator containing 5% CO_2 at saturated humidity for 2 hours. Material was reversed, and 100 μL cell suspension was dropped on the other side of the material at 37 ℃ in an incubator containing 5% CO_2 at saturated humidity for 2 hours. The material was immersed in L-DMEM supplemented with 15% fetal bovine serum at 37 ℃ in an incubator containing 5% CO_2 at saturated humidity. The medium was replaced every 3 days for 14 days.

A half of BMSCs/nHAC stent of even size was fully mixed with VEGF solution. After 2 hours of adsorption, the gas in the stent was aspirated using vacuum machine. VEGF was fully sucked into the stent (the stent implanted in each rat contained about 0.8 μg VEGF), which was separately sealed up in double-layer polyethylene film. The specimen was irradiated by γ ray 2.5×10^5 Gy for 1 hour, dried at −70 ℃ for 24 hours, and then placed at 4 ℃ for use. Another half of BMSCs/nHAC stent was not mixed with VEGF, but freeze-dried for use according to above-mentioned method.

Construction of bone defect models and implantation of tissue-engineered bone

A total of 20 adult Sprague-Dawley rats were equally and randomly assigned to experimental group and control group. After weighing, the rats were intraperitoneally anesthetized with 0.3% sodium pentobarbital 30–35 mg /kg. At prone position, a lateral incision was made after the lower limb was obtained to expose the femur. Bone defect models (including periosteum) at 0.5 cm from the middle part of the left femur using an operating scissor. In the control group, BMSCs/nHAC stent was implanted. In the experimental group, VEGF/BMSCs/nHAC stent was implanted. After surgery, 8×10^4U penicillin was intramuscularly injected for 3 consecutive days, and fed by routine particle forage. Aseptic operation was strictly done during surgery. All models of bone defects were constructed by the same group of person. The length of bone defect and position of cutting bone were the same.

General observation of animals

Foodintake, activity and wound healing were observed after surgery.

X-ray examination

Three rats were randomly selected from each group at 2, 4 and 8 weeks after surgery to take a photograph at the lateral position of the femur to understand the repair of bone defect. Radiological score was assessed according to a previous study[3]. General observation, bone density measurement and pathological examination were conducted in order.

General observation of femur sample

One rat from each group was randomly sacrificed at 2, 4 and 8 weeks after surgery, and numbered. Soft tissue such as adherent muscle was dissociated. General observation was performed to understand bone defect repair. It is important to pay attention to whether the defect region was repaired by new bone; the connection of new bone and broken end; and moulding was good or not.

Bone density measurement

Femur sample was obtained to remove femur soft tissue. Bone density of the bone defect region was measured using CHALLENGE double energy X-ray bone density meter. The position and area of the measure regions were identical in each specimen. Measure value was expressed as $\bar{x}\pm s$, and the obtained value was a relative value.

Hematoxylin-eosin staining of pathological tissues

Femur specimen was obtained from affected side to remove soft tissues of the femur. Bony callus growth was observed in pathological tissue sections using hematoxylin-eosin staining.

Observation of environmental scanning electron microscope

At 8 weeks after surgery, one rat was selected randomly from each group, and sacrificed to remove soft tissues. New bony callus received environmental scanning electron microscope to observe new formed bone trabecula. The sections were equally assigned to nine portions. One point was selected from each portion for scanning.

Statistical analysis

The data were expressed as Mean±SD, and analyzed using two independent samples *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

General condition of experimental animals after surgery

At 3 hours after surgery, the rats were awake in each group. Limb activity was poor in the affected side. The primary wound healing was found at 7 days, and no inflammatory reaction was detected. The activity was limited in two rats of control group, and the activity in rats from experimental group was free 1 week later. A total of 20 rats were included in the final analysis.

Results of general observation of femoral specimen in the affected side

At 2 weeks after surgery, the boundary was clear between graft and host. In the control group, the stability was poor, with obvious abnormal activities. In the experimental group, a few fibriform bony calluses were determined in local region. At 4 weeks after surgery, the boundary between graft and host was unclear. In the control group, activities were still detected. In the experimental group, a large number of bony calluses were

found and could not move when push. At 8 weeks after surgery, a few bony calluses were found in the control group, with abnormal activities. Many bony calluses were found in the experimental groups.

X-ray examination results and radiological score in the femur at affected side

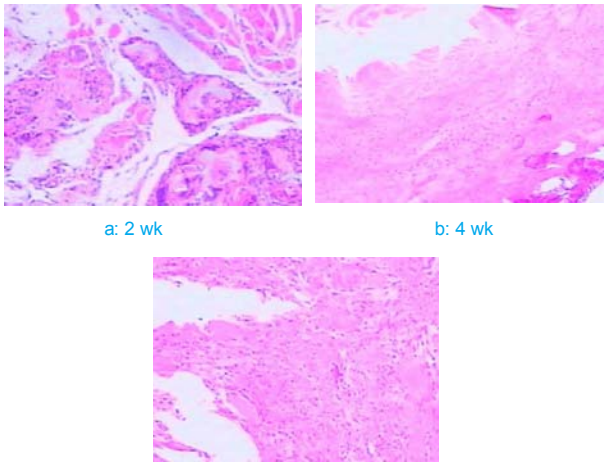
In the control group, no remarkable bony callus formed at defected site at 2 and 4 weeks after surgery, and a few new formed bony calluses were observed at 8 weeks after surgery. In the experimental group, bony callus formed in the bone defected site at 2 weeks after surgery, showing thin cloudiness shape. At 4 weeks after surgery, marked new bony calluses were visible at the two ends of defected site. At 8 weeks after surgery, new bony calluses were observed in the defected site, showing the bridge grafting of defected site. Radiological score results are displayed in Table 1.

Table 1 Radiograph score of each time point after surgery in both groups ($\bar{x}\pm s$)

Group	2 wk	4 wk	8 wk
Control	2.30±0.18	2.87±0.16	3.25±0.48
Experimental	2.63±0.15	3.45±0.21	5.15±0.25
P	< 0.05	< 0.05	< 0.05

Results of pathological tissue sections stained by hematoxylin-eosin staining in the femoral specimen after surgery

Control group: at 2 weeks after surgery, hyperemia, edema, abundant hymphocytes and neutrophil infiltration were found. BMSCs in implanted material did not transformed into osteoblasts, showing fibroblasts-like cells. At 4 weeks after surgery, inflammatory reaction was significantly relieved, showing a few osteoid formation. At 8 weeks after surgery, a few bony tissues were detected, and a great part was fibrous tissues (Figure 1).

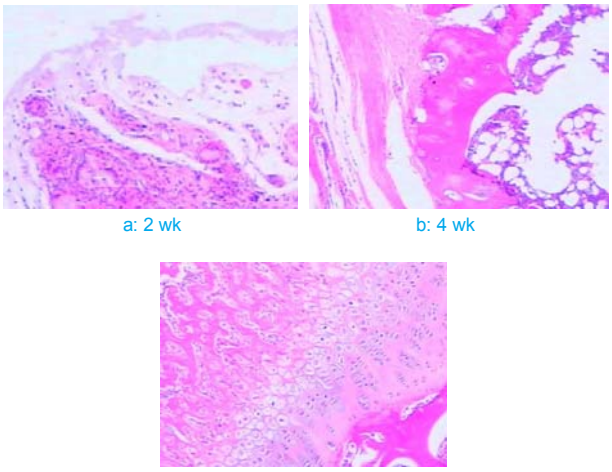


a: 2 wk b: 4 wk

c: 8 wk

Figure 1 Pathological section observation of the control group at 2, 4 and 8 wk after operation (Hematoxylin-eosin staining, ×100)

Experimental group: at 2 weeks after surgery, neutrophil and lymphocyte infiltration was detected in tissues. BMSCs did not transformed into osteoblasts. At 4 weeks, some nHAC degraded in graft. A large number of osteoblasts were determined in spacing, with the presence of osteoid. At 8 weeks, woven bone-like tissue was visible (Figure 2).



a: 2 wk b: 4 wk

c: 8 wk

Figure 2 Pathological section observation of the experimental group at 2, 4 and 8 wk after operation (Hematoxylin-eosin staining, ×100)

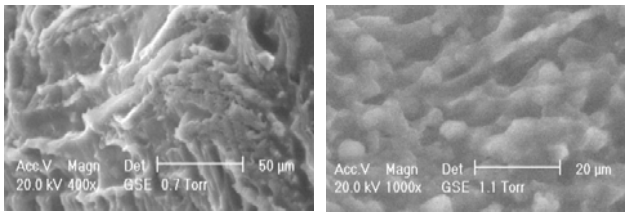
Results of bone mineral density measurement of femoral specimen in the affected side

Significant differences were determined in pairwise comparison of bone mineral density at 2, 4 and 8 weeks after surgery between the control and experimental groups ($P < 0.05$). Measurement results are exhibited in Table 2.

Table 2 Bone mineral density measurement results at different time points between experimental and control groups ($\bar{x}\pm s$, value)

Group	2 wk	4 wk	8 wk
Control	0.448±0.111	0.872±0.361	1.192±0.109
Experimental	0.768±0.122	1.235±0.063	1.489±0.325
P	<0.05	<0.05	<0.05

Results of environmental scanning electron microscope in femoral specimen at affected side (Figure 3)



a: Control group b: Experimental group

Figure 3 Environmental scanning electron microscope observation between control and experimental groups at 8 wk after surgery

In the control group, trabecular pattern was irregular at 8 weeks after surgery, showing bone lacuna structure. In experimental group, bony callus obviously formed at 8 weeks after surgery, showing a larger number of bone trabecularism and osteoblasts.

DISCUSSION

Bone tissue engineering has obtained outstanding progress. Present study mainly focused on stent materials, seed cells and cytokine combined construction.

Stent materials

Ideal stent materials should possess good biological degradation and biocompatibility, suitable surface hydrophilic-lyophobic balance, strong cell specific recognition ability and a certain mechanical strength[4]. Liao *et al*[5] developed nHAC that has close component as natural bone, is beneficial for cell adhesion, growth and collagen secretion, promotes calcification, is characterized by lamellar structure and nanocrystalline, with good biocompatibility. The nanocrystalline on its surface is beneficial for cell phagocytosis and degradation. Results of scanning electron microscopy suggested that materials have many pores, large pore diameter, and high porosity, and are in favor of cell proliferation. nHAC is accorded with the main function of bone tissue engineering stent material, and has been approved to apply in clinic in China. Yu *et al*[6] used nano-hydroxyapatite/collagen bone repair material in cervical vertebra anterior bone graft fusion operation. Its effect was close to autologous bone transplantation. Shen *et al*[7] utilized nHAC in clinic, verified that its effect was close to autologous bone transplantation, and demonstrated that nHAC is an ideal bone repair material. Sun *et al*[8] used nHAC and BMSCs composite to repair rabbit femoral head necrosis defect, verified that nHAC combined BMSCs has strong conduction osteogenesis, and promoted the healing of bone defect. Wang *et al*[9] employed nHAC to repair child skull defect and metacarpal bone defect. Results displayed that bone healing was detected in the experimental group using nHAC at 3 months after surgery, and confirmed that nHAC is suitable bone filling material and can promote the healing of bone defect.

Seed cells

BMSCs are characterized by easy to obtain, low body injury, strong amplification ability *in vitro*, easy to directional differentiation into osteoblasts, easy to fit the physiological, pathological and stress environment and to keep osteogenic activity[10]. BMSCs-induced tissues did not have problems such as tissue matching and immunological rejection during transplantation. BMSCs have tissue differentiation ability, and exogenous gene did not affect BMSC feature[11]. BMSCs are a kind of immune concession cells and cannot induce immunological rejection, and have immunosuppressive function. Some scholars cocultured BMSCs and allogeneic lymphocyte *in vitro* and showed that BMSCs could not lead to significant proliferation of lymphocytes[12-13]. BMSCs were infused into allogeneic baboon, and the living time of transplanted variant skin prolonged to 14 days, and the effect was identical to that of immunosuppressant[13]. Arinzen *et al*[14] have found that host response was not detected at various time points after implantation of allogeneic BMSCs in bone defect site. No lymphocyte infiltration was determined in the level of histology, and anti-allogeneic cell antibody was not detected. The present study transplanted BMSCs/nHAC composite into the bone defect region of animals, no immune rejection was determined. Tian *et al*[15] utilized BMSCs combined with heterogeneous bone matrix gelatin to repair rat radius defect and confirmed that BMSCs/bone matrix gelatin complex has a strong ability of bone formation.

Cytokine

Blood supply at local fracture regions has been considered an important influential factor for fracture healing. Vascular endothelial growth factor (VEGF) has close correlation to bone formation, and plays a positive effect on various bone formation activities. Midy *et al* (1994) during *in vitro* experiment have found that osteoblasts can combine with VEGF165, and induced chemotaxis and migration, and enhanced alkaline phosphatase activities in cells, which exhibited that VEGF165 could reinforce the differentiation ability of osteoblasts. Mayr-Wohlfart *et al*[16] cultured human osteoblasts *in vitro*, treated with rh-VEGF165, and the proliferation ability increased by 70%. Bouletreau *et al*[17] suggested that hypoxia and high expression of VEGF at fracture regions could accelerate bone morphogenetic protein-2 secretion from endothelial cells in local regions, and speed up the healing at injury site, which indicated that effects of VEGF on bone formation were mediated by bone morphogenetic proteins. The increase in VEGF in local regions does not necessarily increase the amount of new bones. Eckardt *et al*[18] used minipump to infuse recombinant VEGF protein into the interspace of the cut bone in rabbit models of tibia limb lengthening, but the results displayed that VEGF did not affect blood flow, torsional rigidity and bone mineral content in local bone. Peng *et al*[19] implanted VEGF165-transfected mesenchymal stem cells at the bone defect site, and bone healing did not occur. The reasons for different results may be associated with different animal models and different intervention levels.

Results from this study demonstrated that no rejection or inflammatory reaction was detected after implantation of stent complex in rats. Moreover, bone rapidly formed using VEGF/BMSCs/nHAC composite, which had better bone regeneration capacity compared with BMSCs/nHAC composite. The method of bone formation was endochondral ossification. It is assumed that VEGF promoted formation of local microvessel and differentiation and proliferation of osteoblasts, accelerated the velocity of endochondral ossification, shortened the time of bone repair, and elevated the quality and velocity of bone regeneration. However, many problems should be solved for clinical application, such as stent strength, seed cells, compounded manner of cytokine and controlled slow release method of VEGF.

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血管内皮生长因子与纳米晶胶原基骨支架复合修复大鼠股骨缺损*★

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摘要

背景：研究证实纳米晶胶原基骨复合间充质干细胞修复骨缺损具有体内成骨能力。

目的：观察血管内皮生长因子与骨髓间充质干细胞、纳米晶胶原基骨复合物修复大鼠股骨缺损的效果。

方法：制作SD大鼠股骨中段骨缺损模型，随机分为2组：对照组植入骨髓间充质干细胞/纳米晶胶原基骨复合物；实验组植入血管内皮生长因子/骨髓间充质干细胞/纳米晶胶原基骨复合物。术后第2，4，8周行股骨标本影像学与组织学观察；术后第8周行新生骨痂环境扫描电镜检查。

结果与结论：纳米晶胶原基骨支架复合物植入大鼠体内后无排斥反应及炎症反应，且血管内皮生长因子/骨髓间充质干细胞/纳米晶胶原基骨复合物成骨更快，较骨髓间充质干细胞/纳米晶胶原基骨复合物具有更好的骨再生能力，其成骨方式主要为软骨内成骨。推测血管内皮生长因子促进了局部微血管的形成和成骨细胞的分化、增殖，加快了软骨内成骨的速率，缩短了骨修复时间，提高了骨再生的质量和速率。

关键词：纳米晶胶原基骨；血管内皮生长因子；骨髓间充质干细胞；骨缺损；环境扫描电镜

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