Bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein in repair of large segmental radius defects in rabbits

Wang Tian-sheng[1], Teng Shou-fa[1], Wang Fa[1], Guo Li[1], Ding Hai-jiao[1], Liu Peng[1], Zhang Ying-xia[1], Cao Yu[2], Wang Zhan[3]

1 Department of Orthopedics, the 463rd Hospital of Chinese PLA, Shenyang 110042, Liaoning Province, China
2 Department of Physiology, China Medical University, Shenyang 110001, Liaoning Province, China
3 The 94-Period 7-Year Department of Clinical Medicine, China Medical University, Shenyang 110001, Liaoning Province, China

Abstract

BACKGROUND: Synthetic bio-ceramics as good biological scaffolds can compound with autologous bone marrow stromal stem cells. Bone tissue engineering of their combination for bone graft brings a new hope for large bone defects.

OBJECTIVE: To observe the therapeutic effects and feasibility of bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein in repair of large bone defects in rabbits.

METHODS: Rabbit models of 1.5 cm-long bone defects were made by implanting the middle radial bone in the upper limbs. Left bone defect region served as the experimental group, implanting with bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein. Right bone defect region served as the control group, only implanting with bio-ceramic. At 4, 8, 12 and 24 weeks after model establishment, general observation was performed in both groups. Histological changes were observed with radiographs to compare the repair and healing in the bone defect region.

RESULTS AND CONCLUSION: At 4, 8, 12 and 24 weeks after model establishment, compared with the control group, the repair of bone defects was faster, new bone formation was more, with complete plasticity, broken absorption was more apparent in the experimental group. At 24 weeks, bone defect region was completely bridged with broken ends of fractured bone. Bone was basically repaired. Results confirmed that bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein as graft materials in treatment of large bone defects of rabbits had a good therapeutic effect, and the effects were better than bio-ceramic alone.

Subject headings: Bone Morphogenetic Proteins; Morphogenesis; Tissue Engineering; Biocompatible Materials; Osseointegration
Funding: the Liaoning Provincial Science and Technology Project, No. 20120234

Wang TS, Teng SF, Wang F, Guo L, Ding HJ, Liu P, Zhang YX, Cao Y, Wang Z. Bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein in repair of large segmental radius defects in rabbits. Zhongguo Zuzhi Gongcheng Yanjiu. 2015;19(3): 347-351.

Wang Tian-sheng, Department of Orthopedics, the 463rd Hospital of Chinese PLA, Shenyang 110042, Liaoning Province, China

Corresponding author: Wang Tian-sheng, Department of Orthopedics, the 463rd Hospital of Chinese PLA, Shenyang 110042, Liaoning Province, China

doi:10.3969/j.issn.2095-4344.2015.03.004
[http://www.crter.org]

Accepted: 2014-10-23

INTRODUCTION

There are many reasons for bone defects in many patients in the clinic. Bone defects are difficult to be cured. The treatment of large bone defects is always a big problem confusing physicians in the department of orthopedics. Therefore, autogenous bone graft is commonly used in the clinic, and obtains ideal outcomes. However, its source is limited, and autogenous bone graft can cause secondary injury and other complications. The source of allogeneic bone is limited. Moreover, there is high risk of cross infection of hepatitis B and AIDS. Allogeneic bone has possible heterologous protein response. Synthetic bio-ceramics avoids above shortcomings. As a good biological scaffold, bone tissue engineering of bio-ceramic combined with bone marrow stromal stem cells and bone graft substance for bone graft brings a new hope for large bone defects, and provides a new pathway for the repair of large bone defects induced by various reasons. In view of this, the present study sought to observe the therapeutic effects and feasibility of bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein in repair of large bone defects in rabbits.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed at the Animal

Experiment Center, China Medical University in China from October 2010 to June 2011.

Materials

A total of 35 purebred male adult Japanese rabbits weighing 2.8–3.2 kg were provided by the Animal Experiment Center, China Medical University in China. Bio-ceramic was produced in the Nanjing Biological Co., Ltd., China. Main reagent STEOSET DBM (containing bone morphogenetic protein-2) particles were provided by Wright, USA.

Methods

Culture and amplification of rabbit bone marrow stromal stem cells in vitro

A total of 30 rabbits were intravenously anesthetized with 3% sodium pentobarbital (30 mg/kg). Bilateral posterior superior iliac spine was a puncture region, which was shaved and sterilized. Puncturation was done using No. 16 bone needle. A total of 6–8 mL of bone marrow was extracted by an aseptical 20 mL-syringe containing 10 mL 10% sodium citrate. Bone marrow was centrifuged with lymphocyte separation medium, and incubated in Dulbecco's modified Eagle's medium (DMEM)/F12. The number of cells was 1×10^9/L. Cells were incubated at 37 °C in 5% CO_2 saturated humidity. Subcultured was terminated until more than 90% confluency (passage 3 or 4).

Combined culture of bio-ceramic combined with bone marrow stromal stem cells in vitro

Bio-ceramic was immersed in DMEM/F12 for 24 hours. The medium was discarded. About 5×10^8 /L bone marrow stromal stem cells were incubated in each scaffold for 24 hours of coculture.

Establishment of rabbit models of large segmental radial bone defects

A total of 30 rabbits were numbered, anesthetized with 3% sodium pentobarbital and fixed in an operating-table. Radial bone region of both upper limbs was shaved and sterilized. An aseptic sheet was laid. The middle segment of bilateral radial bone was exposed. A 1.5 cm-long bone-periosteum defect region was made with a fret-saw and osteotome. Left bone defect region served as the experimental group, implanting with bio-ceramic combined with bone marrow stromal stem cells and OSTEOSET DBM (containing bone morphogenetic protein-2) particles. Right bone defect region served as the control group, only implanting with bio-ceramic. Bone defect region of the remaining five rabbits was not implanted with any material as blank control group. The wound was gradually sutured. After model induction, external fixator was not added (rabbit radius and ulna was synostosis, and ulna could be used as a natural fixator). On the same day, 60 000 U gentamicin was intraperitoneally injected. After model induction, animals were housed in separate cages, with conventional feeds. At 4, 8, 12 and 24 weeks after model establishment, morphological changes of samples in experimental and control groups were observed. Samples in the blank control group were observed at 24 weeks.

X-ray examinations

At 4, 8, 12 and 24 weeks after model establishment, radiographs of transplanted regions of rabbits in each group were examined to observe the repair of bone defects.

Histological observation

At 4, 8, 12 and 24 weeks after model establishment, normal bone tissues were obtained in both ends of bone defect region. Samples were fixed with 10% neutral formalin, decalcified, progressively dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin. Morphological changes in bone tissues were observed under a light microscope.

Main outcome measures

At 4, 8, 12 and 24 weeks after model establishment, general morphology was observed in each group. Radiograph results and histological changes were measured.

RESULTS

Quantitative analysis of experimental animals

A total of 35 rabbits were included in the final analysis, no drop out.

General morphology of bone defect region of rabbits after transplantation of bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein

Experimental group: At 4 weeks after model induction, bio-ceramic tightly connected to surrounding soft tissues. Soft tissues had many scar tissues, with brittle texture. Numerous soft new callus was visible surrounding and in the bio-ceramic. A small quantity of bone bridging was found. At 8 and 12 weeks, callus formation was apparently increased surrounding and in the bio-ceramic. The bio-ceramic was mostly replaced by new bones. Perfect bone bridging was visible in the transplanted region. At 24 weeks, the bio-ceramic was mostly replaced by new bones. Bone defect region was completely connected to bone end. Bone defect region could not be distinguished by naked eyes. Control group: at 4 weeks, very few calluses were seen on both ends of bio-ceramic bone area, and bio-ceramic mostly remained. At 8 and 12 weeks, less new bone formation was observed, and bio-ceramic remained. At 24 weeks, bio-ceramic was partially replaced by new bones. Bridging and moulding were poorer than in the experimental group. Blank control group: at 24 weeks after model induction, no new bones formed in each defect area. Fracture site sclerosis was observed, canal closed. The defect region was filled with a large number of fibrous connective tissue.

Radiograph results in the bone defect region of rabbits after transplantation with bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein

At 4 weeks, more callus formed in the bone defect region, and partial bone bridging formed in the experimental

group. Very few calluses formed in the bone graft region in the control group. The transmittancy was greater in the control group than in the experimental group at the same stage. At 8 and 12 weeks, new bone formation gradually increased in the transplanted region. Bone bridging formed and connected between both bone ends. Bone remodeling was still moderate. In the control group, very few callus formed in the transplanted region. The transmittancy was greater in the control group than in the experimental group in the same period. At 24 weeks, new bone was connected to cortical bone of broken ends. Most marrow cavity was recanalized. Moulding was complete in the bone graft region. In the control group, bone ends of the bone graft region were partially bridged, with the presence of poor moulding and the absence of recanalization of the marrow cavity. In the blank control group, at 24 weeks, no new bone tissues were visible in the bone defect region. Fracture site sclerosis was visible, and bone marrow closed (**Figure 1**).

Histological changes in rabbit bone defect area after implantation of bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein

As displayed in **Figure 2**, in the experimental group, at 2 weeks after model establishment, many soft tissues stuck around bio-ceramic, and chondrocytes were visible. At 4 weeks, a large number of mesenchymal cells, fibroblasts and chondrocytes were observed in and on the edge of bio-ceramic carrier. Original marrow cavity formed. New capillary grew in the bio-ceramic. At 8 and 12 weeks, bone tissue, woven bone, primitive bone marrow and bone marrow cavity formation were found in the implanted area. Obvious boundary line was seen in the juncture of new bones and original bio-ceramic. Moreover, new osteocytes were found. Simultaneously, bio-ceramic carrier fragmentation and absorption were found, and the ingrowth capillary became thick (**Figure 2A**). At 24 weeks, mature bone trabecula were detected among broken bone and new bones. New cortical bone was detectable outside bio-ceramic. New Harvard system was visible. Bone contour units were irregular. “Trim”-like bone cells were detected near the edge of the inner canal. A large number of lamellar bone formed. Marrow cavity and lacunae apparently formed, with the presence of numerous bone marrow cells and fat drop. Original bio-ceramic broke and absorbed evidently. Blood capillary grew into nourishing blood vessels. Simultaneously, broken bone resorption and osteoblastic activity were carried out among external newly-formed cortical bone and bio-ceramic. In the control group: at 4 weeks after model induction, mesenchymal cells, fibroblasts and a few chondrocytes were visible on the edge of bio-ceramic. New blood capillary was occasionally found in bio-ceramic. At 8 and 12 weeks, many chondrocytes were visible. However, obvious bone tissue and woven bone were not seen. No bio-ceramic broke or absorbed. Blood capillary was occasionally found (**Figure 2B**). At 24 weeks, bone tissue and woven bone were observed. Primitive bone marrow and marrow cavity formed. Original bio-ceramic broke and absorbed. Blood capillary grew into nourishing blood vessels. Bonding wire of new bones and bio-ceramic was evident. In the blank control group: at 24 weeks after model establishment, a large number of fibrous connective tissue formed in the bone defect region (**Figure 2C**).

Biocompatibility and adverse reactions after implantation

After model induction, activities in rabbits were normal. Diet, stool and urine were normal. No wound redness, oozing, infection or dehiscence were seen. Wound was healed in the first stage. Bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein after implantation showed good biocompatibility.

DISCUSSION

In the study of bone defect repair materials, osteogenetic potential was significant when experimental bone defects were larger than the self-repair of bone. This kind of experimental bone defect became basic size, also called critical bone defects[1]. Present studies confirmed that the length of critical bone defects was above 1.5–2.5 folds or above 1/10 the length of long bone[2]. The length of Japanese adult rabbit radial defects was about 15 mm, but the total length of Japanese adult rabbit radial bone was about 50.0–60.0 mm, and the critical bone defects were 5.0–6.0 mm. Therefore, the models established in this study have far accordance with critical bone defects. In addition, rabbit radius and ulna were synostosis. Intraoperative ulna was maintained. The implanted ceramic was implanted into radial defect region. After tightly suturing fascia, fixative effects were obtained. In addition, after modeling, rabbits liked to chew external fixator and thrum due to localized itching. Thus, this study did not use internal and external fixators. The thrum was tightly fixed, tied and cut. No graft displacement or skin split infection was visible during the present study.

This study utilized bio-ceramic provided by Nanjing Biotech Co., main component hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], particle diameter 0.5–1.0 μm. Polyvinyl butyral served as adhesives, and dehydrated alcohol as solvent. After treatment with calcinations at 900–1 200 °C, they were made into inner-sparse outer-dense bionic bracket Φ 5 mm×15 mm and porosity of 60%–76%[3-4]. Bio-ceramic as a synthetic material can be easily absorbed, replaced and degraded, with good biomechanical strength. Moreover, there is no risk of cross infection of type B hepatitis or AIDS, no problem of heterologous protein reaction. Suitable seed cells are important factors for forming tissue-engineered bone[5]. Bone marrow stromal stem cells are non-differentiated cells, present good potential of osteogenetic differentiation and activity, and have been considered as an optimal choice for bone tissue engineering[6-7]. Bone marrow stromal stem cells could be used as autologous cells to construct tissue-engineered bone[8-9]. Moreover, bone marrow stromal stem cells had advantages compared with

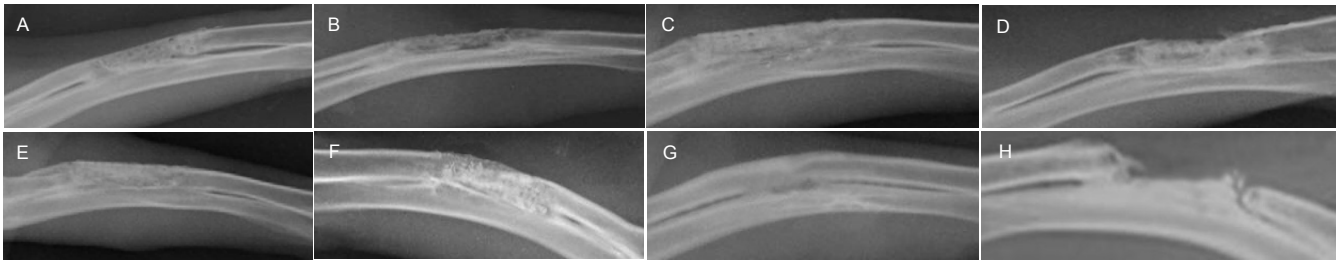

Figure 1 Radiographs of rabbit bone defect areas after implantation of bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein

Note: A: 4 weeks in the experimental group; B: 4 weeks in the control group; C: 8 weeks in the experimental group; D: 8 weeks in the control group; E: 12 weeks in the experimental group; F: 12 weeks in the control group; G: 24 weeks in the experimental group; H: 24 weeks in the blank control group. A 1.5 cm-long bone defect was made in radial bone in the middle of the upper limbs of rabbits. Left bone defect area served as an experimental group. Bio-ceramic and bone marrow stromal stem cells were cocultured *in vitro*, and then bone morphogenetic protein was added, which were implanted in the bone defect area. Right bone defect area served as a control group, only implanting bio-ceramic. At 4–12 weeks after model establishment, the repair of rabbit bone defect area was better in the experimental group than in the control group (images at 24 weeks in the control group are not shown).

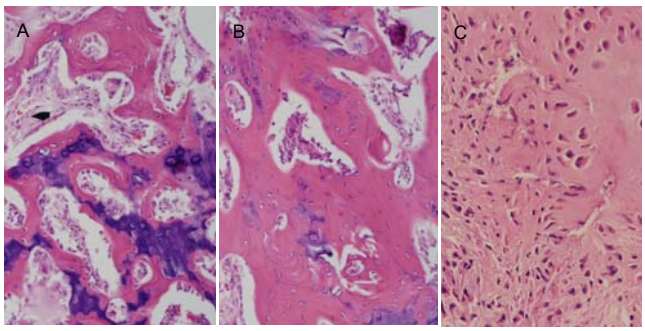

Figure 2 Histological changes in rabbit bone defect region after implantation of bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein (hematoxylin-eosin staining)

Note: A: 8 weeks after model induction in the experimental group (×20); B: 8 weeks after model induction in the control group (×20); C: 24 weeks after model induction in the blank control group (×10). A 1.5 cm-long bone defect was made in radial bone in the middle of the upper limbs of rabbits. Left bone defect area served as an experimental group. Bio-ceramic and bone marrow stromal stem cells were cocultured *in vitro*, and then bone morphogenetic protein was added, which were implanted in the bone defect area. Right bone defect area served as a control group, only implanting bio-ceramic. At 8 weeks after model establishment, bone tissues and woven bone were seen in the implanted region in the experimental group. Simultaneously, bio-ceramic broken and were absorbed. No evident bone tissue or woven bone was found in the control group. No bio-ceramic was broken or absorbed.

other seed cells: (1) easily obtained by bone marrow aspiration; (2) rapid proliferation in a short period. Maniatopoulos *et al* [10] reported that bone marrow stromal stem cells of adult rats could form calcified bone tissue under *in vitro* culture. X-ray diffraction analysis verified that calcified tissue had hydroxyapatite-like structure and confirmed that bone marrow stromal stem cells cultured *in vitro* could differentiate into osteoblasts and formed bone tissues.

Bone morphogenetic protein has two kinds of osteogenetic abilities: (1) increased osteogenetic property of osteoblast-like cells; (2) expression of osteoblast phenotype by inducing osteoprogenitor cell such as bone marrow mesenchymal stem cells. Bone morphogenetic protein is not stable. Single application of bone morphogenetic protein obtained poor outcomes. Bone morphogenetic protein can be easily dissolved and run off. This study used OSTEOSET DBM particles (containing bone morphogenetic protein-2) produced by Wright, USA. Bone morphogenetic protein can be slowly persistently release. A previous study demonstrated that OSTEOSET DBM particles were applied in dog, and confirmed that bone trabecula was thick, showing active osteoblasts[11]. Wang *et al* [12] made bone morphogenetic protein-2 into novel complex repair material, which was implanted into rabbit models of radial defect. Repair experiment of rabbit radial bone exhibited that self-made recombinant human bone morphogenetic protein-2 apparently promoted bone repair, and was fit for clinical application. In the present study, OSTEOSET DBM particles were grounded and scattered on the surface and in the pore of composite bone, and then implanted into the bone defect region. We observed that osteogenetic time of bone marrow stromal stem cells and bone morphogenetic protein was significantly shorter compared with the control group, showing a strong osteogenesis. The time of decomposition and absorption of implanted bone, new bone substitute was shorter. It is verified to be a good bio-derived bone repair material.

Experimental results suggested that bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein could effectively repair big-segment bone defects, and were good bone graft material. Bio-ceramic combined with bone marrow stromal stem cells and bone morphogenetic protein in the repair of segmental bone defects accelerated bio-ceramic substitution. The healing time of bone defects was greatly shortened, exerted complete transformation of materials into bone tissues, avoided the shortcomings of insufficient biomechanical function, shortened therapeutic time and elevated patient's quality of life.

REFERENCES

[1] Rong XF, Wu L, Yang XD, et al. Establishment of an animal model for reconstnlction of mandibular critical-sized defect in rabbits. Zhongguo Yike Daxue Xuebao. 2008;37(1):63-64.
[2] Pei GX. Tissue Engineering: A Laboratory Manual. Beijing: People's Military Publishing House. 2006.
[3] Hu L, Zhao C, Xu L. Fabrication and properties of porous ha ceramics with bionic structure. Shengwu Guke Cailiao yu Linchuang Yanjiu. 2011;8(8):4-7.
[4] Descamps M, Duhoo T, Monchau F, et al. Manufacture of macroporous β-tricalcium phosphate bioceramics. J Eur Ceram Soc. 2008;28(1):149-157.
[5] Jenkins DD, Yang GP, Lorenz HP, et al. Tissue engineering and regenerative medicine. Clin Plast Surg. 2003;30: 581-588.
[6] Bruder SP, Kraus KH, Goldberg VM, et al. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am. 1998;80(7):985-986.
[7] Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. Exp Biol Med. 2001;226(6):507-520.
[8] Jin D, Pei GX, Wang Q, et al. New bone formation by bone marrow stromal cell combined with the bioactive glass ceramic using tissue-engineering methods. Zhonghua Chuangshang Zazhi. 2001;17(3):151.
[9] Li ZH, Peng H, Liao W. Study of the osteogenic ability of mesenchymal stem cells in vivo. Zhongguo Jiaoxing Waike Zazhi. 2006;14(9):680-689.
[10] Maniatopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. 1988;254:317-321.
[11] Turner TM, Urban RM, Gitelis S, et al. Efficacy of calcium sulfate,a synthetic bone graft material,in healing a large canine medullary defect. 45th Annual Meeting, Orthopedic Research Society. Anaheim, CA, USA. 1999.
[12] Wang J, Xiao G, Chen Y, et al. Osteoinductive activity of self-made human bone morphogenetic protein-2 composite bone in bone repairing. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2010;14(21):3806-3819.

生物陶瓷复合骨髓基质干细胞及骨形态发生蛋白修复兔大段桡骨缺损

王天胜[1]，滕寿发[1]，王　法[1]，郭　利[1]，丁海蛟[1]，刘　鹏[1]，张英霞[1]，曹　宇[2]，王　湛[3] ([1]解放军第四六三医院骨科，辽宁省沈阳市　110042; [2]中国医科大学生理教研室，辽宁省沈阳市　110001; [3]中国医科大学 94 期 7 年制临床医学系，辽宁省沈阳市　110001)

王天胜，解放军第四六三医院骨科，辽宁省沈阳市　110042
通讯作者：王天胜，解放军第四六三医院骨科，辽宁省沈阳市　110042

文章亮点：

文章结果发现生物陶瓷复合骨髓基质干细胞及骨形态发生蛋白修复节段性骨缺损，加速了生物陶瓷替代，使骨缺损的愈合时间大幅度缩短，实现了材料向骨组织的完全转化，避免了其生物力学性能不足的缺点，缩短了治疗时间，提高患者的生活质量。

关键词：

生物材料；骨生物材料；生物陶瓷；大段骨缺损；骨髓基质干细胞；桡骨；骨形态发生蛋白

主题词：

骨形态发生蛋白质类；形态发生；组织工程；生物相容性材料；骨整合

基金资助：

辽宁省科学技术基金资助项目(20120234)

摘要

背景：人工合成生物陶瓷作为良好的生物支架材料与自体骨髓基质干细胞复合并联合诱导成骨物质移植的骨组织工程为大段骨缺损的患者带来了新希望。

目的：观察生物陶瓷复合骨髓基质干细胞及骨形态发生蛋白修复兔大段桡骨骨缺损的疗效及可行性。

方法：实验植入兔双上肢桡骨中段制造长约 1.5 cm 的大段骨缺损模型，将左侧骨缺损区作为实验组，植入生物陶瓷与骨髓基质干细胞+骨形态发生蛋白；右侧骨缺损区作为对照组，单纯植入生物陶瓷。建模后 4，8，12，24 周各时间点对各组动物行标本大体观察、X 射线片观察及组织学观察，比较其骨缺损区修复愈合情况。

结果与结论：建模后 4，8，12，24 周，与对照组相比，实验组兔桡骨骨缺损区修复较快，新骨形成量多，塑性完全，原生物陶瓷破碎吸收现象明显。建模后 24 周，桡骨骨缺损区与骨端完全桥接，骨质基本得到修复。结果证实，生物陶瓷复合骨髓基质干细胞及骨形态发生蛋白作为复合移植材料治疗兔桡骨大段桡骨缺损疗效较好，效果优于单纯生物陶瓷移植材料。

作者贡献：实验设计为第一、二作者，干预实施、结果评估为全部作者。文章全部作者均对文章负责。

利益冲突：文章及内容不涉及相关利益冲突。

伦理要求：实验过程中对动物的处置应符合 2009 年《Ethical issues in animal experimentation》相关动物伦理学标准的条例。

学术术语：生物陶瓷-是指用作特定的生物或生理功能的一类陶瓷材料，即直接用于人体或与人体相关的生物、医用和生物化学等的陶瓷材料。

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。

中图分类号: R318　文献标识码: B
文章编号: 2095-4344(2015)03-00347-05

王天胜，滕寿发，王法，郭利，丁海蛟，刘鹏，张英霞，曹宇，王湛. 生物陶瓷复合骨髓基质干细胞及骨形态发生蛋白修复兔大段桡骨缺损[J].中国组织工程研究，2015，19(3): 347-351.

(Edited by Chen M/Qiu Y/Wang L)