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Effect of rhBMP-2 sustained-release nanocapsules on the ectopic osteogenesis process in Sprague-Dawley rats

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ABSTRACT

Objective: To explore the effect of sustained-release recombinant human bone morphogenetic protein-2 (rhBMP-2) on ectopic osteogenesis in the muscle pouches of rats through preparing rhBMP-2 sustained-release capsules by wrapping morphogenesis protein bones-2 (BMP-2) using chitosan nanoparticles, and compositing collagen materials. **Methods:** Twenty four Sprague-Dawley rats were randomly divided into four groups with six rats in each group, that is Group A (control group), Group B (only treated with collagen), Group C (rhBMP-2+collagen treated group) and Group D (rhBMP-2/cs+collagen treated group). The composite materials for each group were implanted in the bilateral peroneal muscle pouches in rats. The peroneal muscles were only separated without implanting any materials in control group. Rats were sacrificed 2 weeks and 4 weeks post treatment and samples were cut off for general observation, Micro CT scans and histological observation. **Results:** General observation showed no new bone formation in Groups A and B mice, while new bones were formed in Groups C and D mice. Two weeks after treatment Micro CT scans showed that The bone volume fraction (BVF), trabecular thickness (Tb.Th), bone mineral density (BMD) in Group C mice were all higher than that in Group D ($P<0.05$). At the fourth week, the BVF, Tb.Th and BMD were significantly higher than that at the second week ($P<0.01$). **Conclusions:** The slow-release effect of rhBMP-2/cs sustained-release capsules can significantly promote ectopic osteogenesis. Its bone formation effect is better than that of rhBMP-2 burst-release group.

1. Introduction

Nonunion induced by oral and maxillofacial tumors or bone defects caused by trauma pose a difficult problem for clinical bone repair at present. Currently, it is a hotspot to study bone repair using tissue engineering bones to repair various kinds of bone defects and nonunion. Growth factors have been confirmed to play an important role in the process of repairing bone defects using tissue engineering bones. By compositing appropriate bone growth factors osteoinduction and osteogenesis process can be promoted so as to obtain a good effect of bone defect repair. Recombinant human bone morphogenetic protein-2 (rhBMP-2) is an important bone growth factor existing in bone matrix. It has been proven to possess an obvious activity in inducing osteogenesis, specifically motivating the directional differentiation of cells, stimulating mesenchymal cells and the precursor cells in bone marrow directionally differentiate into cartilage cells and osteoblast and accelerating the regeneration of bone tissue and repair process. However, no research has been done to study the effect of sustained-release of rhBMP-2 on the whole osteogenesis process. The present study aimed to study the effect of rhBMP-2 sustained-release nanocapsules wraped by chitosan nanoparticles

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on the whole process of ectopia osteogenesis in peroneal muscle pouches in rats, and to provide experimental basis for further studies on materials composited by rhBMP-2 and tissue-engineered bone.

2. Materials and methods

2.1. Animals and materials

Kunming Sprague-Dawley (SD) rats weighing 20 to 24 g were provided by Laboratory Animal Center, Sun Yat-Sen University, Nanjing (License No. SCXK (YUE) 2011-0029). The rhBMP-2 and collagen-1 were provided by the Biological Engineering Research Institute, Jinan University, Guangdong, China.

2.2. Experimental protocol

2.2.1. Preparation of composite materials

0.01 mol/L PBS solution, collagen-1 solution, 0.4 mg/mL rhBMP-2 solution and 4 mg/mL chitosan were used to prepare different solutions in terms of different dosages in different groups. That is Group B (only treated with collagen): 4 810 μL collagen solution+390 μL PBS solution; Group C (rhBMP-2+collagen): 4 810 μL collagen solution+130 μL rhBMP-2 solution+260 μL PBS solution; Group D (rhBMP-2/cs+collagen): 4 810 μL collagen solution+130 μL rhBMP-2 solution+130 μL chitosan solution+260 μL PBS solution. The above solutions for each group were added into 96-well plate with 12 wells for each group and 400 μL solution for each well. The plate was freeze-died in the lyophilizer and sterilized at 60 ℃ for further use.

2.2.2. Animal model

Twenty four Kuming SD rats were kept in cages separately at room temperature with free access to water. They were allowed to acclimatize for one week. The rats were randomly divided into 4 groups with 6 rats in each group and treated with different composite materials. Group A served as blank control, Group B was only treated with collagen, Group C was treated with rhBMP-2+collagen and Group D treated with rhBMP-2/cs+collagen. The rats were anaesthetized by intraperitoneal injection of 2% pentobarbital solution. Their skins at inner side of peroneus on both sides of rats were preserved, sterilized and incised. The intermuscular spaces were bluntly dissected to expose the muscle pouches of peroneus. Then the prepared composite materials were implanted into muscle pouches and the skins were sutured. The intermuscular spaces of rats in control group were only separated without implanting any materials. Each rat was kept in a separate cage.

2.2.3. General observation and sampling

The status of daily activities, feeding and wound healing was observed after operation. The rats in each group were successively sacrificed 2 weeks and 4 weeks post treatment. The parts of peroneus implanted with different drugs were cut off for sampling and then fixed in 10% neutral formalin buffer for further study.

2.2.4. Micro-CT scan

The resected samples were detected with Micro-CT (ZKKS-MCT-Sharp, Guangzhou Zhongke Kaisheng Medical Technology Co., Ltd) and the bone parameters were measured and analized. The scan parameters were scanning resolution 20 μm; rotation angle 360°; rotation angle increment 0.72°; voltage 70 kv; power 40 W; average of frames 2; inter-layer spacing 20 μm. The obtained CT images were transferred into three dimensional reconstructed images by bundled software of ZKKS MCT-Sharp. The regions of interest (ROI) (size 0.5 mm×0.5 mm×0.2 mm) were selected for observation of the effect of bone formation. The following bone parameters were analyzed: 1. bone volume fraction (BVF) refers to the ratio of the volume of bony structure and the total volume of samples, namely the ratio (%) of mineralized tissue; 2. Tb. Th refers to the average thickness of the bone trabeculas (μm); and 3. Bone mineral density (BMD, mg/cc).

2.2.5. Histological observation

The samples that was fixed by the formalin buffer were dyed with HE and the The histologic morphology of samples in each group were observed under inverted microscope.

2.3. Statistical analysis

All the data were presented as mean and SPSS 13.0 statistic package software was used for *t* test analysis. Differences were considered as statistically significant if $P<0.05$ and as significantly different if $P<0.01$.

3. Results

3.1. General observation

Animals of each group were fed under standard condition after operation. Each rat was kept in a separate cage to avoid non-experimental death due to attack between animals, leading to the termination of the experiment. All the rats revived on the day of treatment with reduced activities and feedings which returned to normal 1 to 2 day post treatment. All wounds at hind legs of the rats were primary healing, without swelling and exudation. Sutures fell off 5 to 7 days postoperatively. Weights of rats increased slowly and their activities and feeding were active.

3.2. Micro – CT scan and 3D reconstruction

Micro-CT scan showed no ectopia osteogenesis in group A (blank control group) and group B (collagen group), while

new bone formation could be observed in both Group C (rhBMP-2 + collagen) and Group D (rhBMP -2/cs + collagen). The bones were in irregular shape or long spindle shape. The gray level was obviously lower in the new formed bones than that in normal bones. The outer layers of the newly formed bones were surrounded by continuous cartilage-like sclerotin which discontinuously stretched into the center of caudomedial part, forming a loose bone trabecular-like structure. The 3D reconstruction images of groups C and D are presented in Figure 1. The osteogenesis effect in Group C was better than that of group D at the second week, while this effect is better in Group D than that of Group C at the fourth week. Little changes of osteogenesis was observed in Group C at the second and fourth week, while osteogenesis effect at the fourth week in Group D is obviously better than that at the second week.

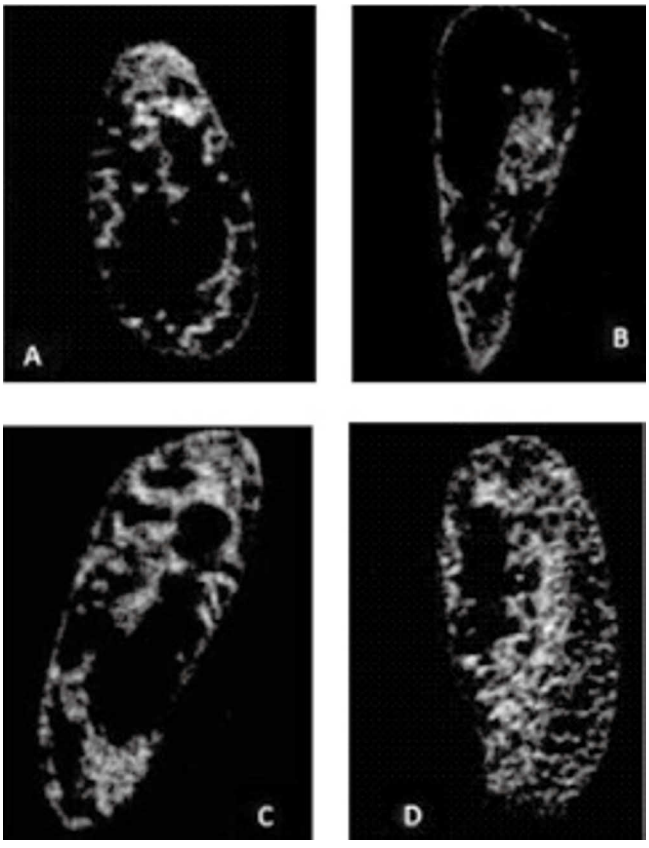

Figure 1. Micro-CT three dimensional reconstructed image of the bones of four groups.
A: Micro-CT image of Group C at the second week;
B: Micro-CT image of Group D at the second week;
C: Micro-CT image of Group C at the fourth week;
D: Micro-CT image of Group D at the fourth week.

3.3. Analysis of Micro-CT bone parameters

Among the bone parameters obtained in Micro-CT, BVF refers to the ratio of the volume of bony structure and the total volume of samples. A greater value represents more content of the bone trabeculas. Tb. Th refers to the average thickness of the bone trabeculas, and a greater value shows solider trabecular bone structures and a better osteogenesis effect. BMD represents the density of the whole bone minerals. A greater value suggests a greater amount of new bone formation. Because no ectopia osteogenesis was observed in Group A and B in Micro-CT scan, bone parameter analysis was performed only in the regenerated bones in Group C and D. The middle regions of cartilage-like bones of the outer layer of regenerated bones were selected as ROI. The results are shown in Table 1.

Table 1
The bone parameters in the ROI of Groups C and D (mean).

Groups	Bone parameters		
	BVF(%)	Tb.Th(μm)	BMD(mg/cc)
Group C (2nd week)	73.51±3.67	127.40±16.55	281.32±15.19
Group D (4th week)	54.05±1.26▲	95.33±11.84▲	207.10±14.82▲▲
Group C (2nd week)	81.69±4.45	135.33±17.84	294.33±13.97
Group D (4th week)	98.52±5.24△△*	161.38±21.60△*	332.71±19.68△△**

▲P<0.05, ▲▲P<0.01,vs Group C(2w), △P<0.05, △△P<0.01,vs Group D(2w), *P<0.05, **P<0.01,vs Group C(4w).

BVF values in Group C were higher than that in Group D ($P<0.05$) at the second week, while BVF values in Group D were higher than that in group C ($P<0.05$) at the fourth week. There is no significant difference ($P>0.05$) between the BVF values at the fourth week and that at the second week in group C, while BVF value in Group D is significantly higher ($P<0.01$) at the fourth week compared to the values at the second week, indicating that the BMD value in Group D is maximum at the fourth week.

3.4. Histological observation

No inflammation or fiber cells infiltrations caused by trauma were observed in muscle tissues of Group A (control group) (Figure 2A). No new bone formation or obvious drug residues was observed in Group B (collagen treated group) within the tissue section, while invasion of a few fiber cells can be seen in the muscle tissues and mesenchymal tissues (Figure 2B). Ectopic bone formations could be observed in Group C at the second week, with clear boundaries between the tissues and the surrounding muscle tissues. The edges of the regenerated bones are surrounded by a circle of thin layer of continuous cartilage-like bone. Most of the cells in bone matrix are present as individual existence, cartilage lacunas are obvious. Scattered discontinuous bone could be seen in the ectopic regenerated bone, and a few capillary lumens and scattered erythrocytes are observed around the bone matrix (Figure 2C). Ectopic regenerated bones were observed in Group D, the thin layers of continuous cartilage-like bones around the regenerated bones are obviously reduced compared with Group C. The cells within the bone matrix exist in the form of single cartilage-like cells with unobvious cartilage lacunas (Figure 2D). The structures of the ectopic regenerated bones at the fourth week were similar to that at the second week in Group C, with the edges

of the regenerated bones surrounded by a circle of thin layer of continuous cartilage-like bones and most of the cells in bone matrix present as individual existence with obvious cartilage lacunas (Figure 2E). There is an obvious increase of continuous bone substance formations in the regenerated bones in Group D compared with that at the second week. The cells inside the bone matrix exist in a form of collective cartilage-like cells and the cartilage lacunas are more clear than that in Group C at the fourth week (Figure 2F).

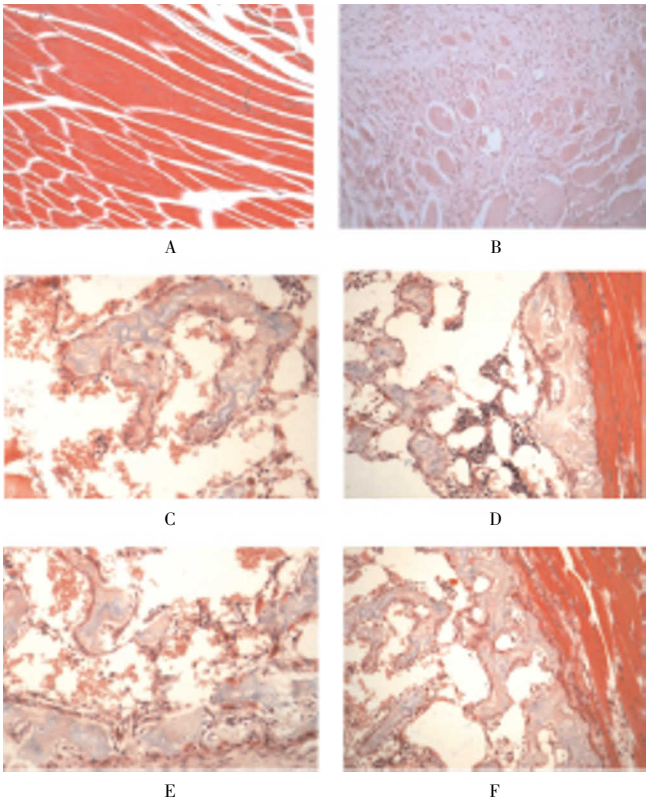

Figure 2. Histological results.

A: Group A (HE, ×100); B: Group B (HE, ×100); C: Group C at 2nd week (HE, ×100); D: Group D at 2nd week (HE, ×100); E: Group C at 4nd week (HE, ×100); F: Group D at 4nd week (HE, ×100)

4. Discussion

There is a good prospect of clinical application in using tissue engineering methods to repair bone defect. Among the studies in this field, the composite of bioactive factors is another research hot spot in the application of bone tissue engineering. There are many known types of bone growth factors, among which bone morphogenetic proteins (BMP) is very effective on inducing osteogenesis. It can specifically induce directional differentiation of the cells, stimulate the directional differentiation of mesenchymal cells and precursor cells in bone marrow into cartilage cells and osteoblasts and accelerate the repair and regeneration process of bone tissue[1]. In the family of BMPs, BMP-2 is one of the most effective bone growth factors in inducing osteogenesis. However, due to the limited body content of endogenous BMP, there is a need to add exogenous rhBMP-2. rhBMP-2 can induce irreversible differentiation of undifferentiated mesenchymal cells into cartilage cells and osteoblasts, promote the proliferation and differentiation of osteoblasts and fibroblasts, thereby inducing the synthesis of bone matrix and motivating new bone formation. rhBMP-2 has no species specificity, it has the ability to induce new bone formation across species. It has been proven in many studies that rhBMP-2 is highly effective in inducing osteogenetic activities[1-7]. Yoneda, *et al*[8]found that BMPs was effective enough in bone regeneration to repair bone tissues in scaffold materials. A previous study showed that BMP-2 could promote bone repair in PLGA/Hap scaffolds[9]. In a study by Wodewotzky *et al*[10], it has been found that after being implanted into bodies of dogs, rhBMP-2 was gradually released along with the degradation of PDLLA, stimulated and induced the differentiation and osteogenesis around the nail paths and the end of broken bones, effectively assisting the healing of the nail paths. Wildemann *et al*[11]studied the effect of combining BMP-2 and PDLLA on delayed union of fracture in animal models. Compared with PDLLA group, there was a significant increase in mechanical stability in BMP-2 and PDLLA composite group, suggesting that regional application of BMP-2 can stimulate the recovery of delayed union of fracture. A study by Tong *et al*[12]has shown that composition of PDLLA and BMP was effective in repairing bone defects. It induced the appearance of new bone formation with the degradation of composite materials. The results of the present study also confirmed that the ectopic osteogenesis could be observed when rhBMP-2 was applied alone, which proved rhBMP-2 can alone induce the differentiation of mesenchymal cells into cartilage cells and osteoblasts in muscle tissues, forming new bone matrix.

The main biological function of BMP is to induce the differentiation of undifferentiated mesenchymal cells into cartilages and bones. However, a regional and alone use of BMP did not show a satisfying treatment effect, for which the main reason was the polypeptide growth factors was diluted, resolved and absorbed regionally in a short period and it could not effectively stimulate the target cells for long[13].

Precursor chitin of chitosan[14]is the main structural polysaccharide in the arthropod keratin. It can interact with matrix proteins and connective tissue components. Chitosan is the product of a partial deacetylation of chitin, so it multiply reacts with the body tissues in the inner environment. Chitosan is positively charged, which induces the PH responsive electrostatic interactions between chitosan and anion glycosaminoglycan, proteoglycan and other negatively charged macromolecular, making these components retain and gather in the stent in the cell culture or after implanted in the body. Studies have shown that growth factors usually combine with

glycosaminoglycans which mediate the effect of growth factors. Chitosan-based biomaterial scaffolds will collect required growth factors from the surrounding tissue fluid. In addition, chitosan is a natural extracted product and it has a good biological compatibility and adaptability without any antagonism effect to human body, so it is often used as a modifying polymer on the surface of materials[15]. In the present study chitosan nanoparticles was used to wrap rhBMP-2 so as to realize the slow release of rhBMP-2 and continuously work in the whole process of ectopia osteogenesis.

The results of the present study showed that at the second week, the effect of ectopia osteogenesis was better in rhBMP-2 burst-release group than that in rhBMP-2 slow-release group, for which the reason may be the incompletely release of rhBMP-2 in slow-release group in 2 weeks time and the function of ectopia osteogenesis of rhBMP - 2 was not fully effected. While at the fourth week, the effect of rhBMP-2 slow-release group was significantly better than that of rhBMP-2 burst-release group, for which the possible reason was rhBMP-2 has been completely released and it quickly worked to induce bone formation in burst-release group by the end of the second week. This is why the osteogenesis effect of rhBMP-2 burst-release group showed nearly no difference betweem the second week and the fourth week. As there were no chitosan nanoparticles to control the slow-release in rhBMP-2 burst-release group, rhBMP-2 was immediately released after contact with body fluids, presenting its effect at the early stages of osteogenesis. However, at the later stages of osteogenesis rhBMP-2 has been completely released, showed no effect of promoting bone formation. On the contrary, the ectopic osteogenesis effect in RhBMP-2 slow-release group was much better at the fourth week than that at the second week, which proved that the slow release of rhBMP-2 ensured rhBMP-2 release in the whole ectopic osteogenesis process, thus maximize its effect on new bone formation.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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