Peri-Implant PLGA Loaded BMP-4 for Bone Regeneration in Rabbit Models

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Abstract: Purpose: This study was designed to incorporate BMP-4 into poly(lactic-co-glycolic acid) (PLGA) implants and evaluate the local release of BMP-4 biological effects. Method: Using rabbit models of a full thickness knee articular cartilage defect filler after surgical core-decompression. Rabbit BMSCs were isolated, cultured and identified by immunohistochemistry. The plasmid pcDNA3.1-BMP-4 was successfully introduced into BMSCs by electroporation. A double layer PLGA scaffold with a 4 mm diameter was set and implanted in the knee. Forty New Zealand white rabbits were randomly divided equally into four groups: the blank group, PLGA group, BMSCs/PLGA group, and PLGA/BMP-4/BMSCs group. Postoperation follow-up were recorded. HE staining of knee joint was performed at the eighth and sixteenth week. Implanted PLGA was observed under scanning electron microscope. The expression levels of chondrogenic markers SOX-9, collagen II and aggreccan were measured by RT-PCR in experimental groups. Result: The results showed there was no record of inflammatory reaction in the knee joint activity postoperatively. HE staining showed that the wound site in PLGA/BMP-4/BMSCs group improved better than the other 3 groups. RT-PCR results showed that the expression levels of SOX-9, collagen II and aggreccan in the PLGA/BMP-4/BMSCs group were significantly higher ($P<0.01$) than that in other groups. Conclusion: This study is one of the first to demonstrate the beneficial effect of BMP-4 delivery system integrated in double PLGA implants, suggesting its great potential in postoperative treatment targeting local bone healing and tissue regeneration.

Abbreviations

BMP-4: Bone morphogenetic protein-4; PLGA: Poly(lactic-co-glycolic acid); MSCs: Mesenchymal stem cells; BMSCs: Bone marrow mesenchymal stem cells

1. Introduction

Orthopedic and dental implant therapies have evolved into important treatments for deranged joints and lost teeth or to provide fixation of bone in the case of fractures. Osteogenesis, i.e. the differentiation of mesenchymal stem cells (MSCs) into mature osteoblasts is essential in bone growth, fracture healing and osseointegration [1]. Moreover, compared with direct transfer of gene vectors, engineering MSCs are associated with less immunologic interference [2]. MSCs have the capacity of enhancing their therapeutic potential and can recognize the location of injury, reach the site, and excrete several soluble factors to accelerate the healing process [3].

The development of growth factor delivery strategies to circumvent the burst release phenomenon prevalent in most current systems has driven research towards encapsulating molecules in resorbable polymer matrices [4]. Bone repair may be necessitated by a number of

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situations including trauma, congenital defects, pathological deformation or revision surgery [5] or even from steroids associated osteonecrosis which might lead to join collapse, a common orthopedic problem [6] and subsequent joint replacement [7]. In some severe situations spontaneous bone regeneration may not occur [8]. Most biomaterials alone lack efficacy and have been proven to perform better if combined with cells or bioactive molecules [9].

Bone morphogenetic protein (BMP) signaling is crucial in skeletogenesis and bone formation during development and repair, and ectopic administration of BMPs is used to enhance local bone regeneration in humans [10-12]. Biocompatibility of particles size was maintained at 4 μm and 6 μm, and their efficient fusion and kinetics were investigated. An initial high dose of BMP is often required to be loaded into the carrier in order to achieve active levels within the therapeutic window leading to elevated costs. Poly (lactic-*co*-glycolic acid) (PLGA) is a biocompatible, biodegradable polymer [13-15] with a history of over forty years use in medical resorbable sutures [16] and evolved into a frequently used synthetic polymer within the field of bone regeneration [17].

The aim of this study was to evaluate the effects of releasing active BMP-4 from PLGA implants on bone healing and to provide a more effective graft material for enhancing bone formation.

2. Materials and Methods

2.1 Animals.

Forty-two New Zealand white rabbits of both genders (weight 3–3.5 kg, age 24 months) were obtained from the Experimental Animal Center of Zhengzhou University. Animals were allowed to range freely single in their labeled cage and feed with a standard diet ad libitum. Surgeries were all performed under sterile conditions. All experiments were approved by the University branch of Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (1996). Animals were randomly divided into 4 groups (n=10): Blank control group, PLGA group, PLGA/BMSCs group, PLGA/BMP-4/BMSCs group. Two separate rabbits were used as donors for BMSCs culture.

2.2 Plasmid and Scaffold.

Plasmid pcDNA3.1-BMP-4 and PLGA scaffolds were purchased from Jinan Banzheng Biotechnology Co., Ltd (Beijing, China).

2.3 Composite Scaffold Fabrication.

The culture, identification, isolation and expansion of bone marrow mesenchymal stem cells (BMSCs) and transfection of pcDNA3.1-BMP-4 plasmid were established successfully in our lab. The BMSCs was identified by immunohistochemistry method. Plasmid pcDNA3.1-BMP-4 was introduced into BMSCs by electroporation. GAPDH gene was used as endogenous reference and the results were identified by RT-PCR as previous study [4]. The PLGA was dissolved in 1, 4-dioxane (85:15) before BMP-4 (MyBioSource, Inc. San Diego, USA) was added to form a uniform paste with a ratio of 3:1 (w/w of PLGA/BMP-4) by vigorous stirring overnight. BMSCs seeded cells were added to form the PLGA/BMP-4/BMSCs scaffold. PLGA has a molecular weight of 150,000 units and 4 mm diameter (Shandong Institute of Medical Instruments, Jinan, China). The upper part has 1 mm thickness, 100-200 μm pore size and 92% porosity; lactic acid (LA) and glycolic acid (GA) molar ratio of 85/15 (Fig.1a). The pore size of the lower part is 300-450 μm, 4 mm thickness, and the porosity is 77% (Fig.1b). The two units were adhered with dichloromethane (Fig.1c).

2.4 Surgery and Scaffold Implantation.

After careful shaving and cleaning with iodophore, the anterior aspect of the distal femoral epiphysis was exposed through an anterolateral skin incision, followed by skin and patella tendon reflection with a blunt scalpel shaft to expose the patella surface. Both right and left side knee joints were operated making a total of 20 surgeries per group. X-ray films were taken at the Orthopedic Department while the animals were under the postoperative anesthetic effect. Using a Tungsten drill

(Hangzhou Anka Cemented Carbide Tools Co., Ltd), was made a full depth knee join defect of 4 mm diameter, 5 mm drill depth at the patella surface to insert the bilayer PLGA scaffolds. PLGA/BMP-4/BMSCs received bilayer PLGA scaffolds with successfully transfected BMSCs; PLGA/BMSCs group got implanted with non-transfected double layered PLGA scaffold; PLGA group was inserted simple empty PLGA; and the blank control group defects was left empty covered with bone wax (Fig.2A). The subcutaneous layer of the wound was closed with resorbable polyglactin sutures (5-0, Vicryl, Ethicon, Johnson & Johnson, Brussels, Belgium) and the skin was closed with transcutaneously placed non-resorbable nylon sutures (4-0, Ethilon, Ethicon, Johnson & Johnson). The animals were allowed free postoperative movement, with food and water ad libitum.

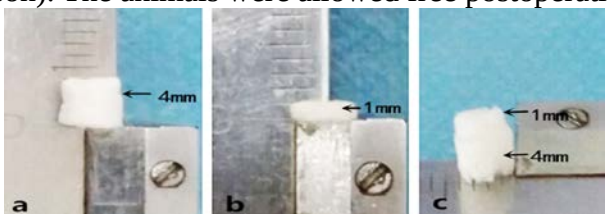


Fig. 1

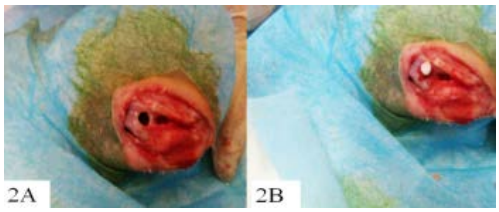


Fig. 2

2.5 Histological Analysis.

The eighth and sixteenth week period of implanted PLGA stent were harvested from five rabbits per group and tissues were washed with PBS and fixed with 10% neutral buffered formalin. They were then embedded in paraffin and 7µm thick sections were cut. The sections were stained with toluidine blue, hematoxilin and eosin, each sample for 5 slices for analysis of newly formed cartilage (Figure 4).

2.6 RT-qPCR Analysis.

Chondrogenesis gene expression of Sox-9, type II collagen, and aggrecan in the eighth and sixteenth week period were analyzed using quantitative real time PCR, respectively, the total RNA was extracted following the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Table 1 summaries the gene primer sequences with 18S rRNA as reference forward primer.

Table 1: Primer sequence for RT-qPCR analysis

mRNA	Oligonucleotide (5'---3')	Product size (bp)	Annealing TM (Cycle)
SOX-9	Fw: GTACCCGCACCTGCACAAC Rv: TCCGCCTCCTCCACGAAG	100	58° C (32)
Collagen II	Fw: GACTGCCTGAGCCCCGAGAT Rv: CTGCCCCTTTGGTCCTGGTTTC	95	58° C (32)
Aggrecan	Fw: ATGGCTTCCACCAGTGCG Rv: CGGATGCCGTAGGTTCTCA	127	58° C (32)
18S rRNA	Fw: GACGGACCAGAGCGAAAGC Rv: CGCCAGTCGGCATCGTTTATG	119	60° C (40)

2.7 Statistical Analysis.

Statistical comparisons were carried out using the SPSS 18.0.1 software package. Comparisons were made using Tukey-Kramer analysis of variance (ANOVA) and results were considered significant at $P<0.05$.

3. Results

3.1 BMSCs Culture and BMP-4 Expression.

BMSCs were cultured for cell transfection and osteogensis induction at the third passage of culture cells. Cells were collected by Trypsin/EDTA treatment. Then washed with serum-free DMEM and once again resuspended in serum-free DMEM at 1×10^6 cell/ml for cell seeding density (Fig 3A1). BMSCs were transfected with pcDNA3.1-BMP-4 plasmid for 48 h and observed under fluorescent microscopy for GFP expression (Fig 3A2), which shows that pcDNA3.1-BMP-4 was transfected successfully.

In order to further confirm BMP-4 expression, we performed RT-qPCR to detect BMP-4 mRNA.

The result shows that BMP-4 mRNA expression in transfected cells was much more significant than that of non-transfected cells (Fig 3B).

Furthermore, BMP-4 protein levels were detected by Western blot. The result was consistent with the BMP-4 mRNA expression in BMSCs. Obviously, BMP-4 expressed in pcDNA3.1-BMP-4 transfected cells was much more than that non-transfected cells (Fig 3C). Decrease in BMP-4 gene expression at 8 and 16 weeks, might be of osteogenic maturation of MSCs as reported by Xu, et al. [24].

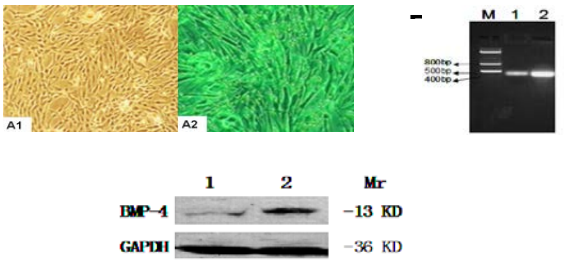
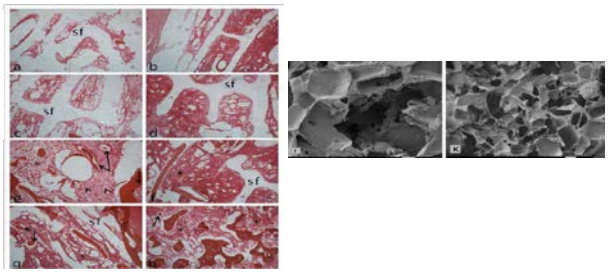


Fig. 3



A B

Fig. 4

3.2 HE Staining and SEM of Engineered PLGA Scaffold.

To analyze the implant while preserving to capture the degree of absorption of the PLGA and its newly developed vasculature, and set its biological activity, the harvested samples were stained in He staining and observed with scanning electron microscopy (SEM). He staining shows that MSCs proliferated to the hybrid scaffold and showed uniform distribution. The cells adhered and produced osteoids more obvious in the transfected scaffold as a result of the degradation of the scaffold (Fig 4A). These results indicate that the PLGA/BMSCs/BMP4 supported cell adhesion and proliferation from the BMP4 induction, less neucrotic cells and dead tissues due to the osteoclast activity. The morphology of the two sets of PLGA was observed with SEM (Fig 4B).

In brief the gross observation in this study recorded no infection, no rejection nor deplacement of any transplant in any group. All animals were fed properly and cleaned duly.

3.3 Appearance of Articular Cartilage Repair at the Implant Surface.

8th week: in PLGA/BMP-4/BMSCs group was observed large number of clustered new translucent cartilage and chondrocytes cells. PLGA scaffold was fibrine like in appearance. In PLGA/BMSCs group, 1 month, visible defect sites by fibrinoid degeneration structured PLGA supporting frame and the bracket in neonatal granulation tissue filled, degeneration of PLGA and the surrounding cartilage boundaries significantly. The PLGA group showed no degradation in the scaffold, subchondral bone defect areas showed neonatal granulation tissue and a small amount of chondrocytes. The blank group support does not see degradation, and embedded fibroblasts or undifferentiated cells (Fig 5A1, A2, A3, A4).

16th week: PLGA/BMP-4/BMSCs group defect repair tissue without PLGA residual, visible columnar arrangement of hyaline cartilage and a large number of filled with the number of chondrocytes of cartilage lacuna, defect new tissue and surrounding tissue closely; PLGA residual PLGA/BMSCs group defect, the defect area filled with granulation tissue, under the microscope, the small number of irregular arrangement of cartilage cells and capillaries, the defect area, the new organization and surrounding tissue with fair (Fig 5B1, B2, B3, B4).

3.4 Scanning Electron Micrograph Observation.

SEM shows that PLGA group defect site, a small amount of PLGA residual, defect area filled with density uneven structure loose fibrous tissue, defect cartilage bone have small amounts of cartilage cell aggregation, the boundaries of the bone defect area and surrounding tissues and cartilage was apparent. The blank group showed a large amount of residual PLGA, defect area of a large number of new elastic fibrous tissue filling, no new cartilage cells under the microscope, as

shown in figure 6.

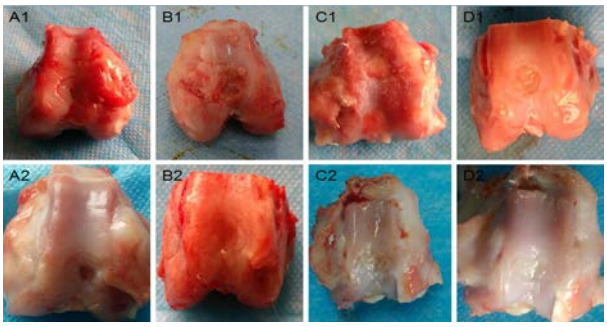


Fig. 5

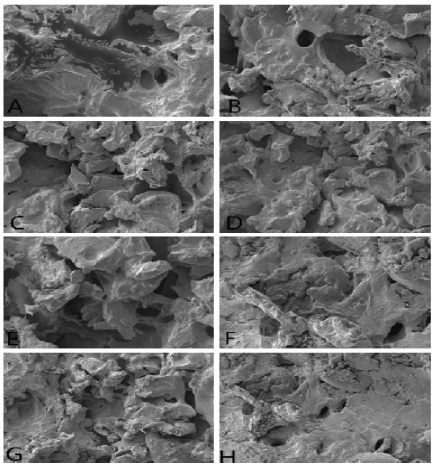


Fig. 6

The PLGA biodegradability and compatibility were perfect in all groups but more dense and homogenous secreting extracellular matrix to fill the pores in the scaffolds and to form compact constructs after the 4 weeks implantation. There was no difference among all of the PLGA groups regarding the HE staining results except the quantity of necrotic cells.

3.5 Analysis of SOX-9, Collagen II and Aggrecan by RT-qPCR.

After 8 weeks implantation, the expression of genes encoding SOX9, collagen II and Aggrecan in cells PLGA/BMSCS/BMP4 scaffold was statistically higher than that of other groups. Under the influence of BMP-4, the expression of the cartilage-specific markers collagen II, Aggreccan and Sox9 was upregulated; the secretion of collagen type II was increased. In vivo, the chondrogenesis effect of BMP-4 was also played (Table 2).

BMSCs cultured and transfected with PLGA/BMP4-immobilized PLGA scaffold showed higher expression of genes encoding SOX9, collagen II, and aggreccan compared with MSCs cultured in the collagen-PLGA scaffold treated with simple non transfected PLGA after 8 and 16 weeks implantation (Table 2, 3).

Table 2: Analysis of SOX-9, Collagen II and Aggrecan by RT-qPCR at 8th week (*mean ±SD*)

Groups	N	SOX-9	Collagen II	Aggrecan
Blank control*	10	12.37±0.09	4.32±0.58	3.37±0.45
PLGA**	10	31.57±0.04	6.39±1.12	5.78±1.37
PLGA/BMSCs***	10	41.57±0.04	17.34±0.13	12.16±0.08
PLGA/BMP-4/ BMSCs****	10	56.12±0.01	21.20±0.43	19.77±1.19

NB: comparing SOX-9 in group1 and group 2: * and ** no statistical significance (P > 0.05); but significant between group 2 an group 3: ** and *** P<0.01. Type II Collagen: statistical significance between group1 and group 2: and between group2 and group 3, respectively * and ** P<0.01; ** and ***, P<0.01. Aggrecan: * and **, P<0.01; ** and ***, P<0.01. *** and ****, P<0.01

Table 3 Analysis of SOX-9, Collagen II and Aggrecan by RT-qPCR at 16th week (*mean ±SD*)

Groups	N	SOX-9	Collagen II	Aggrecan
Blank control*	10	14.23±0.16	5.74±0.23	4.89±1.21
PLGA**	10	45.24±0.03	19.78±0.98	13.56 ±0.46
PLGA/BMSCs***	10	63.54±0.09	30.32±0.98	21.22±0.34
PLGA/BMP-4/ BMSCs******	10	80.38 ±0.02	40.56 ±0.76	43.42 ±1.12

NB: comparing SOX-9 in group1 and group 2, no statistical significance (P > 0.05): * and ** ; but significant between group 2 an group 3: ** and *** P<0.01. Type II Collagen: statistical significance between group1 and group 2: and group2 and group 3, respectively * and ** P<0.01; ** and ***, P<0.01. Aggrecan: * and **, P<0.01; ** and ***, P<0.01. *** and ****, P<0.01 all are statistically significant.

4. Discussion

The baseline in treating articular cartilage disease is to regenerate the joint surface with extracellular matrix produced by chondrocytes, which is the specialized cells in producing the cartilage matter [18]. Various methods and or techniques have been developed to achieve this goal.

This study showed that hyaline cartilage regeneration was delicate in the 16th week and is in line with the in-vivo findings at 12 weeks of Junjun et al. [19] where MSCs were induced by BMP-4 to secrete collagen type II and glycosaminoglycan and differentiate into chondrocytes. MSCs are the mostly used stem cells for tissue engineering as they can differentiate into osteoblasts, chondrocytes, and adipocytes in vivo, and in vitro with high expansive potential [2].

Growth factors are a group of bioactive molecules currently receiving large research attention in the field of tissue regeneration [20]**.** However, most delivery systems involve harsh chemical environments during manufacture which may not pose a problem for small drug molecules but may denature bioactive proteins and thereby reduce the activity of the growth factor [21-23].

This study is in line with previous studies which have shown that BMP-4 promotes chondrocyte differentiation, the extracellular matrix composition of cartilage, collagen II deposition, and cartilage regeneration [20]. Also, to correlate BMP-4 properties with PLGA, we found that [21] is in favor to our study when he stated that PLGA internal erosion mechanisms is in slower release rate and it is characterized by good biocompatibility and without immunoreaction in vivo, and could release drugs by surface erosion.

The spatial locations of periostin and calcitonin receptors were studied in decalcified paraffin-embedded sections of the tissue defect, 7 and 28 days postoperatively. Periostin-positive cells were distributed throughout the regenerated tissue and osteoblast bone-lining cells were strongly stained for periostin. Positive staining for the calcitonin receptor was observed at 7 days, on both mono- and multinucleated cells localized at osteoblast seams on the bony surfaces facing the bone marrow and at the bone-implant interface. Similar patterns were detected 28 days postoperatively.

No qualitative or quantitative histological differences were observed between the implant groups. The HE-stained sections revealed that there is significant differences in cartilage and bone area between PLGA/BMP-4/BMSCs, PLGA and Blank control groups at any time point of the screening. BMP-4 has positive effects as shown by the return of the regular histological features, thus enhancement of expression of BMP-4 in PLGA in the synovial membrane.

5. Conclusion

The treatment of PLGA/BMP-4/BMSCs scaffolds implantation in local area is effective in recovering rabbits full thickness knee articular cartilage with respect to degree of thickness, surface smoothness, faster in time frame, cartilage binding stability and in integration with the surrounding normal cartilage without abrasions or infections in defective areas. The BMSCs/PLGA/BMP4 scaffold could be useful for bone tissue engineering and regeneration. Extended studies to large

animal models and longer duration postoperative follow up is needed prior to clinical application of this promising PLGA/BMP-4/BMSCs scaffolds.

Conflicts of Interest

All authors declare that there is no conflict of interest.

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