British Biotechnology Journal
4(11): 1223-1237, 2014
ISSN: 2231–2927

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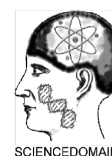

Enhancement of Bone Formation by Bone Morphogenetic Protein-2 Released from Poly (L-lactic-co-glycolic acid) Microsphere

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Authors' contributions

This work was carried out in collaboration between all authors. Author WZ designed the study, wrote the protocol and performed the experiments. Author YL performed the statistical analysis, managed the literature searches and the analyses of the study and wrote the first draft of the manuscript. Authors YL and XH edited and revised manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2014/12949
Editor(s):
(1) Laura Pastorino, Dept. Informatics, Bioengineering, Robotics and Systems Engineering (DIBRIS), University of Genoa, Italy.
Reviewers:
(1) Masoud Mozafari, Bioengineering Research Group, Nanotechnology and Advanced Materials Department, Materials and Energy Research Center (MERC), Tehran, Iran.
(2) Anonymous, Mayo Clinic, USA.
(3) Anonymous, Pontifical Catholic University of São Paulo, Brazil.
(4) Anonymous, Osaka Dental University, Japan.
(5) Anonymous, Jilin University, China.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=688&id=11&aid=6577

Original Research Article

Received 25th July 2014
Accepted 23rd September 2014
Published 22nd October 2014

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ABSTRACT

An appropriate carrier acting as a sustained delivery vehicle for bone morphogenetic proteins (BMPs) is required for the maximal clinical effectiveness of these osteogenic proteins to enhance bone formation. The purpose of this study was to evaluate a low-molecular-weight poly(L-lactic-co-glycolic acid) (PLGA) copolymer as a synthetic, biodegradable carrier for the sustained delivery of bone morphogenetic protein-2 (BMP-2), and then to address the hypothesis that BMP-2 delivery from this vehicle could promote cell proliferation in vitro and ectopic bone formation in vivo. The BMP-2 was entrapped in microspheres of PLGA by using an improved water-in-oil-in water double-emulsion-solvent-extraction technique. The in vitro release kinetics of rhBMP-2 was determined by ELISA. Then we verified the effect of the sustained delivery vehicle on MSC cell proliferation. The ectopic bone induction in intramuscular implants of mice was evaluated at 2 and 4 weeks post-implantation. The results showed the PLGA microsphere released a total of 14.2%±0.71% rhBMP-2 at the initial phase followed by a prolonged release for 28 days. The rhBMP-2 released from the PLGA microsphere stimulated an increase in alkaline phosphatase (ALP) activity of MSC cells for 5 days in vitro, suggesting that the delivery vehicle releases BMP-2 for a prolonged period in an active form. Moreover, the released rhBMP-2 from the PLGA microsphere significantly promoted MSC cells proliferation after days 5 in culture. In vivo bone formation studies showed the rhBMP-2-loaded PLGA microsphere induced ectopic bone formation to a much greater extent than did rhBMP-2 treated mice. These results demonstrated that the PLGA copolymer material is capable of potentiating the osteogenic efficacy of BMP-2 and, as such, represents a promising delivery vehicle for BMP-2 for orthopedic and dental repair.

Keywords: rhBMP-2; PLGA; osteogenesis; bone formation.

1. INTRODUCTION

For the hundreds of thousands of cases of bone defect that occur annually and that result in impaired, improper or no healing of the bone[1], engineered biomaterials combined with growth factors or cells have emerged as an important new treatment alternative in bone repair and regeneration. Existing treatment modalities, including autogenous, allogenic and synthetic bone grafts with autograft being the clinically preferred choice of bone grafting material [2-5], all have limitations. Not only is the supply of autograft tissue physically limited, but disease transfer and histo-incompatibilities are also very likely to occur in the case of allografts [6,7]. As an alternative, therefore, a number of different growth factors with important roles in skeletal repair have been isolated, and the advances in recombinant DNA technology can make possible their large scale production for research and therapeutic purposes [8]. In particular, the bone morphogenetic protein (BMP) family is a kind of well used growth factor that plays a crucial role in bone formation and repair [9,10].

BMP regulates cell growth and the differentiation of a variety of cell types including osteoblasts and chondrocytes [9,11,12]. In vivo, BMPs improve bone regeneration; in fact, BMP-2 has been found to induce replacement for repairing segmental bone defects [13,14]. So far, studies have resulted in two commercially available BMP-based products, BMP-2 and BMP-7, for orthopedic indications [15,16].

Although BMPs can be potent osteogenic growth factors, their application in orthopedic indications is complicated by their short biological half-lives, rapid local clearance and

localized actions [17]. Studies have proved that a single exposure to an exogenous BMP-2 may not be sufficient to stimulate and sustain bone growth in vivo [18]. To overcome these problems, effective BMP treatments in bone defects can be obtained by incorporating BMP into a biomaterial for its local sustained delivery at the target site. The delivery vehicle should not only possess adequate biodegradation rate, mechanical strength and morphological structural but also maintain a local BMP concentration within the therapeutic window for a sufficient period of time to guide and control cell attachment, migration, proliferation and differentiation [19]. Micro-particles made of biodegradable polymers have been widely utilized as vehicles for drug delivery. They can be loaded at an afflicted site during surgery or injected as a suspension to a wound area, or, alternatively, micro-particles can be impregnated into polymer scaffolds that can be used in repairing bone defect to provide bony bridging [20]. Among different polymers, poly (DL-lactic-*co*-glycolic acid) (PLGA) copolymers are some of the most prospective matrices studied as micro-particle carriers for many kinds of bioactive molecules [21]. PLGA copolymers are biocompatible, biodegradable, and approved for human clinical use, [22] and they possess good mechanical property, low immunogenicity, nontoxicity and an adjustable degradation rate [23]. Kim et al. fabricated the polycaprolactone/PLGA scaffolds conjugating with a heparin-dopamine to control the release of BMP-2 [24]. They found that this scaffolds enhanced osteoblast activity in vitro and promoted new bone formation in vivo. In order to optimize the tissue engineering scaffolds, Nazemi et al. fabricated chitosan/58S-bioactive glass scaffold incorporated with PLGA nanoparticles which were homogenously distributed in the scaffolds [25]. In addition, Lee et al developed a 3D scaffold embedding with PLGA-loaded with BMP-2 microspheres by solid free-form technology [26]. They found that the 3D scaffold could continually release BMP-2 which regulated pre-osteoblasts differentiation in vitro and promoted bone formation in vivo. However, the methods used in fabricating the above scaffolds are complicated which are difficult to apply these methods for clinical bone tissue engineering.

The purpose of this study was to deliver BMP-2 via a controlled release vehicle and to investigate in vitro whether it is possible to improve osteogenic activity of canine bone marrow MSC and, in vivo, whether it can promote the osteogenesis by intramuscular implantation in mice. Using an improved simple water-in-oil-in water (W1-O-W2) double-emulsion-solvent-extraction technique, the BMP-2 was entrapped in microspheres of PLGA. The kinetics of BMP-2 release from the microspheres was studied by enzyme-linked immunosorbent assay (ELISA). The proliferative and differentiative effects of BMP-2 were determined by MTT and alkaline phosphatase assays, respectively.

2. MATERIALS AND METHODS

2.1 Materials

The PLGA used in this study had a 50:50 lactic to glycolic acid copolymer ratio (Medisorbt, Alkermes, Cincinnati, OH). The polymer weight average molecular weight (Mw) was measured by gel permeation chromatography as 30,000. Poly (vinyl alcohol) (PVA), 88% mole hydrolyzed, with an Mw of 25,000, was supplied by Aldrich. Recombinant human BMP-2 (rhBMP-2) was supplied by Medtronic Sofamor Danek (MN). All other reagents were of analytical grade. Distilled deionized water was utilized in all studies.

2.2 Microparticle Preparation

Using the previously described water-in-oil-in water (W1-O-W2) double-emulsion-solvent-extraction technique, the BMP-2-loaded PLGA microspheres were fabricated [14,27]. Briefly,

100ml of a 10mg/ml BMP-2 solution was emulsified in a solution of 500 mg of PLGA in 1.25ml of dichloromethane. The mixture was re-emulsified for 30s in 2ml of 1% w/v aqueous PVA solution to create the double emulsion. The content was then added to 100ml of a 0.3% w/v aqueous PVA solution and 100ml of a 2% w/v aqueous isopropanol solution and stirred for 1h. The microspheres were collected by centrifugation, washed twice with double distilled water (ddH_2O) , vacuum dried into a free flowing powder and stored at -80°C before use.

2.3 Microparticle Characterization

The size distribution of the microparticles was measured with a Coulter counter multisizer (model 0646, Coulter Electronics, Hialeah, FL) after suspending the microparticles in an Isoton II solution (Coulter Electronics). The microparticle diameter is reported in the diameter range. Using an established solvent-extraction technique, the entrapment efficiency of the proteins was determined by normalizing the amount actually entrapped with the starting amount [28].

2.4 In vitro Release Kinetics of rhBMP-2

In vitro Release Kinetics of rhBMP-2 was allowed to release from the 10mg of rhBMP-2 loaded microspheres at 37°C into a phosphate buffered saline (PBS) solution with a pH 7.4. At 2h，8h，24h，2d，4d，7d，14d，21d，and 28d, 1ml of conditioned medium was sampled, and an equal volume of fresh release medium was added back to maintain the total volume. Samples were then immediately frozen at -20°C until measurement. Each release condition was performed in triplicate.

2.5 RhBMP-2 Measurement with Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

The amount of rhBMP released was quantified by a sandwich ELISA using an assay from a commercially available kit (R&D Systems). Briefly, 100μL of the sample was added into the designated ELISA wells and incubated at 4°C overnight. After the incubation, the plate was washed four times with a wash buffer. Biotin antibody was then added into each well, and the plate was incubated at room temperature for 1h. The plate was washed, and 100μL of Streptavidin solution was added and incubated for 45 min at room temperature. The substrate was added to the wells and the absorbance was measured at 450nm. The total accumulated release of rhBMP-2 was calculated by integration of the individual measurements over the cumulative time of the experiment.

2.6 Isolation and Culture of Mesenchymal Stem Cells (MSC) from Bone Marrow

Bone marrow MSCs were isolated by density-gradient centrifugation with Ficollpaque (Amersham Bioscience, No. 17-1440-02) from bone marrow obtained from the femoral diaphysis of canines as described previously [29,30]. Briefly, samples were diluted 1:2 with Hank's and centrifuged at 400×g for 30 min to obtain mononuclear cells. The supernatant was discarded, and the middle layer was washed three times in Hank's. Cells were cultured in complete cell culture medium (DMEM/Hams-F12 1:1, 15% FCS, 75μg/mL ascorbic acid, 1% essential amino acids and 1% Glutamine) and incubated at 37°C in a humidified 5% CO_2 atmosphere. At day 4 of culture, non-adherent cells were removed, and adherent cells were incubated in a medium for 3 days before initiation of the experiments.

2.7 MTT Assay

The cell proliferation was evaluated during 12 days via 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction based on a cell growth determination protocol [12]. Cells were seeded in 96-well plates with a cell density of 1×10^4 cells/cm^2 for at least 16 hours, after which the culture medium was replaced with serum-free DMEM. Cells were then incubated with rhBMP-2 (100ng/ml) or rhBMP-2/PLGA for 1, 3, 5 days. After that, 20μL MTT stock solution (5mg/mL) was added to each well, followed by incubation at 37°C for 4 hours to MTT formazan formation. The upper medium was removed and the converted dye was solubilized with acidic anhydrous isopropanol (0.1N HCl in absolute isopropanol). The absorbance of produced formazan was measured at 570nm with a microplate reader (ZS-2, Beijing).

2.8 Alkaline Phosphatase (ALP) Activity

The MSCs were seeded on the 24-well plate and cultured with osteogenic media in the presence or absence of microparticles. The levels of ALP activity were determined on day 1, 3 and 5. Cells were rinsed twice with PBS, and 1.5ml of 0.1% Triton X-100 was added to each well. After the MSCs were frozen and then thawed repeatedly for four times, the solution was collected to measure the ALP activity that was determined at 405nm using p-nitrophenyl phosphate (sigma, USA) as the substrate [31]; protein concentration was measured by Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories) and expressed as units per mg of protein.

2.9 ALP Staining

After 7 days culture of MSCs, ALP staining was performed by a standard procedure according to the manufacturer's instructions (Sigma). The MSCs were washed three times with PBS, and fixed in 4% paraformaldehyde (PFA) for 15min. Cells were then incubated with naphthol-ASBI phosphate (N-ASBI-P) for 20min. After immersing into acetic acid for 1 min, cells were washed and cover slipped with glycogelatin. The cells staining positive for ALP were viewed with a light microscope.

2.10 Animals and Surgical Procedure

According to an approved protocol by local animal care and use committee, 24 male 8-week-old KunMing mice (weight 20-24g) were used for the experiment. Prior to surgery, the mice were anesthetized with pentobarbital (50mg/kg). After shaving and disinfecting, small skin incisions were made in the proximal part of each thigh. At each site, a subcutaneous pocket was created and filled with one implant according to a randomized scheme. One pocket was filled with rhBMP-2 (20mg/ml) or rhBMP-2/PLGA (20mg/ml) and the other pockets with negative controls (containing only PLGA). The animals were sacrificed after 2 and 4 weeks to evaluate bone formation using histology.

2.11 Histologic Examination

Each excised specimen with surrounding muscle was fixed in 10% formalin and decalcified in 15% EDTA. After the fixed and decalcified specimen was embedded in paraffin, four-micrometer sections were cut and stained with hematoxylin and eosin for light microscopic observation.

2.12 Statistical Analysis

Data were analyzed using standard statistical methods and ANOVA followed by Fisher's PLSD and Bonferroni post hoc tests where appropriate. Group data are expressed as means ± SD. Values of all parameters were considered significantly different at a value of $P<0.05$.

3. RESULTS

3.1 Initial Microparticle Characterization

PLGA microparticles were fabricated using the double-emulsion, solvent-extraction technique. The microparticles were observed via scanning electron micrograph, confirming their spherical shapes and sizes. The low and high molecular weight of PLGA microparticles had a diameter range of 110-140μm and 300-340μm, respectively and the average size of microparticles was 242.2±31.3μm (Fig. 1). The initial microparticles were spherical with smooth and nonporous surfaces according to the scanning electron micrograph analysis (Fig. 2). The entrapment efficiency of rhBMP-2 in the microparticles was 64.52±4.31%, and the load density of rhBMP-2 was 2.12±0.15%.

3.2 *In vitro*rhBMP-2 release Kinetics

The in vitro rhBMP-2 release profiles of the PLGA microspheres were studied in a cell culture model. The microspheres showed a typical initial burst release followed by a sustained S-shaped release profile for the rest of the in vitro study (Fig. 3). The release profiles were partitioned into four phase in accordance with previous investigation [32,33]. A burst release at 24h (phase 1) was observed with a released total of 14.2%±0.71%. Then the moderate release was followed, and the release rates for phases 2 (days 1-4), 3 (days 4-14) and 4 (days 14-28) were calculated. The microspheres released a total of 44.6%±3.5% at the end of 28 days.

3.3 Effect of Immobilized rhBMP-2 on MSC Cell Proliferation

Proliferation of MSC cells were determined by MTT assay after cultured for 1, 3 and 5 days, as shown in Fig. 4. After 1, 3 and 5 days of culture, the proliferation of cells on the rhBMP-2 (100ng/ml) and rhBMP-2/PLGA (100 ng/ml) groups were significantly higher than that on the PLGA group (Fig. 4A). Moreover, there was significant difference in proliferation between the rhBMP-2 group and rhBMP-2/PLGA group ($p<0.05$) after days 5 in culture, indicating rhBMP-2/PLGA treatment prolonged the life span of rhBMP-2 and enhanced the effect of rhBMP-2 on the proliferation of cells. We also detected the effects of different concentrations of rhBMP-2 and rhBMP-2/PLGA on the cell proliferation, and it could be seen that the cell proliferation under the concentration of 100ng/ml and 200ng/ml was almost equal (Fig. 4A&C). However, further increasing rhBMP-2 immobilized on PLGA microspheres to 400ng/ml induced a clear difference in cell proliferation between the rhBMP-2 group and rhBMP-2/PLGA group (Fig. 4C).

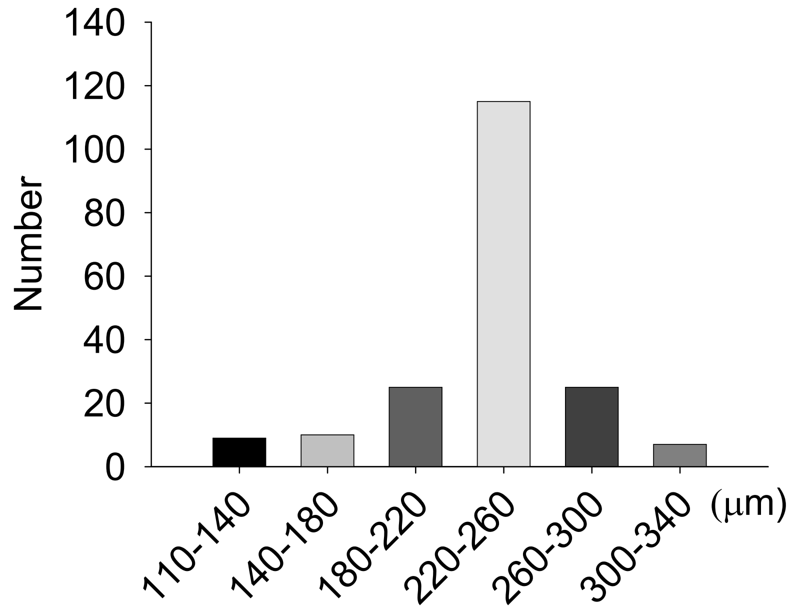

Fig. 1. The size distribution of the microparticles. The main size of microparticles is 220-260μm

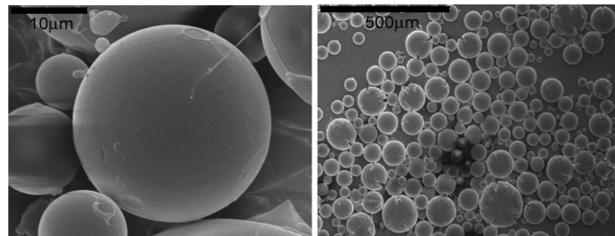

Fig. 2. Scanning electron micrographs PLGA microparticles. The initial microparticles were Spherical with smooth, nonporous surfaces

3.4 Effect of Immobilized rhBMP-2 on MSC Cell Differentiation

The differentiated function of MSC cells on rhBMP-2/PLGA microsphere was evaluated by determining the ALP activity as shown in Fig. 5. It could be seen that ALP was expressed at low levels in the PLGA-treated group, while rhBMP-2 or rhBMP-2/PLGA treatment significantly increased the ALP activity compared with PLGA treatment after days 1, 3 and 5 of culture (Fig. 5A). Moreover, after days 5 of culture, rhBMP-2/PLGA notably enhanced the activity of ALP compared with the rhBMP-2 treatment, suggesting rhBMP-2 immobilized on the PLGA microsphere stimulated the expression of ALP and enhanced ALP activity. We also detected the expression of ALP by histochemical staining. As shown in Fig. 5B, rhBMP-2/PLGA significantly increased the expression of ALP in MSC cells after treatment for 7 days.

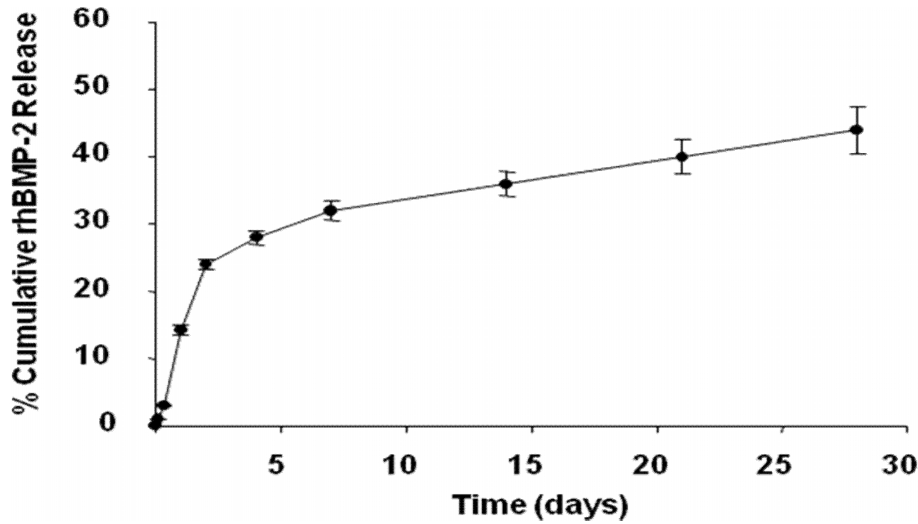

Fig. 3. The cumulative release kinetics of rhBMP-2 from PLGA microparticles. Error bars represents the mean ± SD for n=4

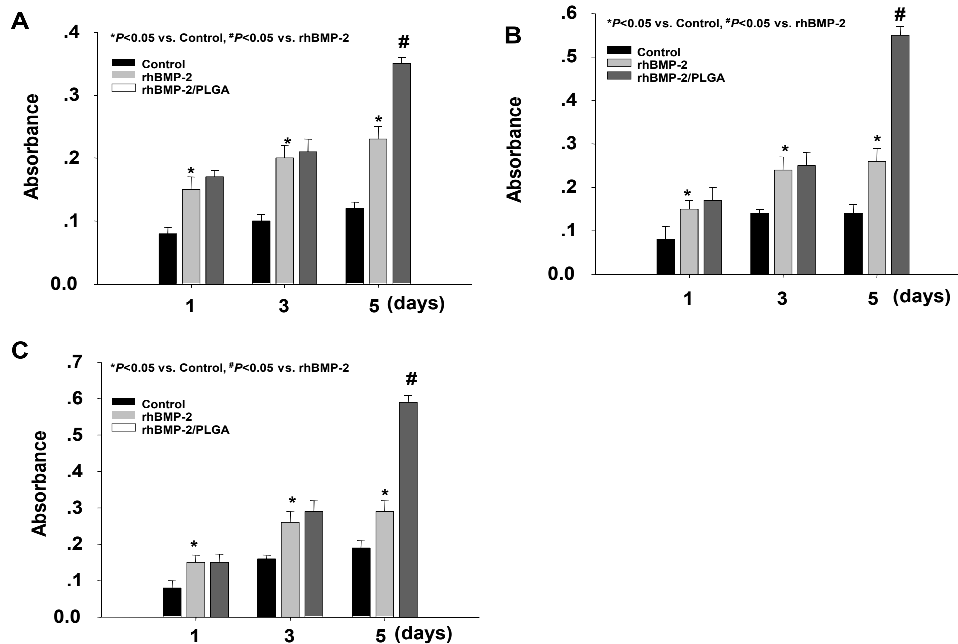

Fig. 4. MTT-tetrazolium assay of MSC cells cultured on various PLGA microparticles within different Period. Values are expressed as means ± SD (n=4)

3.5 Effect of Immobilized rhBMP-2 on Bone Formation *In vivo*

The histological analysis of the intramuscular implants of PLGA, rhBMP-2 and rhBMP-2/PLGA at 2 and 4 weeks post-surgery was undertaken with H&E staining, and the results showed that there was no bone formation in PLGA implants, while rhBMP-2 implants

promoted the chondrogenesis at 2 weeks post-surgery (Fig. 6A). Compared with the rhBMP-2 implants, the rhBMP-2/PLGA implants further increased the chondrogenesis, which was characterized as diffusely scattered calcified cartilage as shown in Fig. 6A. At 4 weeks after implantation, rhBMP-2/PLGA implants promoted the ectopic bone formation as shown by the formation of the trabecular bone and marrow cavities compared with that found in rhBMP-2 implants (Fig. 6B), indicating that, compared to rhBMP-2, biodegradable rhBMP-2/PLGA is a better favorable treatment for bone formation.

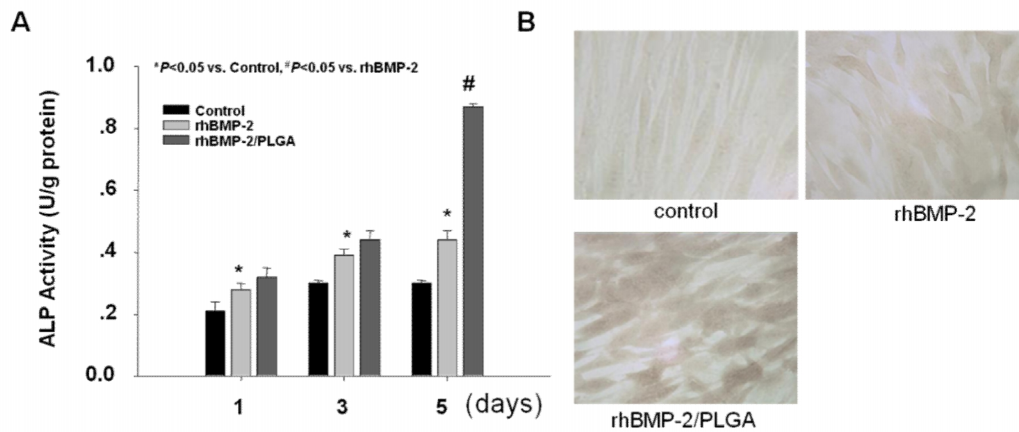

Fig. 5. A ALP activity assay of MSC cells cultured on various PLGA microparticles after cultured for different period. ALP activity was determined as the enzyme activity unit (U) per gram of protein. Values are expresser as means ±SD(n=4).B, ALP staining after 7 days cell culture of MSCs

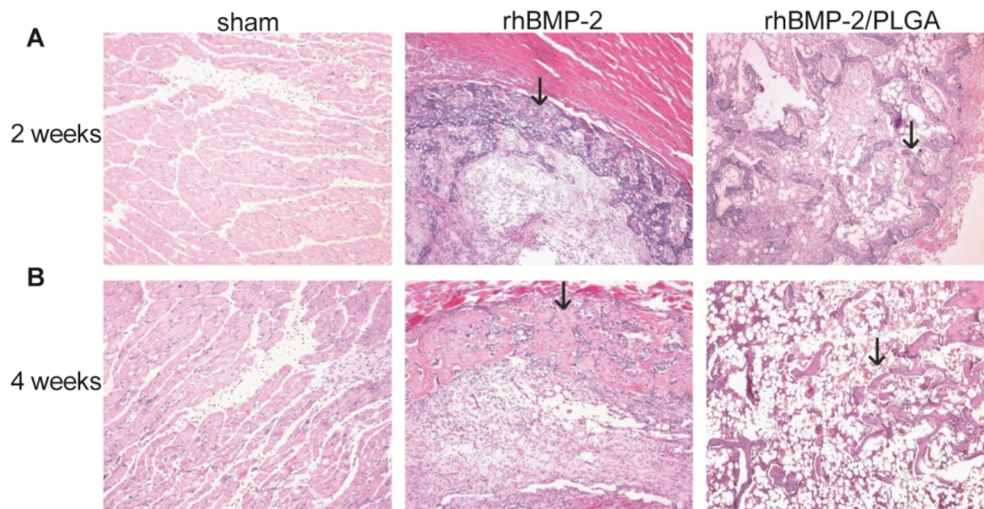

Fig. 6. Bone formation after 2 (A) and (B) weeks of implantation by HEstained historical evaluation in a rat subcutaneus model. rhBMP- 2/PLGA treatment promote thee subcutaneous bone formation. Arrow indicated THE formation of cartilage (A) or bone (B)

4. DISCUSSION

This study clearly demonstrated that biologically active rhBMP-2 can be released from sustained delivery microspheres over a prolonged period of time. The delivery microspheres, based on PLGA, exhibited an in vitro release of biologically active rhBMP-2 for at least 21 days. rhBMP-2/PLGA microspheres significantly promoted MSCs proliferation in a dose-dependent manner. Moreover, rhBMP-2/PLGA treatment time-dependently enhanced ALP activity in cultured MSCs. In vivo, rhBMP-2/PLGA administration markedly induced bone formation after 2 weeks and 4 weeks of intramuscular implantation.

BMP-2 has strong bone-inductive activity and is being evaluated as a bone growth inducer for dental and orthopedic indications. However, the short half-life and instability of BMP-2 require the administration of physiologically high doses or multiple dosages of the protein [34,35], which may cause adverse effects [36] and be costly. Therefore, a controlled and localized delivery system for a planned amount of BMP-2 would be appropriate for effective bone regeneration, given that in humans a large dose of BMPs is required [34]. A number of systems have been designed and evaluated for the delivery of BMP-2, such as porous hydroxyapatite [37,38], absorbable collagen [39,40], PLGA [26,41] and demineralized bone powder and bovine collagen type sponges [42]. Of these, PLGA is preferentially used as it offers many advantages over biological materials, such as biocompatibility, minimal immunogenicity, biodegradability and the ability to be manufactured with high reproducibility. In this study, we loaded rhBMP-2 into PLGA microspheres. SEM showed that the shapes of the macropores from PLGA microspheres were round and neighbored each other. The average sizes of the PLGA microspheres were 242.2±31.3um. Evidence showed that pores of this size are optimal for bone formation [43,44]. Moreover, the size of interconnection is the main limiting factor for vascularization and bony ingrowth rather than the size of the pores themselves [45].

An efficacious delivery system is still needed to localize BMP-2 at the defect site for prolonged periods of time. In vitro, the longer cells were exposed to BMP-2, the more fully they expressed and sustained osteoblastic traits [46]. As the same, in vivo, the longer release duration of bioactive BMP-2 resulted in faster and more complete bone healing[14, 47-49]. Byung et al. developed a sustained delivery system for rhBMP-2 that can prolong release of rhBMP-2 for 21 days and an immediate release implant that only released rhBMP-2 for 7 days. Results showed the sustained delivery of BMP-2 based on the PLGA microsphere system enhanced bone growth more effectively than immediate release within bone defects [50]. Consistent with the results, Oju et al proved that long-term delivery of BMP-2 enhances in vivo osteogenic efficacy of the protein compared to short-term delivery at an equivalent dose [47]. Also, Diederik et al. showed that biologically active BMP-2 can be released over a prolonged period of time from sustained delivery vehicles that exhibit an in vitro release of biologically active BMP-2 for at least 42-84 days [14]. In vivo subcutaneous implantation showed bone formation after 42 days of surgery. In the present study, we fabricated the PLGA microsphere to deliver rhBMP-2. rhBMP-2 was released in a multiphasic fashion; a typical initial burst release at 24h was followed by a sustained S-shaped release profile for 28 days. At the end of 28 days, the microspheres released a total of 44.6%±3.5%. The release of rhBMP-2 from a PLGA microsphere was found to significantly enhance the ALP activity in MSCs after culturing for 5 days compared with that in rhBMP-2 treatment group, but there was no difference on the day 1 and day 3 release in medium, suggesting that rhBMP-2 retained its activity during the microspheres fabrication process and could be released not only in a controlled fashion but also in a bioactive form. Moreover, there was significant difference in MSC cells proliferation between the rhBMP-2

group and rhBMP-2/PLGA group after day 5 of culture, suggesting rhBMP-2/PLGA microspheres prolonged the life span of bioactive rhBMP-2. Indeed, in vivo histological analysis demonstrated a significant increase in bone formation in the rhBMP-2/PLGA treated group after 2 and 4 weeks of intramuscular implants compared with that found in the rhBMP-2 treated group. Moreover, the fabrication is easy, so it has potential application in clinical bone tissue engineering.

Although rhBMP-2 possesses strong osteoinductive actions and can induce ectopic bone formation at subcutaneous, intramuscular and intra-fatty sites [51], no study has reported on the suitable dose of rhBMP-2-carrying products as material for bone repair. As known, exogenously delivered BMPs turn over rapidly in acute wounds, necessitating the delivery of high doses [35]. In a previous study, it appeared that a dose of 50mg of rhBMP-2 could induce ectopic bone formation in intramuscular sites in rats [52]. Generally speaking, physiologically high doses of BMPs are required for human applications compared to animal applications. A commercialized rhBMP-2 graft (INFUSE, Medtronic Sofamor Danek, Minneapolis, MN) has been applied for spine fusion that possesses a burst release within 1 h of implantation without any controlled releasing property. The concentration of rhBMP-2 is 1.5mg/ml with a half-life of only 7-16 min. Shields et al. reported that a significant rate of complications resulted after the use of a high dose of INFUSE in anterior cervical fusions [53]. Therefore, a controlled and localized delivery system for a small amount of rhBMP-2 would be appropriate for effective ectopic bone regeneration. In the present study, comparison of the cell proliferation in the three different rhBMP-2 concentration conditions with the control showed the dose of above 100ng/ml rhBMP-2 had the obvious effect. In vivo, a therapeutic dose of 20mg/ml, which was 50-fold lower than that applied in the clinical trials, induced extensive bone formation. Therefore, this study demonstrated that long-term delivery of rhBMP-2 by PLGA microsphere enhanced the osteogenic efficacy or rhBMP-2 in intramuscular implants. However, additional studies are still needed to determine the threshold dose of rhBMP-2, and whether the PLGA microsphere delivery vehicle would be efficacious in large animal orthotopic bone repair models and clinical trials so as to develop more effective BMP-2 therapy with fewer complications.

5. CONCLUSION

We designed a delivery system using a PLGA microsphere to sustain rhBMP-2 release for at least 28 days, and the rhBMP-2 released from the delivery vehicle retained its biological activity and significantly promoted MSC cells proliferation. The extended rhBMP-2/PLGA delivery showed a much more extensive ectopic bone formation than rhBMP-2 administration alone in intramuscular implants, suggesting that the sustained and controlled delivery vehicle for rhBMP-2 is appropriate for effective bone formation and may become a useful modality for bone regeneration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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