Critical assessment of rhBMP-2 mediated bone induction: An in vitro and in vivo evaluation

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Understanding the influence of formulation and storage conditions on rhBMP-2 bioactivity is extremely important for its clinical application. Reports in the literature show that different research groups employ different parameters such as formulation conditions, storage, doses for in vivo applications etc. that makes it difficult to correlate results from different experiments. We therefore decided to rationalize these anomalies by performing a basic study on such parameters using two commercially available BMPs. Our in vitro experiments suggest that BMPs from different sources have significant differences in their bioactivity. The clinically approved rhBMP-2 (InductOs®; BMP-P) showed superior stability, compared to rhBMP-2 from R&D Systems (BMP-R) at physiological pH (determined by ALP assay). This BMP-P also showed lower binding to polypropylene Eppendorf tube. The BMP-R almost lost its bioactivity within 30 min at physiological pH and also shows more adhesion to plastic surfaces. This aggregation behavior was unequivocally ascertained by performing light scattering studies of the two BMPs, which revealed linear aggregation with time for BMP-P unlike BMP-R. The in vitro results were also reflected in the in vivo experiments, in a rat ectopic model with injectable hyaluronic acid (HA) hydrogel as BMP carrier. After 7 weeks post-implantation we observed larger bone volume with oriented collagen in the BMP-P group but a smaller bone with disoriented collagen in the BMP-R case. Our results highlight the large difference in activity between seemingly identical substances and also the importance of proper handling of such sensitive proteins.

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1. Introduction

Bone fracture is a common event that affects both young and old individuals. Our own innate bone regeneration potential heals most of the fracture; however, larger defects could not be repaired by this process. Current methods to treat patients with large bone defects include autologous bone transplantation surgery with donor tissue obtained from another site such as the iliac crest of the same patient [1], or artificial fixation of bone in case of lack of available donor tissue [2]. Both of these procedures are painful, while the bone grafting often causes donor site morbidity and increases the risk of infection. Moreover, the overall success is also dependent on the quality of transplanted bone from the patient [3].

One of the alternative procedures involves bone regeneration utilizing growth factors (proteins) such as bone morphogenetic proteins (BMPs) that promotes differentiation of endogenous stem cells towards bone phenotype, mimicking the natural bone healing process. This method requires development of technologies to deliver such sensitive proteins at the defect site with minimal invasive procedures. BMPs were originally identified as key proteins required for skeletal development, maintenance of adult bone homeostasis and are known to play an important role in fracture healing [4–7]. BMPs are expressed in an inactive pro-form, and proteolytic cleavage by proteases releases the mature BMP proteins [8].

Several groups including ours are working on designing efficient delivery vehicles for rhBMP-2, due to its short half-life in vivo. BMP-P is approved for clinical use by the FDA and the European Medicines Agency (EMEA) for specific clinical assessment in spinal fusion and fracture healing [9]. A recent review on clinical use of rhBMP-2 shows an increasing concern over the safety of this protein due to serious complications observed in some cases [10]. It is due to the fact that rhBMP-2 is not functionally specific for osteoblasts and its high dose often resulted in soft tissue inflammation, native bone resorption and implant fracture. Thus finding ways to reduce the dose of BMP-2 in clinical practice will be of great significance. In order to find such methods to improve BMP-2 function, in vivo, we need to understand proper handling conditions for this protein. A close survey of the published literature related to BMP-2 research reveals that several groups employ different parameters in their experiments...
such as different doses of protein, different reconstitution solutions, temperature, etc. as instructed by the manufacturer. These differences can have profound effect in protein bioactivity, which limits direct comparison of results from different experiments. We therefore decided to test how crucial such handling conditions are for in vitro and in vivo experiments. We performed basic study with clinically approved BMP-P and for comparison we tested commercially available BMP-R. Both of these BMPs were obtained from genetically modified Chinese hamster ovary (CHO) cells.

We have recently reported injectable hydrazone crosslinked HA hydrogel as an efficient rhBMP-2 delivery vehicle that can promote bone regeneration at ectopic [11] and orthotopic sites [12,13] without adding any cells to the scaffold. HA is a natural extracellular matrix (ECM) glycosaminoglycan that is known to have diverse biological function which include proliferation and differentiation of stem cell, wound healing etc. [14] but can also retain BMP-2 and increase its stability in vivo [15]. These systems also recruit endogenous stem cells from the surrounding tissue and support BMP-2 mediated osteogenic differentiation [15].

In this article, we showed for the first time that the stability and bioactivity of BMPs from different commercial sources possess profound differences. We found that BMP-P has a higher stability at physiological pH and has a lower adhesive property towards polypropylene Eppendorf tube as compared to the BMP-R. The higher stability of BMP-P was also reflected in the in vivo experiments, which showed larger however less compact bone formation with BMP-P whereas smaller and compact bone, in the case of BMP-R. There were also visible differences in collagen orientation between two groups with BMP-P-induced bone to have an ordered structure similar to the native bone while the BMP-R group had a disordered collagen orientation.

2. Materials and methods

Hyaluronic acid (HA, 150 kDa) was purchased from Lifecore Biomedical, LLC (Chaska, MN). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Dialysis membranes Spectra/Por 6 (3500 g/mol cut off) were purchased from Spectrumlabs Europe. The NMR experiments (δ scale; J values are in Hz) were carried out on a Jeol JNM-ECP Series FT NMR system at a magnetic field strength of 9.4 T, operating at 400 MHz for 1H.

2.1. Synthesis of hydrazide and aldehyde modified hyaluronic acid

For hydrogel preparation we have developed a new hydrazide derivative of HA using commercially available dihydrazide, namely carboxyhydrazide (CDH). The synthetic procedure were as follows: 400 mg HA (1 mmol of disaccharide repeating units) was dissolved in 50 ml de-ionized water, followed by 90 mg (1 mmol) of CDH linker. N-hydroxybenzotriazole (HOBt; 153 mg, 1 mmol) was dissolved separately in a 2 ml solution of 1:1 (v/v) mixture of acetonitrile–water and was added to the HA solution. The pH of the resultant solution was adjusted to 4.7 and to this solution 19.17 mg (0.1 mmol) of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) was added and stirred overnight. The solution was transferred to a dialysis tube (Mw cutoff 3500) and dialyzed first against dilute HCl (3.5 pH) containing 0.1 M NaCl for 48 h, and then against dilute HCl (3.5 pH) for 24 h. The solution was lyophilized to get 360 mg of hydrazide derivative of HA (HA hydrazide). The degree of modification was 7%, as determined by TNBS (2,4,6-trinitrobenzenesulfonic acid) assay [16]. 1H NMR analysis also showed clean product with no structural difference from native HA.

The aldehyde modified HA (HA aldehyde) was prepared according to the recently reported procedure [13].

2.2. Cytotoxicity studies of HA-aldehyde and hydrazide derivatives

To determine toxicity of HA hydrogel components Cell-Titer 96AQueous One Solution Cell Proliferation Assay (MTS, Promega), a tetrazolium salt assay was performed. First the mouse embryonic fibroblast cell line (NIH-3T3, ATCC) was expanded in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; PerBio Science), 100 U/l penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) (culture medium) in T75 flasks (Nunc, VWR International) until 80% confluence at 37 °C in a humidified atmosphere of 5% CO2. The culture medium was changed every second day. The cells were detached by the addition of trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco, Invitrogen) and were suspended in culture medium. The cells were plated in flat-bottomed 96 well plates at 5 × 103 per well in 200 μl culture medium overnight. Components were dissolved in culture medium and add to the wells at concentrations 100 and 200 μg/ml (n = 5) for 48 h. Then MTS substrate was added to the wells. After 4 h of incubation at 37 °C an absorbance at 450 nm was measured. Cell viability was shown in percentage (standard deviation from three samples) with controls as cells only considered as 100%.

2.3. Hydrogel preparation

HA hydrazide (7% modification) and HA aldehyde derivatives (7% modification) were dissolved in sterile phosphate buffer saline (PBS, Sigma-Aldrich) at pH 7.4 to a final concentration of 16 mg/ml followed by sterile filtration using 0.45 μm syringe filter (Whatman). The reconstituted stock solutions of BMP-P or BMP-R were added to the HA-hydrazide solution to maintain final concentration of 16 mg/ml of polymer and 20 μg/ml of BMP-2s. HA hydrogel matrix was formed by mixing (20 times) equal volume of HA-aldehyde and HA-hydrazide solutions in two syringes linked by a connector under sterile conditions in a laminar flow hood.

2.4. Rheological evaluation

An AR2000 Advanced Rheometer (TA Instruments) was used to determine the shear storage modulus, G′. In a typical experiment 1 ml gel (1.6 wt.%) was prepared as mentioned earlier and injected to cylindrical mold immediately after mixing and kept for 24 h at room temperature. The hydrogels were then carefully transferred and applied to the bottom plate of AR2000 rheometer. The mechanical properties were measured at a frequency of 0.1 to 10 Hz at 25 °C using 8 mm aluminum plate geometry. The gap was adjusted starting from the original sample height and compressing the sample to reach a normal force of about 50 mN (gap sizes were between 7 and 8 mm).

2.5. Stability studies of recombinant BMPs

Growth factors were reconstituted according to the manufacturers’ protocols. BMP-P (Pfizer, Inductos® former Wyeth Europe Ltd., Berkshire, UK) was reconstituted in sterile water. BMP-R (R&D System) was reconstituted in 4 mM HCl (final pH is 0). Both proteins are delivered in lypophilized form containing glycine, sucrose and glutamic acid. The stock solution of BMP-P at a concentration of 1.5 mg/ml was prepared and stored at 4 °C, while the stock solution of BMP-R was prepared at the concentration of 100 μg/ml and stored at −20 °C.

We evaluated the stability effects of BMP-P and BMP-R at two pHs stored in two types of vials. For that purpose BMPs stock solutions were diluted in different buffers namely at 7.4 pH (PBS) and 4.5 pH (BMP-P formulation buffer recommended by Pfizer) till concentration of 100 ng/ml. Those samples in triplicate were stored either in glass or polypropylene tubes (Eppendorf) and incubated at two time point as, 24 and 0 h at room temperature. The 0 h time point was
approximately 30 min and stated as ‘0 h’ throughout the text. The stability of the BMPs was quantified using an alkaline phosphatase activity (ALP) assay, which gives a measure of bioactivity for the BMP.

2.6. ALP activity assay

The mouse stromal cell line W20 clone 17 (W20-17) was obtained from the American Type Culture Collection (ATCC—LGC Standards, Sweden). The cells were expanded in DMEM supplemented with 10% FBS, 100 U/l penicillin and 100 μg/l streptomycin (Sigma-Aldrich) (culture medium) in T75 flasks (Nunc, VWR International) until 80% confluence at 37 °C in a humidified atmosphere of 5% CO2. The medium was changed every second day. The cells were detached by the addition of EDTA (Gibco, Invitrogen) and suspended in culture medium and counted using Trypan blue (Gibco, Invitrogen). Then the cells were seeded as a cell suspension at 5 × 10⁴ cells per well in 96-well plates with culture medium. After 24 h media was replaced with 100 μl of sample (preparation described in previous section) supplemented with 100 μl of culture medium. After 48 h the cells were washed with PBS (Sigma-Aldrich) and lysed by two freeze-thaw cycles (30 min − 80 °C, 20 min 37 °C). ALP activity in this extracted solution was determined by calculating the p-nitrophenol (pNP) concentration obtained from 100 μl of substrate (p-nitrophenyl phosphate; Sigma-Aldrich) in 0.1 M glycine, 1 mM MgCl2, 1 mM ZnCl2, (10.4 pH) at 37 °C. After 20 min reaction was stopped with 50 μl of 0.1 M NaOH. The absorbance at 405 nm, which corresponds to pNP concentration was determined using microplate reader (Kinetic Microplate Reader; Molecular Devices). ALP activities were measured in triplicate and the average of three values were used to determine the standard deviation in each group.

2.7. Western blot analysis

Samples with different amounts of BMP-P and BMP-R (100, 250, 500 ng) were mixed with sample buffer (ClearPAGE, CBS Scientific) and loaded on 5–10% SDS polyacrylamide gels (CBS Scientific) and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) under non-reducing conditions. Membranes were exposed to anti-BMP-2/4 (1:1000 dilutions, R&D System) antibody and incubated overnight at 4 °C. Thereafter, blots were treated with secondary rabbit anti-goat IgG (1:5000 dilutions, Abcam) antibody conjugated to horseradish peroxidase. Immunodetection was performed by chemiluminescence using Immuno Star™ Western™ Kit (Bio-Rad) in a ChemiDoc XRS system (Bio-Rad).

2.8. Dynamic light scattering

The particle size distribution was carried out using Malvern laser Zetasizer Nano ZS, United Kingdom. Both BMP-P and BMP-R were suspended either in PBS 7.4 pH or formulation buffer pH 4.5 recombinant horseradish peroxidase. Immunodetection was performed by chemiluminescence using Immuno Star™ Western™ Kit (Bio-Rad) in a ChemiDoc XRS system (Bio-Rad).

2.9. In vivo rat model

2.9.1. Animal preparation and anesthesia

All animal handling and surgical procedure were approved by the Uppsala (Sweden) local committee for experimental animal research ethics and conducted according to the Helsinki guideline for the use and care of laboratory animals and approved by institutional ethical protocol (246/8). Six adult male Sprague-Dawley rats (Taconic M&B, Lille Skensved, Denmark), 280–300 g in weight were housed with two rats per cage with 12/12 h light/dark cycles in a temperature (22 °C) and humidity (45 ± 10%) controlled environment with ad libitum access to food and water. All surgical procedures were performed in aseptic conditions. Animals were anesthetized by using isoflurane (4.5% induce; 2.5% maintain; Forene®, Abbott Scandinavia) starting with 4 l min⁻¹ oxygen and 4 l min⁻¹ isoflurane in an induction chamber and then by mask at 1.5 l min⁻¹ oxygen, 1.5 l min⁻¹ air and 3 min⁻¹ isoflurane.

2.9.2. Surgical technique

The HA hydrogel matrices with either BMP-P or BMP-R (20 μg/ml) were prepared as described earlier. After incubating the gels for 3 h at room temperature, the lumbar region was shaved and surgically prepared with three washes of alcohol. Then hydrogel constructs of 0.2 ml volume containing physically loaded 4 μg of BMP-P (n = 6) or 4 μg of BMP-R (n = 6) or only hydrogel (n = 6) were injected subcutaneously with a 21 G needle, at a minimal distance of 15 mm apart. Immediately after intervention, animals were administered buprenorfin (Temosigic, Schering-Plough, 0.05 mg/kg) subcutaneously for pain mitigation. Rats were sacrificed after 7 weeks by CO2 asphyxiation. The implants were dissected, extracted and analyzed by micro computed tomography (micro CT). The samples were also retrieved for histological observation (n = 5 per group).

2.10. Micro computed tomography (μCT) analysis

To quantify ectopic bone formation, the harvested samples were placed in 4% paraformaldehyde in PBS, after 24 h replaced by 70% ethanol, at room temperature. Micro CT analysis was performed using a Skyscan 1072 (Kontich) with X-ray source of 100 kV/98 μA. For quantitative 3D analysis, the specimens were placed vertically onto the sample holder of a micro-CT imaging system. Subsequently, a high-resolution scan was recorded at a 1416-μm-voxel resolution (Magnification 20×; exposure time 3.9 s; 1 mm filter applied). Then, using Nrecon V1.4 (SkyScan), a cone beam reconstruction was performed on the projected files. Opaque tissue was calculated using CT Analyzer software (Version 1.10.1.0; SkyScan). Finally, by using 3D creator software (3D-DOCTOR 4.0, Able Software Corp.), 3D-reconstructions of the samples were obtained. Tissue formation was assessed by analyzing reconstructed coronal cross-sectional images. In addition, bone volume/total volume (BV/TV) of ectopic bone was calculated with CT-based morphometric analysis.

2.11. Statistical analysis

All experiments were performed in triplicate, and data were expressed as the means ± standard deviation, unless otherwise noted. Statistical analysis was performed with PASW Statistics 16.0 (SPSS Inc.). Student t-test was used to analyze the statistical differences between two groups (BMP-P and BMP-R) with consideration that p-value less than 0.05 was significant.

2.12. Histological evaluation

Specimens were stored in 70% ethanol, completely decalcified using an electrophoresis system (Tissue-Tek Miles scientific, Histolab) with formic acid, dehydrated, and embedded in paraffin wax. Serial cross sections were cut with a microtome to 5 μm, deparaffinized and stained. Representative slides were stained with hematoxylin/ eosin (H&E, Merck), Masson’s trichrome (Merck) [13], Sirius Red (Fluka) [17] as well as Alcian Blue (Sigma-Aldrich) solution [18]. For collagen observation the cross-sections (five sections per bone sample) were stained 0.1% Sirius Red dissolved in a saturated aqueous solution of picric acid (Fluka) for 1 h and then observed and photographed using polarized light microscopy with the slides inclined at 45° to the incident light. The amount of organized collagen fibers was quantified by measuring the area of green color using imaging software (BioPix AB). For Alcian Blue staining, sections were incubated in phosphate buffer (6.0 pH) or in presence of ovine testicular hyaluronidase.
mixed for 20 cycles at room temperature (Scheme 1). We have also shown its use for bone augmentation in rat calvarial model [13]. In this work, we developed a novel hydrazone crosslinked HA hydrogel derived from CDH, anticipating a superior mechanical property and hydrogel stability. The synthesis of this HA hydrazide derivative was achieved by employing EDC coupling chemistry with 10-fold excess of carbohydrates as the aldehyde units were grafted on HA backbone under mild conditions. The hydrogels were prepared by mixing the two components (HA-aldehyde and HA-hydrazide) between two syringes connected by luer-lock adapter and the two solutions were added to HA hydrazide residue before mixing with the two-syringe system. Rheological evaluation of this material showed the soft materials modulus than the loss modulus ($G''$) implies that the material is elastic and has a typical gel like characteristics. This storage modulus also corresponds to an average molecular weight between crosslinks of ~20 kg/mol forming stable networks [19] (Scheme 1).

3.1. Synthesis of HA derivatives, hydrogel preparation and characterization

Designing biomaterials for bone tissue engineering requires bio-compatible 3D scaffold that could deliver sensitive growth factors such as BMP-2 in vivo. We have recently developed HA based injectable hydrogel using hydrazone chemistry and have also shown its use for bone augmentation in rat calvarial model [13]. In this work, we developed a novel hydrazone crosslinked HA hydrogel derived from CDH, anticipating a superior mechanical property and hydrogel stability. The synthesis of this HA hydrazide derivative was achieved by employing EDC coupling chemistry with 10-fold excess of commercially available CDH with respect to HA disaccharide units to obtain 7% modification of HA carboxylates. Next, we synthesized HA aldehyde following our previously described protocol with 7% modification [13]. This method is superior to conventional NaIO oxidation of carbohydrates as the aldehyde units were grafted on HA backbone under mild conditions. The hydrogels were prepared by mixing the two components (HA-aldehyde and HA-hydrazide) between two syringes connected by luer-lock adapter and the two solutions were mixed for 20 cycles at room temperature (Scheme 1). The mixture was injected to cylinder mold immediately after mixing and was kept for 24 h at room temperature (gels were formed within 30 s). This method of hydrogel preparation is crucial to obtain reproducible data and also represents the scenario for material preparation during in vivo experiments. For gels with encapsulated BMP-2, the protein was added to HA hydrazide residue before mixing with the two-syringe system. Rheological evaluation of this material showed the soft materials to have average storage modulus $G'$ and loss modulus $G''$ of $1196±65$ Pa and $3.1±0.3$ Pa respectively. This significantly higher storage modulus than the loss modulus ($G'>G''$) implies that the material is elastic and has a typical gel like characteristics. This storage modulus also corresponds to an average molecular weight between crosslinks of ~20 kg/mol forming stable networks [19] (Scheme 1).

3.2. In vitro toxicity studies with HA derivatives

We further decided to test the cytotoxicity studies of our chemically modified HA using MTS assay, which measures the mitochondrial activity of live cells. As shown in Fig. 1, HA derivatives did not show any toxicity on NIH-3T3 fibroblast cells at the concentrations tested. On the other hand we observed increase in cell proliferation upon addition of HA derivatives.

This increase in cell proliferation with HA has been observed previously [20].

![Scheme 1. Schematic representation of HA-hydrazide synthesis and hydrazone crosslinked hydrogel preparation.](image)

3.3. In vitro stability studies of recombinant BMPs

In order to evaluate critical aspects of handling BMPs, we decided to perform in vitro bioactivity assay (ALP) when they are stored under different conditions. We incubated BMPs at two pHs (physiological pH 7.4 and in pH 4.5 formulation buffer, recommended for BMP-P) and also in two different vials (glass and Eppendorf tube). The ALP levels were tested within 30 m and after 24 h under these conditions. We anticipate that the presence of hydrophobic residues in the protein will favor binding to hydrophobic surfaces (plastic), thereby reducing the protein concentration in the medium whereas hydrophilic surfaces (glass) will prevent such association. It has been reported earlier that BMP-P stability is pH dependent and aggregation occurs at pH 6.5 [21]. This is due to the change in pH that affects the net charge of the protein, resulting in its precipitation or aggregation [22]. The buffers with excess of acidic or basic residues can also affect hydrogen bonding between amino acids and consequently can disrupt the tertiary structure of protein causing biological de-activation [22].

Since in vitro ALP assay depends on BMP-2 binding to its cell-surface receptor and signaling, only bioactive BMPs are detected with this method. We employed this assay with W20-17 cells that are known to induce BMP-2 dependent osteogenic differentiation [23,24].

Our in vitro results are shown in Fig. 2, which can be summarized as follows: (i) Both BMPs showed identical ALP activity at 0 h, indicating almost identical initial concentration of the two proteins. (ii) BMP-P remained bioactive under all conditions tested for up to 24 h. (iii) Bioactivity of BMP-R was found to depend on pH and hydrophilic/hydrophobic nature of the storage vial. Higher stability was observed at pH 4.5 and hydrophilic glass vials; however, at
physiological pH the stability was instantaneously lost within 30 min (0 h). Increased binding of BMP-R on polypropylene Eppendorf tube probably suggests that there are more hydrophobic residues on BMP-R as compared to BMP-P. Since both proteins preferred glass surface and acidic pH indicates presence of basic amino acid residues (such as lysine and arginine) that require acidic pH for their protonation and solubility in aqueous medium. These differences in BMP-2 stability could also arise from differences in glycosylation pattern of BMP-2 as it was indicated recently that non-glycosylated BMP-2 has higher stability resulting in increased bioactivity [25].

3.3.1. Western blot analysis

Since the BMP-R showed almost rapid aggregation at physiological pH, we decided to test if this difference could be observed using the Western blot analysis. Since this experiment was performed with basic running buffer (pH 8.5) we anticipated that BMP-R could be lost or aggregated during the experiment. As shown in Fig. 3, we could detect BMP-P at all concentrations tested; however the density of BMP-R on the other hand were substantially lower. These experiments also demonstrated that one should be careful with sensitive proteins such as BMPs especially if they are from natural sources.

3.4. Dynamic light scattering studies of recombinant BMPs

Since we observed substantial difference in the in vitro bioactivity assay, we decided to investigate protein aggregation behavior using dynamic light scattering technique, at two pHs (4.5 and 7.4). Interestingly, we observed a time dependent steady aggregation in BMP-R sample in both buffer conditions, while no aggregation was observed with BMP-P. In PBS 7.4, BMP-R acquired a size of 295 nm within 15 min, which gradually increased to 750 nm in nearly 2 h with good polydispersity of ~0.2 (Fig. 4a). After 15 h, this sample attained an average size of 2.7 μm. However, in formulation buffer (pH 4.5) the particle formed was bigger with an initial size of 700 nm, which gradually increased to 2.8 μm in nearly 2 h (Fig. 4b). This is presumably due to the presence of surfactant polysorbate 80 and other stabilizers present in formulation buffer, which form complex with BMP-2. This was evident upon analyzing the DLS of neat formulation buffer without any added protein, which showed presence of particles of ~700 nm unlike in PBS. When BMP-P was suspended in formulation buffer pH 4.5, we observed particles of ~1 μm, which did not change with time.

3.5. In vivo bone induction in a rat ectopic model

3.5.1. Evaluation of total bone volume in the ectopic model

After observing surprising difference in bioactivity of two proteins we decided to evaluate its in vivo efficacy by employing our HA hydrogel in a rat subcutaneous model. We used this model because it is quite straightforward and reproducible system to evaluate bone regenerative potential of BMPs. The subcutaneous surgical intervention also allows reducing the number of animals necessary for evaluation. We employed same amount (4 μg/implant) of BMPs from the two sources to compare its bioactivity. This is therefore the first study of direct comparison of BMP-2 with identical doses.
In our experimental model, we injected 200 μl of hydrogel with either physically trapped BMP-P/BMP-R in gel or gel alone as negative control (n=6). After 7 postsurgical weeks we sacrificed the animals and opened the implantation site for inspection. As expected, we did not observe any bone tissue at the implantation site in the control group lacking rhBMP-2. In the groups with BMPs, ectopic bone tissue was found. Our intraoperative observation revealed that ectopic bones were limited to the implantation site and firmly attached to the surrounding tissue with no sign of inflammation or fibrosis. To quantitatively determine their bone volume of the neo-bone, they were subjected to micro CT analysis. The 3D reconstructions of one of the representative samples are shown in Fig. 5a (BMP-P) and 5b (BMP-R). Micro-CT-based morphometric analysis showed that the average ectopic bone volume induced by BMP-P was significantly higher (almost twice) as compared to that observed for BMP-R group (Fig. 5c). The statistical analysis revealed the ratio of BV/TV to be 43.87±6.38 mm$^3$ for BMP-P group as compared to 27.48±5.24 mm$^3$ for the BMP-R group (p<0.01). The low standard deviations indicate that the experimental procedure to be highly reproducible.

3.5.2. Histological evaluation of ectopic bone

The morphology of ectopic bones induced by BMP-P and BMP-R after 7-week post-implantation was examined by histology (Fig. 6).

The representative cross sections stained with H&E (Fig. 6a and b) and Masson’s trichrome (Fig. 6c and d) showed woven bone distribution within newly formed tissue with no inflammatory cells. Both groups showed abundant bone marrow and collagen matrix (collagen is stained blue with Masson’s trichrome). We however observed substantial differences within ectopic bone architecture. Ectopic bone in BMP-P group was larger in diameter with more cortical bone than trabecular bone as compared to BMP-R group. The BMP-P group also had larger void space towards the middle of the scaffold, unlike BMP-R group. This result is corroborated with the in vitro stability differences observed between the two BMPs. The more stable BMP-P presumably differentiated the recruited stem cells that formed mineralized outer surface.

This could prevent migration of cells towards the core forming void space. The BMP-R on the other hand, being less stable did not differentiate cells on the scaffold periphery, which resulted in migration of cells to the core of the scaffold. Therefore BMP-R formed bone with smaller volume but with less void space.

3.5.3. Qualitative and quantitative evaluation of collagen orientation

Another important aspect to access the quality of regenerated tissue is the type of matrix laid down by the recruited cells. One of the main components of bone ECM is collagen that serves as the main structural protein of bone. During healing process newly formed callus tissue undergoes changes in its structural properties due to initial

Fig. 5. Representative μCT images of 3D reconstruction of ectopic bones induced by (a) BMP-P and (b) BMP-R after 7 weeks of implantation. (c) Quantitative analysis of bone volume/total volume (BV/TV) showed a statistical significant difference between both groups (*p<0.01; error bars indicate SD for n=6).

Fig. 6. Histological observation of paraffin-embedded cross sections of ectopic bone induced by BMP-P and BMP-R after 7 weeks of implantation. (a,b) hematoxylin/eosin (H&E) stained and (c,d) Masson’s trichrome stained. BMP-P represented by (a,c) and BMP-R by (b,d). In the magnified images: BM shows bone marrow; B shows trabecular bone structures.
deposition of less oriented ECM, which is replaced by bone tissue with organized collagen fibrils [26]. The formation of a new layer is dependent on the pre-existing bone, which serves as an endogenous scaffold for lamellar matrix [27]. Moreover a higher degree of collagen organization also improves the mechanical load-bearing capacity of the bone. We therefore decided to investigate the role of different BMPs on collagen fiber orientation within the newly formed bone tissue. We performed Sirius red staining of the histological sections, which enabled us to visualize natural birefringence of collagen in polarized light microscope [28]. As shown in Fig. 7a and b, the ectopic bone induced by BMP-P showed a homogenous green layer that indicates structured collagen orientation in the inner periphery of the new bone. The BMP-R group on the other hand showed red layer indicative of dispersed and disoriented collagen matrix within the mineralized tissue. Quantitative assessment (Fig. 7c) of the oriented collagen area demonstrated that bone formed by BMP-P had nearly 3 folds higher amount of oriented collagen as compared to the BMP-R group (n = 5). This striking difference could also be due to improved stability of BMP-P allowing controlled mineralization and matrix formation.

3.5.4. Estimation of remaining HA-hydrogel within ectopic bone

Finally, we attempted to elucidate the remnants of HA gel within the histological sections obtained from the two groups after 7 weeks in vivo implantation using Alcian blue staining. This reagent specifically stains glycosaminoglycan within the neo-tissue (Fig. 8). As observed in Fig. 8a the BMP-P group showed evidence of scaffold remaining in the inner part of specimen (red arrows in Fig. 8a). This observation was confirmed in control experiment where similar section was pre-treated with hyaluronidase (enzyme known to degrade HA) before staining with Alcian blue (Fig. 8c). The absence of staining in the control experiment unequivocally proves that HA gel was indeed remaining in this group. Similar experiment with samples obtained from the BMP-R (Fig. 8b and d) group however, did not show any remaining hydrogel. This observation also proves the above-mentioned hypothesis that stability of BMP-2 can result in efficient mineralization of the scaffold at the periphery that corroborate with limited tissue formation and scaffold degradation at the core.

4. Conclusions

In this article we attempt to critically evaluate the bioactivity of two most common commercially available BMPs following standard assay conditions. As we are actively involved in developing hydrogel scaffold for delivery of BMP-2 for bone tissue regeneration, this evaluation is extremely important. We have discovered that bioactivity of BMP-2 is strongly influenced by storage conditions and pH. Our in vitro results show that there were significant differences in bioactivity between the two proteins with BMP-P being more stable under all conditions and also show lower binding to the plastic surfaces. On the other hand, BMP-R was extremely adhesive to hydrophobic surfaces and sensitive to pH as it almost lost its activity in less than 30 min at pH 7.4. This was also confirmed by dynamic light scattering studies of the two BMPs, which revealed gradual and progressive aggregation of BMP-R in both 4.5 and 7.4 pH conditions. To further understand the impact of this behavior in an in vivo setting, we developed hydrazone

Fig. 7. Sirius red staining of ectopic bone induced by (a) BMP-P and (b) BMP-R after 7 weeks implantation to show collagen orientation. Green color identified thicker and organized fibers while red-thinner fibers identified as unorganized collagen. (c) Quantitative analysis of green area representing organized collagen fibers. Results are shown as mean and standard error of minimum n = 5.

Fig. 8. Paraffin embedded cross sections of ectopic bone after 7 weeks of implantation stained with Alcian blue to demonstrate remaining hydrogel. (a) BMP-P group, red arrows shows remaining hydrogel; (b) BMP-R group; (c,d) controls respectively.
crosslinked hydrogel that was used as a BMP carrier. Same dose of BMPs was loaded in this carrier and in vivo experiments were performed in a rat ectopic model. Similar to in vitro observation, we found significant differences in bone formation in vivo between the two proteins. Higher stability of BMP-P resulted in bone formation with larger BV/TV ratio with void space at the core of the implant having remnants of hydrogel. The BMP-R groups, showed smaller but compact bone without any traces of gel. The BMP-P group also showed to be effective to form bone with an organized collagen matrix unlike BMP-R group. Our qualitative evaluation of trabecular/cortical bone and collagen supported with quantitative evaluation of bone volume and collagen fibers clearly suggest that BMP-P has also better in vivo bone induction capability. These experiments also indicate that rhBMP-2 mediated bone regeneration could be obtained if one can find ways to improve BMP-2 stability under physiological conditions and also that experimental results employing BMPs from different sources cannot be correlated by any means. These experiments also highlight the importance of handling techniques that are to be considered while performing BMP-2 related research.

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References