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Combination of BMP-2 and relaxin to induce osteogenesis in vivo

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COMBINATION OF BMP-2 AND RELAXIN TO INDUCE OSTEOGENESIS *IN VIVO*

by

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ABSTRACT

Bone grafts are a common procedure for treating craniofacial injuries such as trauma, surgery, and reconstruction. Autografts and allografts have limitations which encourages the use of synthetic bone grafts. While synthetic bone grafts, such as hydroxyapatite (HA) microspheres, are advantageous for their cost and reproducibility, they lack the cells and growth factors needed for proper osteoinduction. Bone morphogenetic protein-2 (BMP-2) has been shown to increase the osteoinduction of grafts but the effective dose is above physiological levels and causes undesired effects on the body. Relaxin has been shown to enhance BMP-2's ability to induce bone formation in vitro and thus lower the dose of BMP-2 required for effective bone regeneration. In this study, HA microspheres sized 212-250µm were synthesized; the in vitro release of BMP-2 and relaxin into a medium of fetal bovine was measured. The release of both was slow and controlled and no hindrance to release was seen when both proteins were loaded on the microspheres. When implanted into rat calvarial defects for 6 weeks, an enhancement to new bone percent was seen when loading HA microspheres with 0.5µg BMP-2 and 0.05µg of relaxin compared to microspheres loaded with 0.5µg BMP-2 alone. A 50% reduction in BMP-2 dose, compared to a previously published dose, was seen when relaxin (at 0.05, 0.1, and 0.25µg per defect) was loaded on the HA microspheres. A 75% reduction in BMP-2 dose was seen when 0.05µg per defect of relaxin was loaded. These results suggest that a combination of relaxin and BMP-2 loaded onto HA microspheres will reduce the dosage of BMP-2 required to provide significant bone regeneration.

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NOMENCLATURE

Symbol	Description
BSA	Bovine Serum Albumin
HA	Hydroxyapatite
PBS	Phosphate Buffer Saline
BMP-2	Bone Morphogenetic Protein-2
H&E	Hematoxylin and Eosin
FBS	Fetal Bovine Serum
EDTA	Ethylenediaminetetraacetic acid

1. INTRODUCTION

Approximately 3 million bone grafts are performed annually in the United States for treatment of large bone defects [1]. Bone defects can result from trauma, malignancy or congenital diseases and are a significant clinical problem. Trauma in the craniofacial region poses a great challenge to bone growth due to the location of highly important anatomic structures [2]. Bone has a high capability to regeneration on its own with a three stage bone repair process. These three stages are the early inflammatory stage, the repair stage and the late remodeling stage [3]. During the inflammatory stage a hematoma develops allowing inflammatory cells and fibroblasts to enter and form granulation tissue [3]. In the repair stage, fibroblasts form a stroma to support vascular growth [3]. A collagen matrix is laid down and mineralized leading to the formation of a soft callus [3]. The callus eventually ossifies and forms a bridge of bone over the fracture [3]. The remodeling stage is the longest and results in the bone being restored to its original shape and mechanical strength [3].

When it is impractical to allow bone to naturally heal on its own bone grafts can be used to treat bone defects. Bone grafts can have osteoconductive or osteoinductive properties. Osteoconduction is the process in which the graft allows osteogenic cells to have a biological role, while osteoinduction involves the molecules, such as growth factors, that influence the fate of osteogenic cells [4]. Autografts are considered the gold standard for treatment because they have osteoinductive effects and do not cause an adverse immune response in the patient. However, donor site limitations and morbidity remain obstacles for using them [5]. Allografts lack the risk of host site morbidity, but are costly and risk a negative host immune response [1]. A variety of synthetic bone grafts are being developed

to circumvent limitations of both autografts and allografts. These include bioceramics (hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP), and biphasic calcium phosphate (BCP)), bioactive glass, and polymers (e.g. hyaluronic acid) [1] . β-TCP is known for its high strength similar to cancellous bone but has a low replacement ratio (more β-TCP is resorbed than replaced with new bone) which has limited its clinical use [6]. BCP are mixtures of β-TCP and HA and release calcium and phosphate ions into the defect as the scaffold is resorbed [6]. Bioactive glass, such as 45S5 glass, reacts in the body to form a layer of hydroxyapatite and supports the regeneration of bone [1]. Polymers like hyaluronic acid have the ability to combine with other materials to enhance the properties but natural polymers have a risk for an immune response [7]. Hyaluronic acid has also been used as a delivery method for the release of growth factors [7]. Synthetic bone grafts can be made in large quantities, have reproducible quality, and typically do not have an adverse host response [8]. Unfortunately, synthetic bone grafts are limited by their osteoconductive rather than osteoinductive capabilities because they lack the osteoprogenitor cells [8, 9]. Since they do not produce significant bone regeneration compared to autografts, the addition of growth factors is needed.

Hydroxyapatite (HA) is an ideal biomaterial for synthetic bone grafts because it consists of the inorganic elements found in natural bone [6]. Its chemical formula is $Ca_{10}(PO_4)_6(OH)_2$ and therefore it consists of calcium and phosphate ions. This makes HA highly biocompatible since it produces no toxicity or immune response. [9]. HA has also been known to promote cell adhesion and growth when produced with adequate pore sizes, typically 100μm [6, 10]. Another quality of HA is its ability to adsorb different types of chemical species on to its surface [11]. Disadvantages of using HA include its low

mechanical strength, limiting its use in load bearing bones, and its low degradation rate [6, 12]. Microspheres composed of HA have a high affinity to growth factors and can be used as a delivery vehicle [13]. Delivery systems are typically composed of porous particles, granules, or scaffolds in which the bioactive protein is either adsorbed to the surfaces of the porous material, or encapsulated within the pores [14]. Hollow HA microspheres have a large surface area due to their mesoporous shell [14]. This allows for an enhanced capability to release growth factors because absorption can occur in the shell and within the hollow core [13]. This mesoporous structure can also enhance cell adhesion [15]. The absorption and release of growth factors from HA microspheres make them a model carrier.

Bone morphogenetic protein 2 (BMP-2), a member of the TGF-β superfamily, is a strong inducer of osteogenesis [16]. BMP-2 is naturally found at a concentration of 2ng/g of bone and in serum at a concentration of about 90pg/mL [17, 18]. BMP-2 is one of two BMP proteins currently used in clinical settings for orthopedic and dental applications [18]. Adversely, BMP-2 has a tendency to rapidly degrade (half-life of about 7 to 16 minutes) when injected directly into a defect site, due to proteases. Therefore, a supraphysiological dose of the protein is typically required. This supraphysiological dose can cause some undesired side effects like swelling and an increased risk for cancer (more than three times the incident rate) [7, 19, 20]. Because of this, it is preferable to have controlled release of BMP-2 in a localized environment since it will lower the required dose and reduce side-effects. At similar doses it has been shown that controlled, long-term delivery of BMP-2 is more effective and safer compared to short-term delivery [21]. Currently, the only carriers clinically approved for drug delivery are collagen scaffolds [18]. Unfortunately, growth factors have a low affinity to collagen scaffolds and tend to have a high initial burst

release [22]. HA microspheres have been shown as a novel system for controlled drug release due to a decreased initial burst and sustained release of BMP-2 [23, 24].

In additional to providing the controlled release of BMP-2 to reduce undesired effects, an enhancer to BMP-2 can be used to potentially decrease the required dose of BMP-2. Relaxin is an example of such an enhancer. Relaxin is a pleiotrophic hormone of the insulin family [25]. It is typically known as a pregnancy hormone, in which it has a role in promoting cervical softening to facilitate birth [25]. Relaxin also has roles in connective tissue metabolism, collagen turnover, angiogenesis, and tumor metastasis [26, 27]. Relaxin 2, which is the only relaxin protein found circulating in the blood, acts equally through the Relaxin/insulin-like family peptide receptors (Rxfp) 1 and 2, although the Rxfp2 receptor is known to play a larger role in osteogensis [25, 28]. These receptors have been recently found on bone tissue, namely osteoblasts, notably on mouse calvarial tissue [29, 30]. While examining the effects of relaxin on mesenchymal stem cell differentiation into osteoblasts and bone formation, Moon *et al* found that relaxin augmented BMP-2-induced osteogenesis [26]. When determining the effects that relaxin played on C3H/10T1/2 mouse embryonic fibroblast cells it was found that relaxin was not an inducer of osteogenesis but an enhancer to BMP-2. It is believed that a combination of BMP-2 and relaxin may provide satisfactory bone regeneration therefore leading to a reduction of the necessary BMP-2 dose.

Previous studies have investigated bone regeneration using HA microspheres and the growth factors transforming growth factor beta (TGF-β) and BMP-2. Loading the microspheres with 1µg per defect of BMP-2 provided considerable bone regeneration at six weeks [8]. TGF-β was not as successful in inducing osteogenesis compared to BMP-2

and required a higher dosage (5µg per defect) [13]. In a quick comparison, this dose of BMP-2 is lower than the dosage used by other types of calcium phosphates and other biomaterials [8]. However, continuing to lower this dose will contribute to a reduction in clinically-relevant, unwanted side effects.

The present study hypothesizes that the use of relaxin will lower the dose of BMP-2 required to achieve sufficient bone regeneration. Bone regeneration in rat calvarial defects was analyzed using histomorphometric analysis. The rat calvarial defect was used because it is a standard technique for studying bone regeneration [6] [4].

2. MATERIALS AND METHODS

2.1. PREPARATION AND CHARACTERIZATION OF HA MICROSPHERES

Closed HA microspheres were prepared by reacting glass microspheres in a phosphate buffer as described in a previous publication [23]. Briefly, borate glass with a composition of 15CaO, 11Li_2O and 74B_2O_3 was prepared by melting reagent grade $CaCO_3$, Li_2CO_3, and H_3BO_3 in a Pt crucible at 1200°C for 45 minutes and quenching the melt between cold stainless steel plates. Particles of size 212-250 μm were obtained by grinding the glass in a hardened steel mortar and pestle, and sieving through 212 and 250 mesh sieves. The glass particles were dropped down a vertical tube furnace at 1000°C to obtain glass microspheres. The glass microspheres were reacted for two days in 0.02M K_2PO_4 solution at 37°C at a starting pH of 9.0 to produce hollow HA microspheres. The convert microspheres were washed three times with distilled water, soaked in ethanol to remove residual water and dried for twelve hours at room temperature ad for twelve hours at 90°C.

Characterization of the converted microspheres was performed using scanning electron microscopy (SEM). Briefly, the microstructure of the surface and cross-section of the microspheres was examined using scanning electron microscopy (SEM; S4700; Hitachi, Tokyp, Japan). The specific surface area of the microspheres and pore size was measured using nitrogen adsorption (Autosorb-1; Quantachrome, Boynton Beach, FL) as previously described [8]. The carbon content was measured, previously, using a combustion technique at a commercial laboratory (LECO Corp., St Joseph, MI). X-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy was used to check the phase composition of the microspheres. XRD was performed using Cu K_α radiation

(λ=0.15406 nm) at a rate of 1.8°/min in the 2θ range 20-70°, while FTIR was performed in the range of 400-4000 cm^{-1}.

2.2. RELEASE PROFILE OF BMP-2

Human recombinant BMP-2 was purchased from Shenandoah Inc. (Warwick, PA) and diluted in sterile water to a concentration of 10µg/mL. 10mg of HA microspheres were placed in the BMP-2 solution. A small vacuum was used to remove any air trapped in the microspheres then allowed to dry in 4°C overnight. The microspheres were placed in a buffer consisting of fetal bovine serum (FBS) and phosphate buffered saline (PBS) at a 1 to 1 ratio and kept at 37°C with gentle agitation. After 1 hour, 1 day, 3 days, 5 days, 7 days, and 14 days, the entire release buffer was collected. The microspheres were then rinsed with additional release buffer and the rinse was collected and pooled with the previous collection, making sure not to collect any microspheres in the process. Fresh buffer was then added to continue the experiment. The amount of BMP-2 released was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Peprotech, Rocky Hill, NJ). Absorbance was measured with a microplate reader (BMG LABTECH, FLUOstar Omega). The concentration of the unknown samples was compared to a standard curve ran at the same time.

2.3. RELEASE PROFILE OF RELAXIN

The release profile of relaxin was conducted like the release profile for BMP-2 mentioned above. Human recombinant Relaxin-2 was purchased from R&D systems (Minneapolis, MN) and diluted in sterile water to a concentration of 1µg/mL. 10mg of HA

microspheres were placed in the relaxin solution. The remaining procedure was followed exactly as mentioned above. Samples were collected after 1 hour, 1 day, 2 days, 3 days, 5 days, 7 days, and 14 days. The amount of relaxin released was measured using an ELISA kit (R&D systems). The concentration of the unknown samples was compared to a standard curve ran at the same time.

2.4. RELEASE PROFILE OF A COMBINATION OF BMP-2 AND RELAXIN

The combined release was conducted in a similar manner to the individual release profiles mentioned above. Briefly, 10mg of HA microspheres were placed in a solution containing both 10µg/mL of BMP-2 and 1µg/mL of relaxin. The remaining procedure will be followed exactly like the individual release profiles mentioned above. Samples were collected after 1 hour, 1 day, 2 days, 3 days, 5 days, 7 days, and 14 days. The amount released of either protein was measured using their respective ELISA.

2.5. ANIMALS

All animal procedures were approved by the Missouri University of Science and Technology Institutional Animal Care and Use Committee (IACUC), in compliance with the NIH Guide for Care and Use of Laboratory Animals [31]. Sixty male Sprague Dawley rats of three months old were purchased from Envigo (Indianapolis, IN) (weighing between 350 and 400g) and housed under a 12h/12h light/dark cycle. They were allowed to acclimate to diet, water and housing for 14 days before the experiment was conducted. All animal procedures followed the groups previous publications [8, 13, 32].

2.6. SURGERY

The rats were anesthetized with 3-5% isoflurane in oxygen administered by inhalation or an intraperitoneal injection of ketamine (70 mg/kg), xylazine (7 mg/kg). The surgical area was shaved, scrubbed with 70% ethanol, and draped with a sterile drape. Using aseptic technique, a cranial skin incision was made in an anterior-to-posterior direction. The subcutaneous tissue, musculature, and periosteum were dissected to expose the calvarium. Bilateral full-thickness defects 4mm in diameter were created using a trephine burr and an electric drill. The site was constantly irrigated with sterile PBS to prevent overheating. The defects were implanted with 13 groups of implants consisting of closed hollow HA microspheres (10 mg per defect) loaded with varying amounts of Relaxin and BMP-2 and are detailed in Table 2.1.

Care was taken to avoid mixing implants containing different proteins in the same animal. The animals were monitored daily and after 6 weeks the animals were sacrificed by CO_2 inhalation. The calvarial defect sites and surrounding bone were harvested.

2.7. HISTOLOGY

The calvarial samples were washed and fixed in a 10% formalin solution for 5 days. The fixed samples were cut transversely in half; half of each sample was for paraffin embedding and the other half was for methyl methacrylate embedding.

The paraffin embedded samples were decalcified for 10 days in 14% EDTA and later dehydrated in ethanol and embedded in paraffin using standard histological techniques. Sections of 5μm were made from the samples and stained with hematoxylin and eosin.

Table 2.1. Detailed description of the amount of BMP-2 and relaxin loaded in the 13 groups used in the study.

Group	BMP-2 (µg)	Relaxin (µg)	Description
1	0	0	As prepared microspheres
2	0	0.05	Relaxin alone
3	0	0.1	Relaxin alone
4	0	0.25	Relaxin alone
5	0.25	0	BMP-2 alone
6	0.5	0	BMP-2 alone
7	1	0	BMP-2 alone
8	0.25	0.05	0.25µg BMP-2 + Relaxin
9	0.25	0.1	0.25µg BMP-2 + Relaxin
10	0.25	0.25	0.25µg BMP-2 + Relaxin
11	0.5	0.05	0.5µg BMP-2 + Relaxin
12	0.5	0.1	0.5µg BMP-2 + Relaxin
13	0.5	0.25	0.5µg BMP-2 + Relaxin

The methyl methacrylate embedded samples were dehydrated in ethanol and embedded in Poly(methyl methacrylate) (PMMA). The sections were affixed to acrylic slides, ground down to 40µm using a surface grinder (EXAKT 400CS, Norderstedt, Germany) and stained using the von Kossa method to observe mineralization [33].

2.8. HISTOMORPHOMETRIC ANALYSIS

Transmitted light images of the stained sections were taken with an Olympus BX 53 microscope connected to a CCD camera (DP70, Olympus, Japan) and were analyzed using ImageJ software (National Institutes of Health). The percentage of new bone formed

was determined from the H&E stained samples. The total defect area was identified by measuring one edge of the old calvarial bone to the other edge including the implants and tissue within. The new bone within the defect area was outlined and expressed as a percentage of the total defect area.

2.9. STATISTICAL ANALYSIS

Data sets are presented as means ± standard deviation. Data were compiled in Microsoft Excel and statistical analysis was performed using Minitab 17. A student's t-test was conducted to determine statistical significance between two samples. One-way analysis of variance (ANOVA) with Dunnett's method pairwise comparison plots was used to determine and identify statistically significant values. Values were considered significant if the *p*-value was less than 0.05 ($p<0.05$).

3. RESULTS

3.1. CHARACTERIZATION OF HA MICROSPHERES

Scanning electron microscope (SEM) images of the hollow HA microspheres are shown in Figure 3.1A and B. The hollow HA microspheres had a diameter of 212-250µm, with a surface area of 168.0 m^2/g and a mesoporous shell wall with a pore size of 10.12 nm. The total pore volume of the HA microspheres was determined, by nitrogen adsorption, to be 0.425cm^3/g.

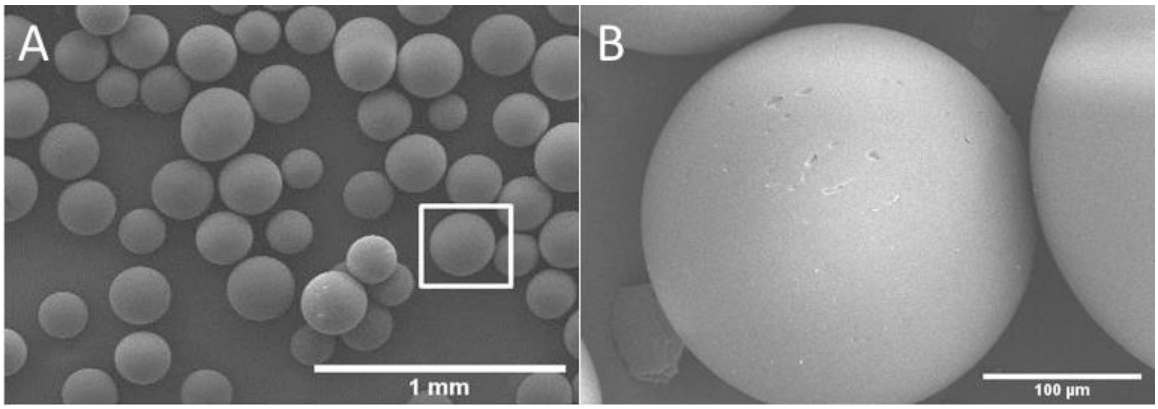

Figure 3.1. SEM images of as prepared HA microspheres. A) low magnification, B) high magnification.

The x-ray and Fourier transform infrared spectroscopy (FTIR) patterns of the hollow HA microspheres were described in previous publications[8, 23]. The X-ray powder diffraction (XRD) patterns confirmed that the hollow HA microspheres were composed of nanometer-sized HA crystals, while the resonances in the FTIR spectrum were associated with crystalline HA. The FTIR results also showed a weak resonance corresponding to the CO_3^{2-} group that may be produced during the glass conversion

process. The carbonate content was determined to be 2.1% based on the combustion analysis.

3.2. RELEASE PROFILE

The amount of BMP-2 released from hollow HA microspheres, at particular time points, into the FBS:PBS release buffer is shown in Figure 3.2A. The medium was completely removed at each time point and fresh medium was added. The release at select time points was used to determine the cumulative release of BMP-2 (Figure 3.2B).

HA microspheres loaded with BMP-2 alone showed an initial burst release at 1 hour then continued release at the following time points. Microspheres loaded with a combination of BMP-2 and relaxin did not show a burst release and instead had a more continuous but lower release throughout the course of the experiment. Regardless of the high initial burst or continuous release over the time points, both groups, BMP-2 alone and BMP-2 combined with relaxin group, had comparable final release amounts at the termination of the experiment (p=0.287; n=3). The final released amount of the alone group was 13.1ng when compared to the initial load amount. Similarly, the final released amount of the combination group was 10.3ng when compared to the initial load amount.

The amount of relaxin released from hollow HA microspheres, at particular time points, into the release buffer is shown in Figure 3.2C. As previously described, the medium was completely removed at each time point and fresh medium was added. The release of relaxin at select time points was used to determine the cumulative release (Figure 3.2D).

When loaded with relaxin alone and relaxin simultaneously loaded with BMP-2, microspheres showed an initial burst release of relaxin similar to microspheres loaded with BMP-2 alone. Continued release was noted in both samples; however, the relaxin alone group had a greatly decreased release after 1 day. Microspheres loaded in combination with BMP-2 showed significantly higher cumulative release compared to microspheres loaded alone (p=0.001; n=3). The final released amount of relaxin alone was 150pg when compared to the initial loaded amount. For relaxin with BMP-2, the final released amount was 270pg of the initial loaded amount.

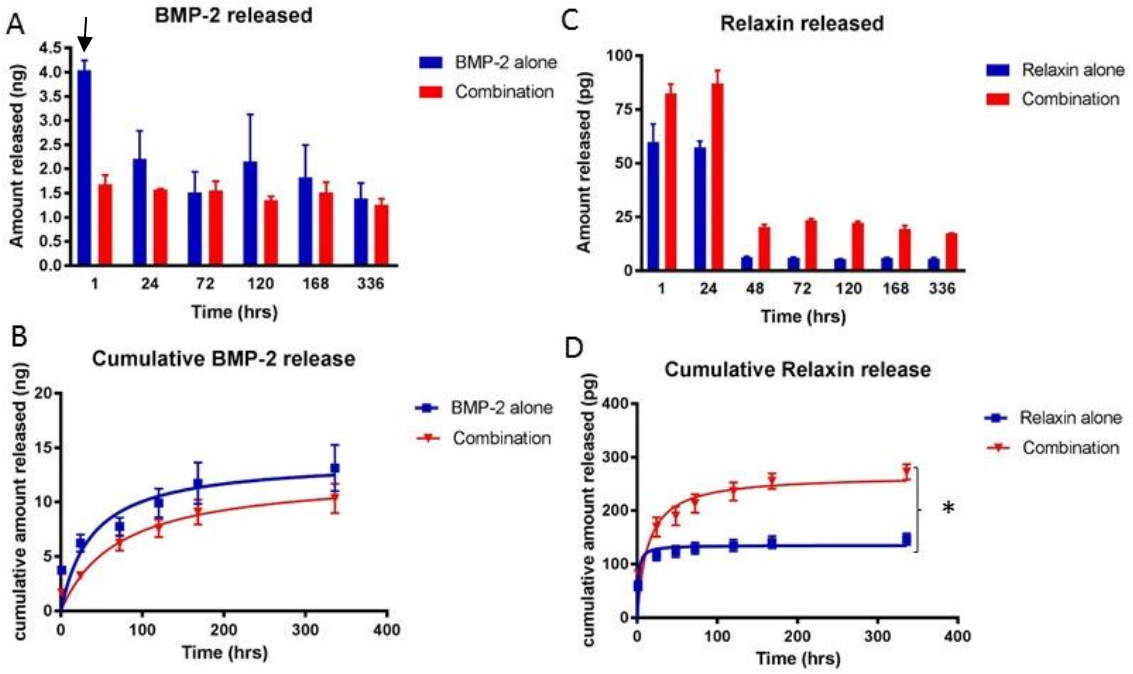

Figure 3.2. Release profiles of A-B) BMP-2 and C-D) Relaxin from hollow HA microspheres. A) amount of BMP-2 release when loaded alone or in combination with relaxin at selected time points, B) cumulative amount of BMP-2 released as a function of time C) amount of relaxin released when loaded alone or in combination with BMP-2 at selected time points, D) cumulative amount of relaxin released as a function of time. Arrow indicates time points with burst release. Results are displayed as mean ± SD. (*p<0.05; n=3).

3.3. H&E STAINING

H&E stained sections of rat calvarial defects implanted with as prepared HA microspheres and microspheres loaded with different amounts of BMP-2 are shown in Figure 3.3. The as prepared microspheres showed little new bone growth (4±6%) which occurred along the edges to the host bone (Figure 3.3A). The defect area instead consisted of fibrous connective tissue. The addition of 0.25µg of BMP-2 per defect did not significantly increase new bone growth (p=0.129; n=6) (Figure 3.3B). The addition of BMP-2 only significantly increased the new bone growth seen in the defect at amounts greater than 0.5µg per defect (24±12%; p=0.006; n=6) (Figure 3.3C). The greatest new bone growth was seen when using microspheres loaded with 1µg per defect (40±6%) (Figure 3.3D). New bone bridged the defect in samples loaded with 1µg BMP-2 within the 6-week period.

H&E stained sections of rat calvarial defects implanted with HA microspheres loaded with different amounts of relaxin are shown in Figure 3.4. Little new bone growth was seen in defects loaded with 0.05µg, 0.1µg, and 0.25µg of relaxin and was not significantly different than as prepared HA microspheres with p-values of 0.177, 0.541, and 0.401 (n=7), respectively (Figure 3.4B, C, and D). Bone growth was only seen adjacent to host bone and from the dura mater, which is a source of osteogenic cells and growth factors.

Images of H&E stained sections loaded with 0.25µg of BMP-2 and varying amounts of relaxin are shown in Figure 3.5. Defects loaded with all tested concentrations of relaxin (0.05µg, 0.1µg and 0.25µg) in combination with 0.25µg BMP-2 showed no

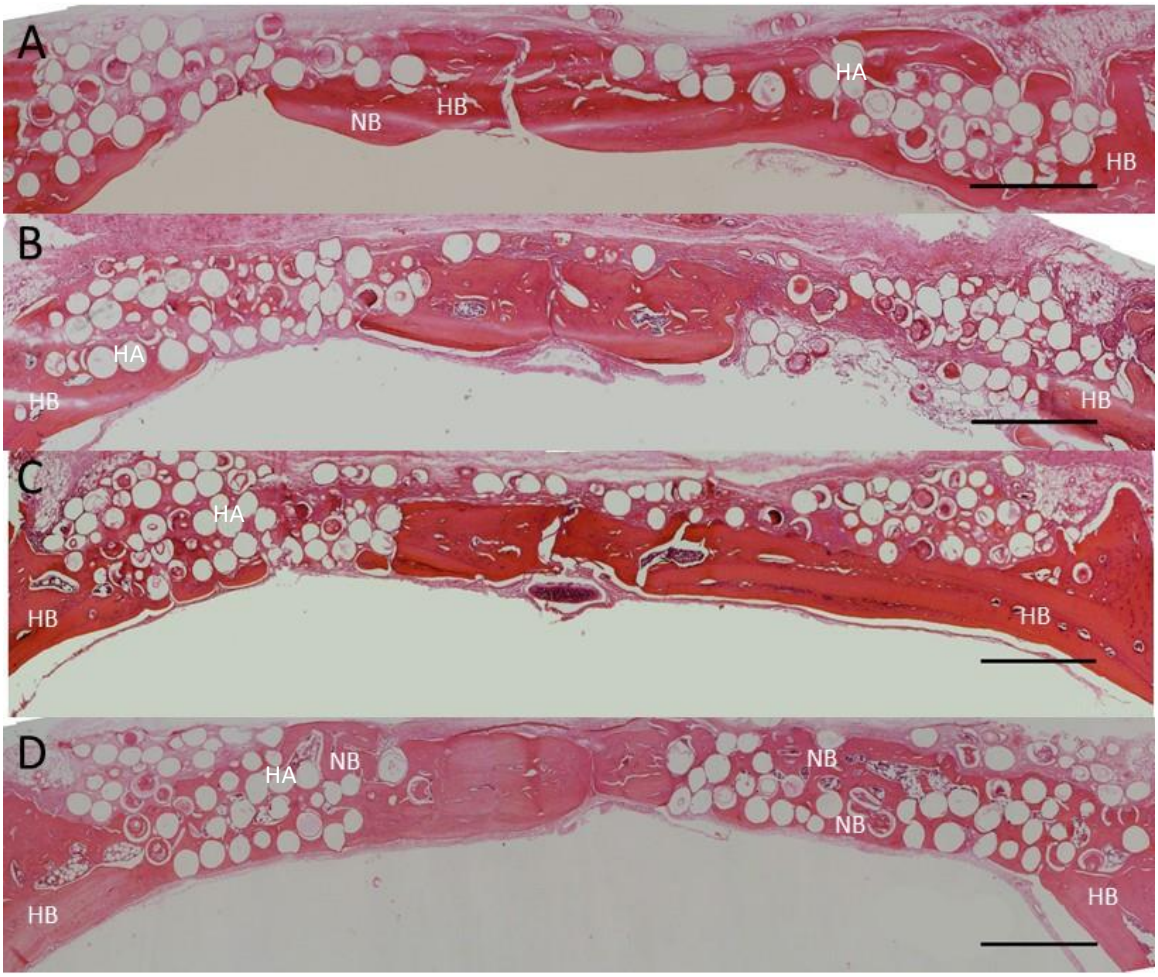

Figure 3.3. H&E stained sections of rat calvarial defects implanted for 6 weeks with A) as prepared HA microspheres, B) HA microspheres loaded with 0.25µg of BMP-2, C) loaded with 0.5µg of BMP-2, D) loaded with 1µg of BMP-2. HB= host bone; NB= new bone; HA= hydroxyapatite. Scale bar= 1mm.

significant improvement in new bone growth compared to defects loaded with 0.25µg BMP-2 alone (p=0.105, 0.856, and 0.800, respectively, n=7). New bone growth bridged the gap of the defect in samples of microspheres loaded with 0.25µg BMP-2 and 0.05µg relaxin (Figure 3.5B).

Figure 3.6 shows H&E stained sections of rat calvarial defects implanted with HA microspheres loaded with 0.5µg of BMP-2 and different amounts of relaxin. All amounts

of relaxin (0.05µg, 0.1µg, and 0.25µg) showed significant new bone growth as new bone bridged the defect for all of the groups (Figure 3.6B, C, and D). However, only defects

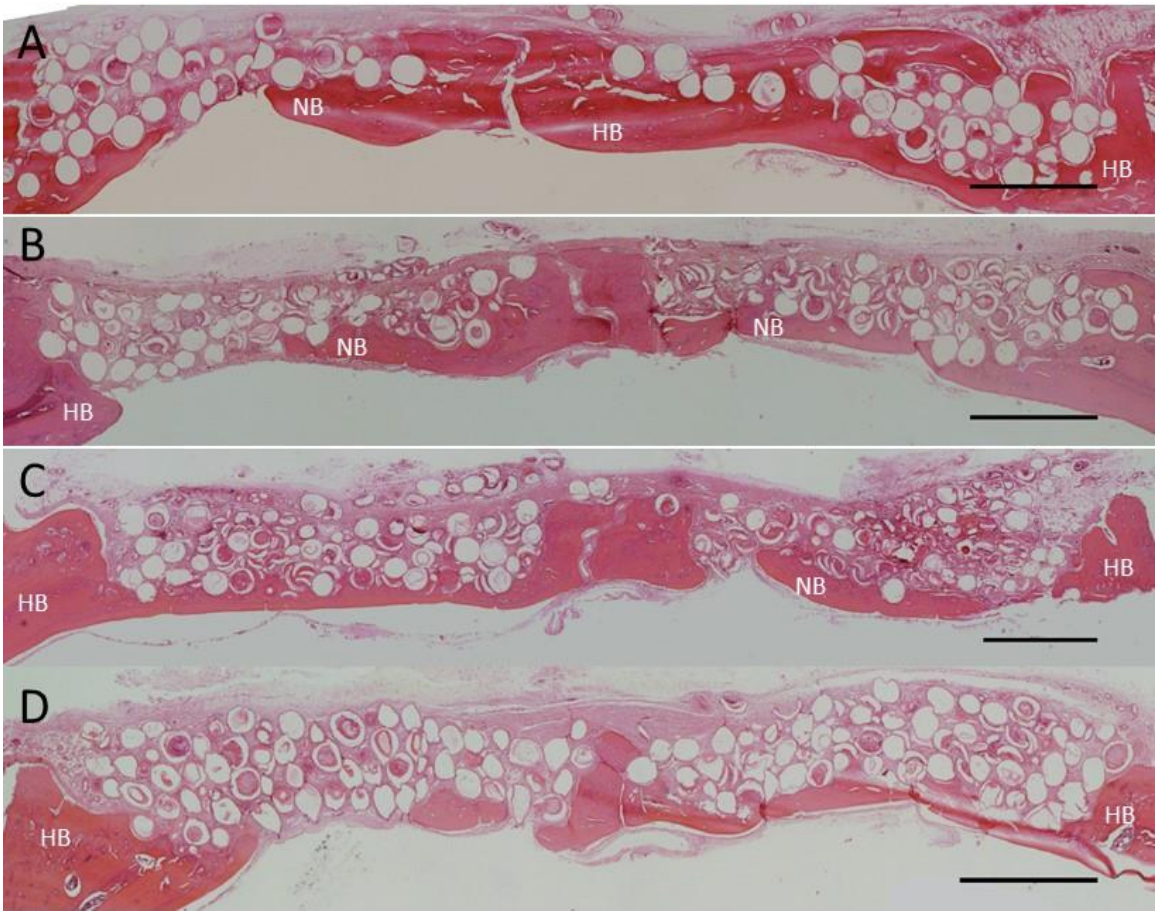

Figure 3.4. H&E stained sections of rat calvarial defects implanted for 6 weeks with A) as prepared HA microspheres, B) HA microspheres loaded with 0.05µg of relaxin, C) loaded with 0.1µg of relaxin, D) loaded with 0.25µg of relaxin. Scale bar= 1mm.

loaded with 0.5µg of BMP-2 and 0.05µg of relaxin showed significant enhancement of bone growth compared to 0.5µg BMP-2 alone (p=0.002; n=7) (Figure 3.6B).

High magnification images of select groups are shown in Figure 3.7. The image of a defect loaded with as prepared HA microspheres confirmed that the defect area

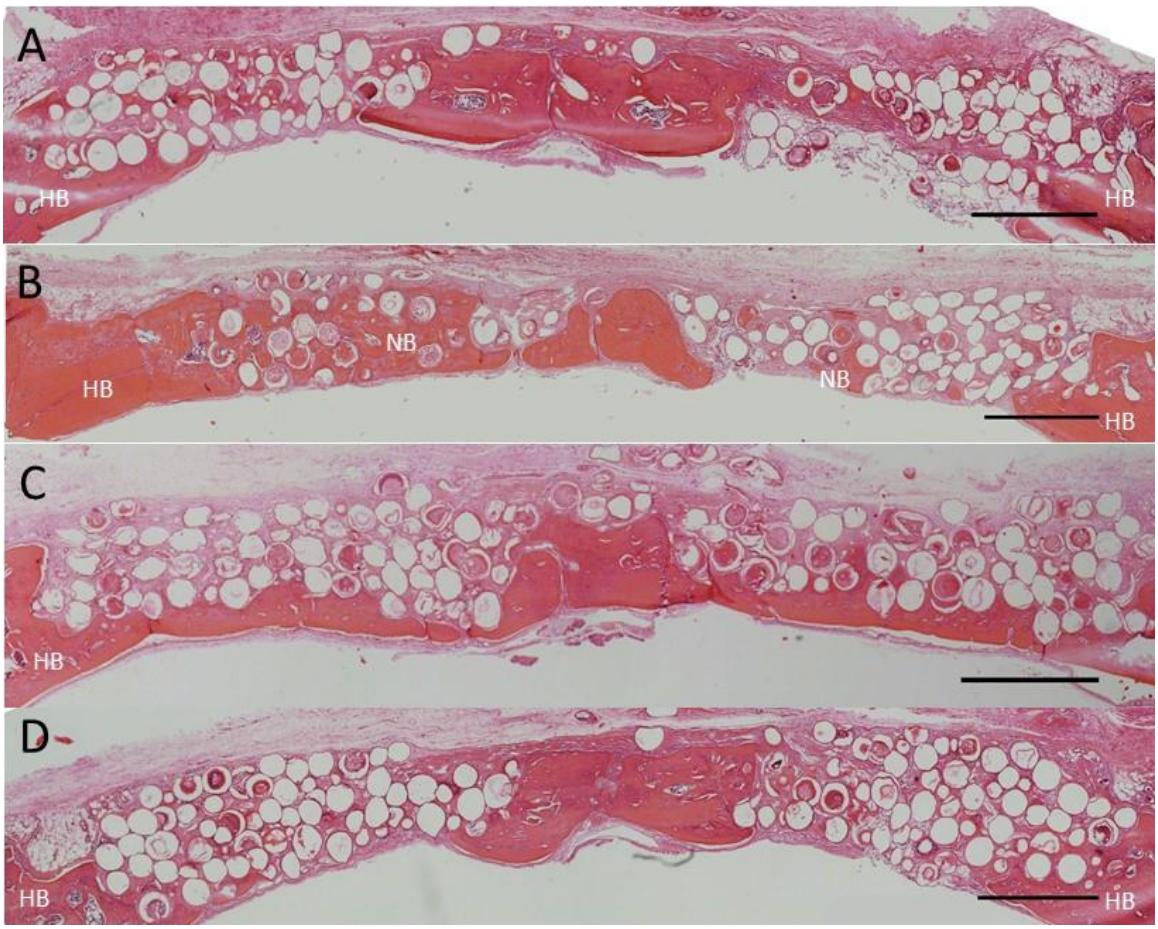

Figure 3.5. H&E stained sections of rat calvarial defects implanted for 6 weeks with HA microspheres loaded with A) 0.25µg of BMP-2, B) 0.25µg of BMP-2 + 0.05µg of relaxin, C) 0.25µg of BMP-2 + 0.1µg of relaxin, D) 0.25µg of BMP-2 + 0.25µg of relaxin. Scale bar= 1mm.

consisted of mostly fibrous connective tissue (Figure 3.7A). More new bone area is seen in defects loaded with 0.5µg BMP-2 along with 0.05µg relaxin when compared to those loaded with 0.5µg BMP-2 alone. This confirmed an enhancement effect of relaxin on BMP-2 at this amount (Figure 3.7C and D). High magnification images of defects loaded with 1µg of BMP-2 looked histologically comparable to defects loaded with a combination of 0.5µg BMP-2 and 0.05µg relaxin (Figure 3.7B and D).

Figure 3.8 shows the dose response in new bone growth with varying amounts of BMP-2 or relaxin. Bar graphs depicting the enhancement effects of relaxin on 0.25µg BMP-2 and 0.5µg BMP-2 are shown in Figure 3.9. The mean ± SD of the new bone percent for all groups are summarized in Table 3.1.

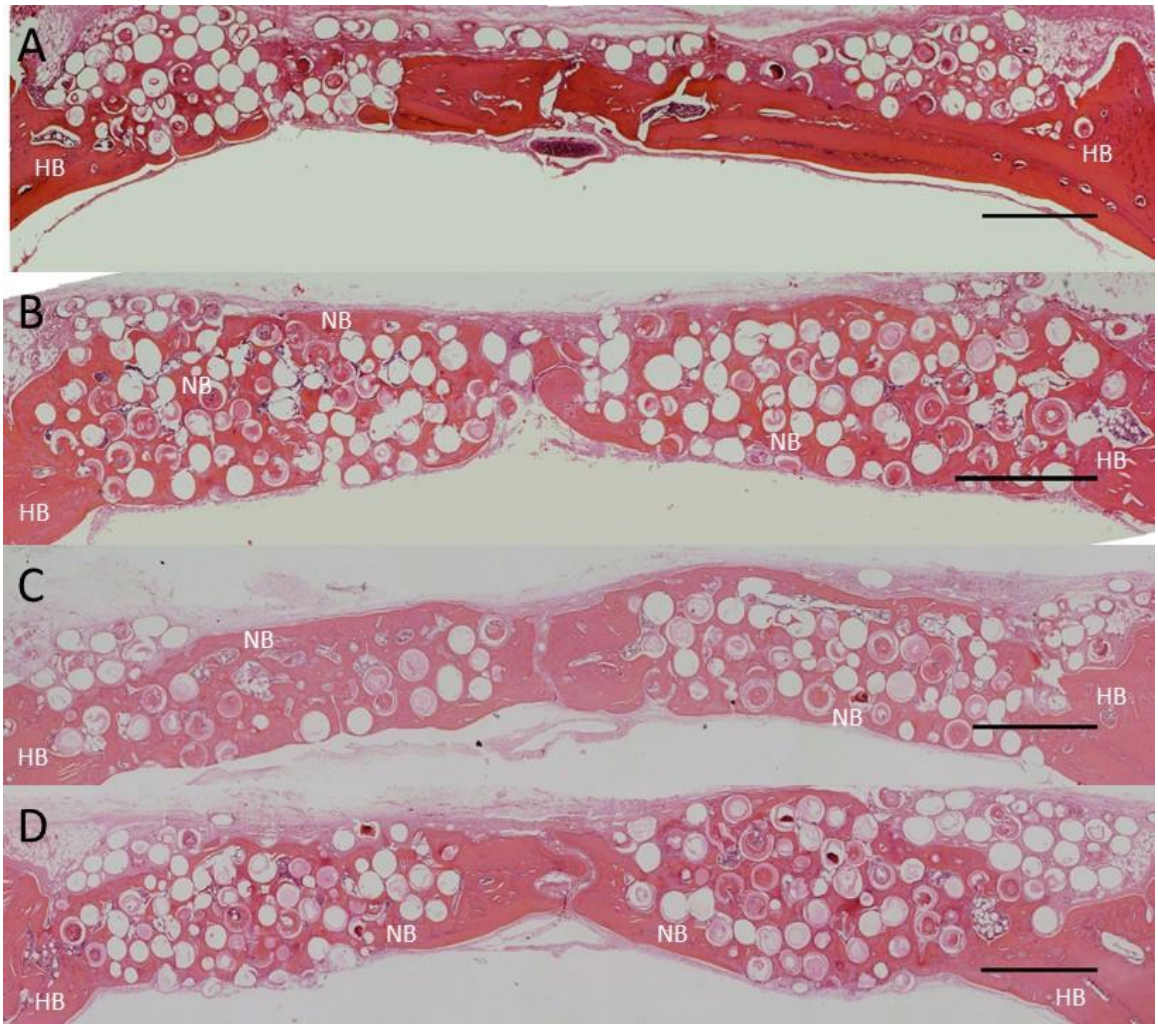

Figure 3.6. H&E stained sections of rat calvarial defects implanted for 6 weeks with HA microspheres loaded with A) 0.5µg of BMP-2, B) 0.5µg of BMP-2 and 0.05µg of relaxin, C) 0.5µg of BMP-2 and 0.1µg of relaxin, D) 0.5µg of BMP-2 and 0.25µg of relaxin. Scale bar= 1mm.

3.4. COMPARISON TO 1µg OF BMP-2

Comparisons of BMP-2 and relaxin groups to a previously published effective dose of 1µg per defect are shown in Figure 3.10. No significant difference in new bone percent was seen when 0.5µg of BMP-2 were loaded in HA microspheres with 0.05µg, 0.1µg, and 0.25µg of relaxin when compared to those loaded with 1µg of BMP-2. The p-values of these are 0.458, 0.723, and 0.985, respectively (n=8). No significant difference

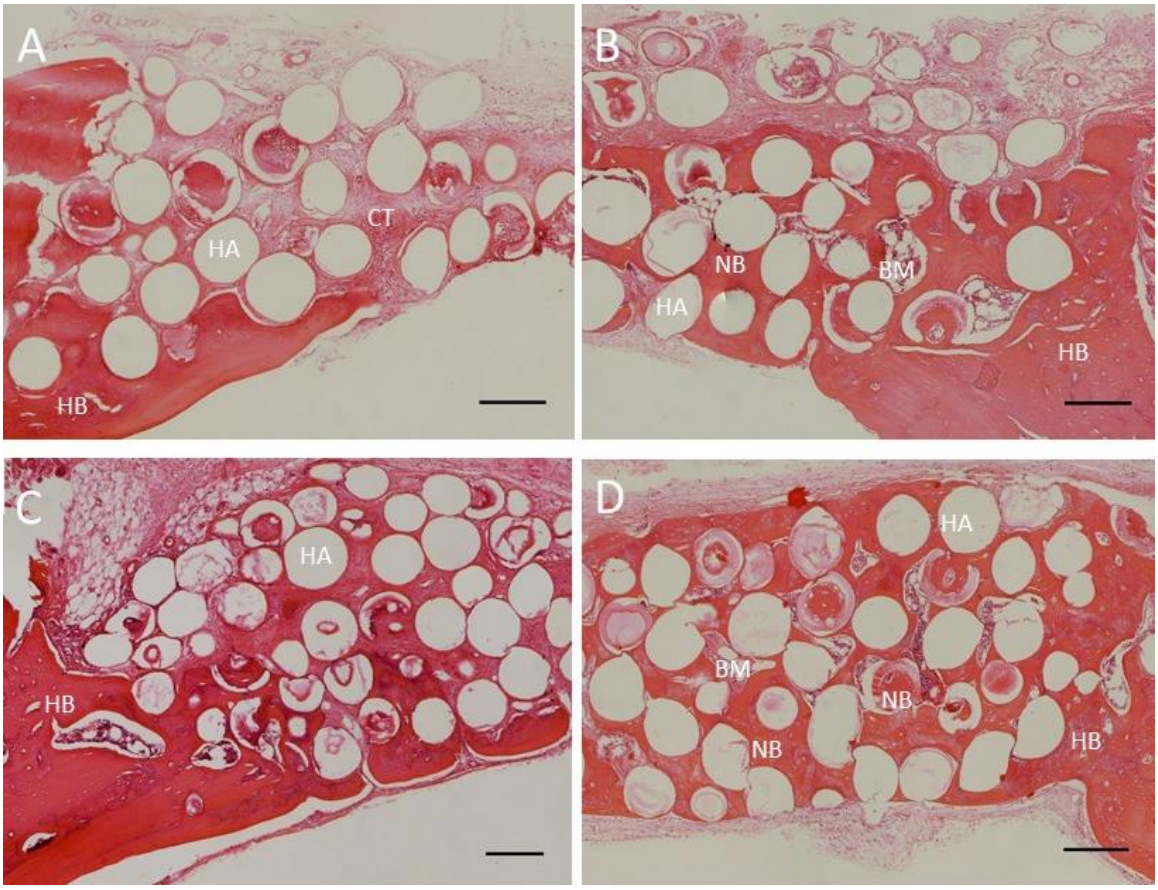

Figure 3.7. High magnification images of H&E stained sections of rat calvarial defects implanted for 6 weeks with A) as prepared HA microspheres, B) HA microspheres loaded with 0.5µg of BMP-2, C) HA microspheres loaded with 1µg of BMP-2, D) HA microspheres loaded with 0.5µg of BMP-2 and 0.05µg of relaxin. HB= host bone, NB= new bone, HA= hydroxyapatite, BM= bone marrow-like tissue, CT= fibrous connective tissue. Scale bar= 250µm.

in new bone percent was observed between HA microspheres loaded 0.25µg of BMP-2 and 0.05µg of relaxin when compared to microspheres loaded with 1µg of BMP-2 ($p=0.357$; $n=6$). This demonstrates that the BMP-2 dose can be reduced with the addition of relaxin

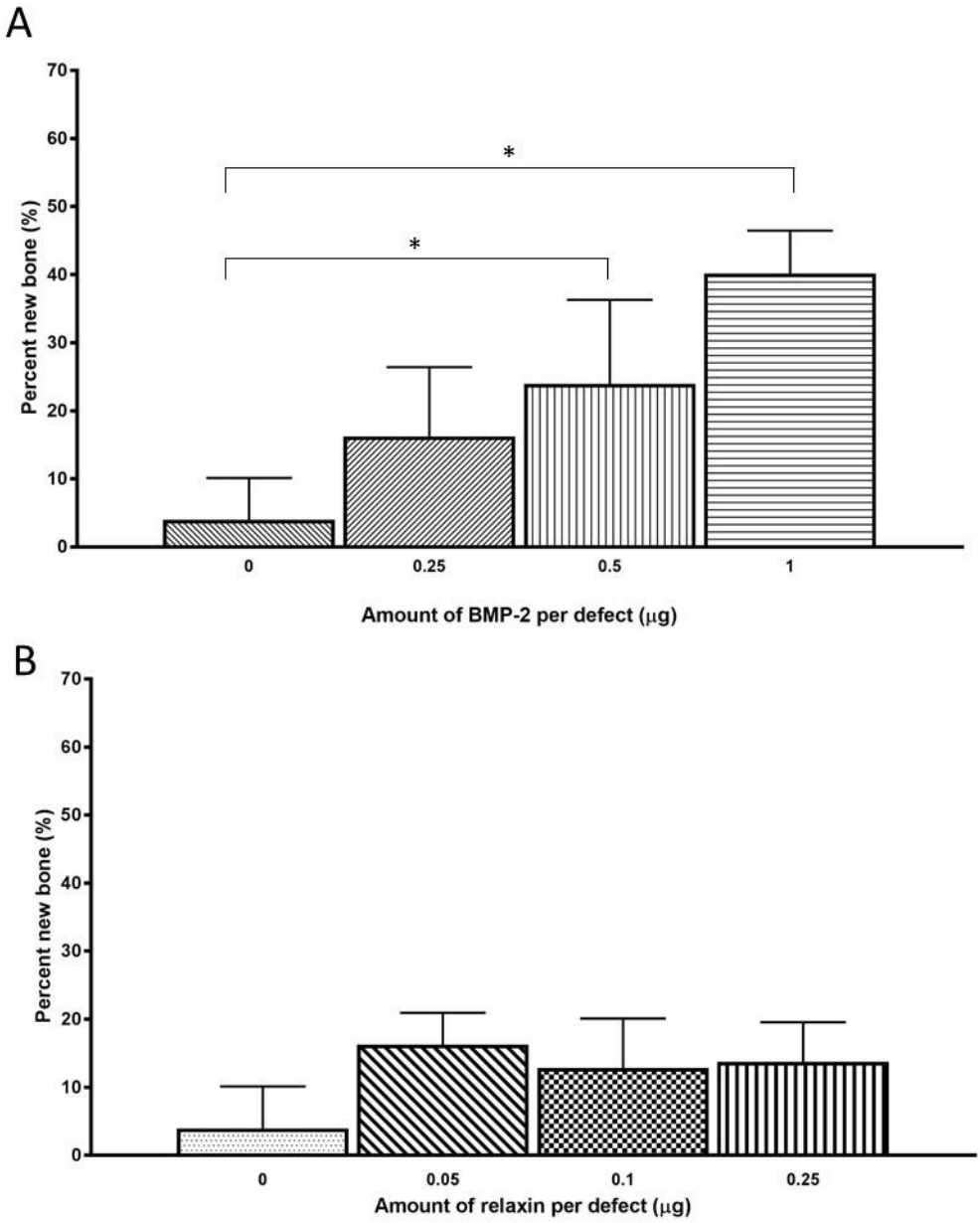

Figure 3.8. The percent new bone formed in rat calvarial defects implanted for 6 weeks with HA microspheres loaded with A) doses of BMP-2, B) doses of relaxin. Graph is shown as mean ± SD (*$p<0.05$).

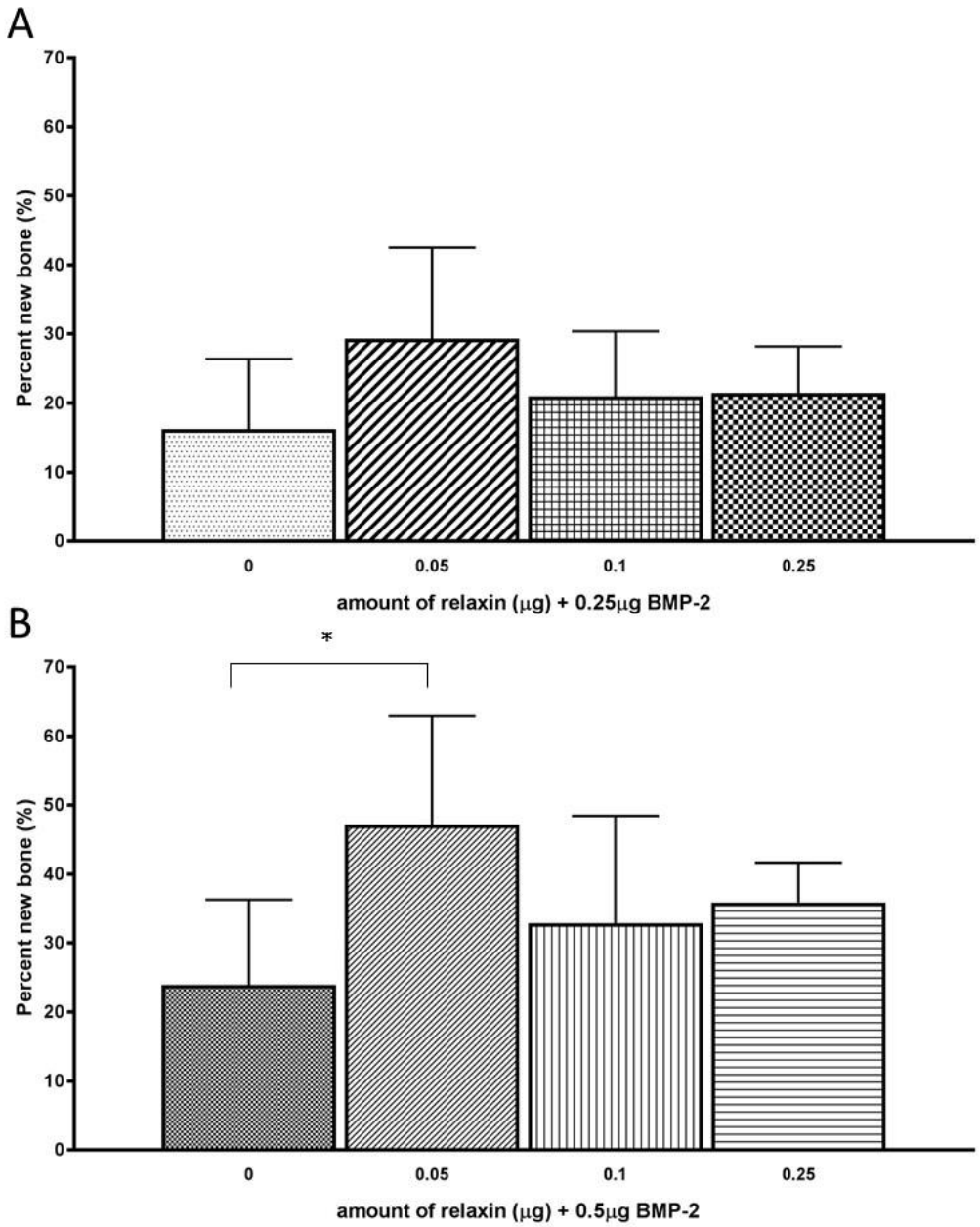

Figure 3.9. The enhancement effect of relaxin on rat calvarial defects implanted with A) HA microspheres loaded with 0.25μg of BMP-2 B) HA microspheres loaded with 0.5μg of BMP-2. Data is shown as mean ± SD (*p<0.05).

and can still provide effective bone regeneration.

New bone percent for microspheres loaded with 0.25μg and 0.5μg of BMP-2 were significantly lower than microspheres loaded with 1μg of BMP-2 (p=0.003 and 0.042,

respectively). New bone percent for microspheres loaded with 0.05µg, 0.1µg, 0.25µg of relaxin was significantly lower when compared to microspheres loaded with 1µg of BMP-2 with all p-values<0.001. New bone percent for microspheres loaded with 0.25µg of BMP-2 along with 0.1µg and 0.25µg of relaxin was significantly lower compared to microspheres loaded with 1µg of BMP-2 (p=0.006 and 0.008, respectively).

This demonstrates that use of either a lower dose of BMP-2 or relaxin alone will not have a comparable effect for bone regeneration compared to the published dose. A combination of 0.25µg of BMP-2 and either 0.1µg or 0.25µg of relaxin cannot produced comparable bone regeneration compared to 1µg of BMP-2 alone.

Table 3.1. Percentage new bone (mean ± SD) in rat calvarial defects with varying amounts of BMP-2, relaxin, and combinations of both.

			Relaxin (µg)			
			0	0.05	0.1	0.25
			New bone (%)			
BMP-2 (µg)	0	New bone (%)	4±6	16±5	13±7	14±6
	0.25		18±8	29±13	21±9	21±7
	0.5		24±12	50±15	33±16	36±6
	1		40±6	n/a	n/a	n/a

3.5. VON KOSSA STAINING

Figure 3.11 shows von Kossa stained sections of rat calvarial defects implanted for 6 weeks with as prepared HA microspheres and microspheres loaded with different amounts of BMP-2. Von Kossa stains phosphate ions, a dark brown-black, which can be found, within the defect, along the surface of the microspheres as well as in the new bone area. Von Kossa is used as a verification of new bone area within the defect. An increase in the amount of stained area correlates to the increase in amount of BMP-2. The von Kossa stained area corresponds, histologically, to the new bone growth seen in the H&E stained images of Figure 3.3.

Figure 3.12 shows von Kossa stained sections of rat calvarial defects implants for 6 weeks with as prepared HA microspheres and microspheres loaded with different

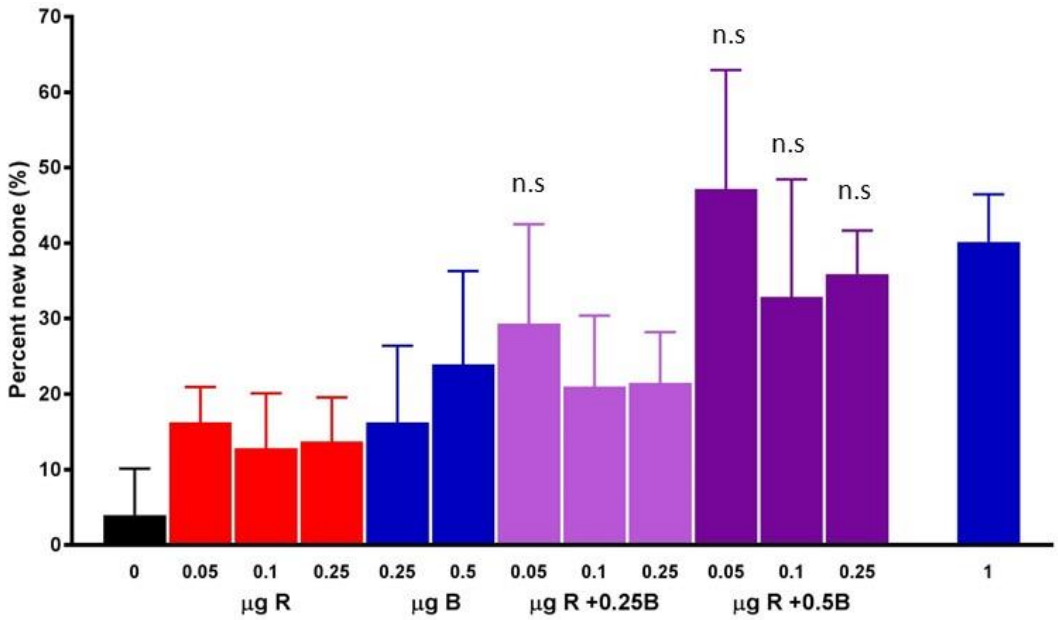

Figure 3.10. Comparison of the percent new bone formed in rat calvarial defects implanted with BMP-2 and relaxin to a previously published dose of 1µg per defect (1B). B=µg of BMP-2, R=µg of relaxin. Data is shown as the mean ± SD (n.s.= not significant $p>0.05$).

amounts of relaxin. Minimal new bone growth is seen in these samples and corresponds to the H&E images in Figure 3.4 and the new bone percent shown in Table 3.1.

Von Kossa stained sections of defects implanted using HA microspheres loaded with 0.25µg of BMP-2 and various amounts of relaxin are seen in Figure 3.13. Defects loaded with 0.25µg of BMP-2 and either 0.25µg or 0.1µg of relaxin did not show a difference in von Kossa stained area compared to defects loaded with 0.25µg of BMP-2 alone (Figure 3.13A, C, and D). Defects loaded with 0.25µg of BMP-2 and 0.05µg of

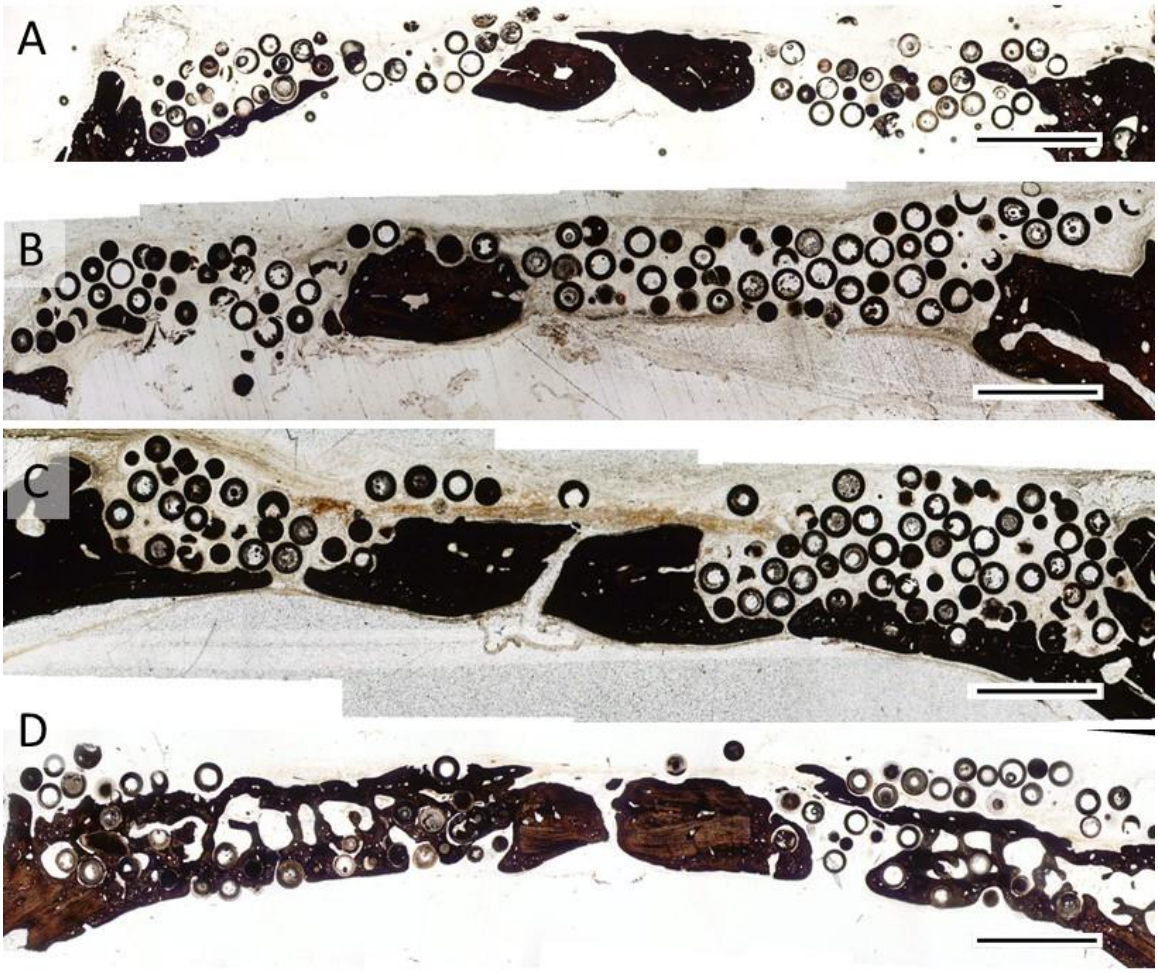

Figure 3.11. Von Kossa stained sections of rat calvarial defects implanted for 6 weeks with A) as prepared HA microspheres, B) HA microspheres loaded with 0.25µg of BMP-2, C) loaded with 0.5µg of BMP-2, D) loaded with 1µg of BMP-2. Scale bar= 1mm.

relaxin showed an increase in von Kossa stained area with brown shades seen between microspheres (Figure 3.13B).

Von Kossa stained sections of defects implanted with HA microspheres loaded with 0.5µg of BMP-2 and various amounts of relaxin are seen in Figure 3.14. In defects with

Figure 3.12. Von Kossa stained sections of rat calvarial defects implanted for 6 weeks with A) as prepared HA microspheres, B) HA microspheres loaded with 0.05µg of relaxin, C) loaded with 0.1µg of relaxin, D) loaded with 0.25µg of relaxin. Scale bar= 1mm.

0.5µg of BMP-2 along with either 0.25µg or 0.1µg of relaxin, von Kossa stained area did not exceed 50% to 75% of the within microsphere area (Figure 3.14 C and D). Defects

loaded with a combination of 0.5µg of BMP-2 and 0.05µg of relaxin showed von Kossa staining in more than 90% of the in between microsphere area (Figure 3.14B). This correlates to the H&E staining results shown in Figure 3.6 and Table 3.1.

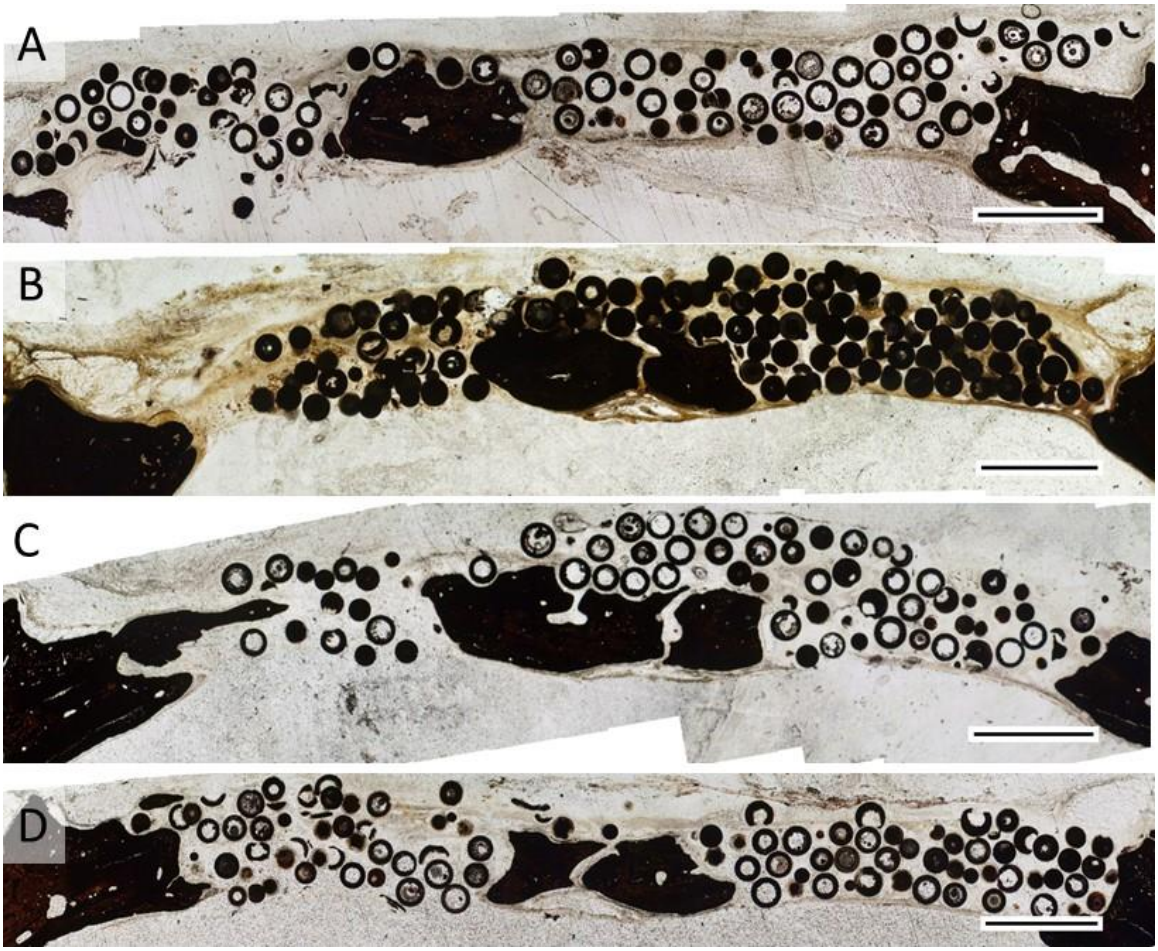

Figure 3.13. Von Kossa stained sections of rat calvarial defects implanted for 6 weeks with HA microspheres loaded with A) 0.25µg of BMP-2, B) 0.25µg of BMP-2 + 0.05µg of relaxin, C) 0.25µg of BMP-2 + 0.1µg of relaxin, D) 0.25µg of BMP-2 + 0.25µg of relaxin. Scale bar= 1mm.

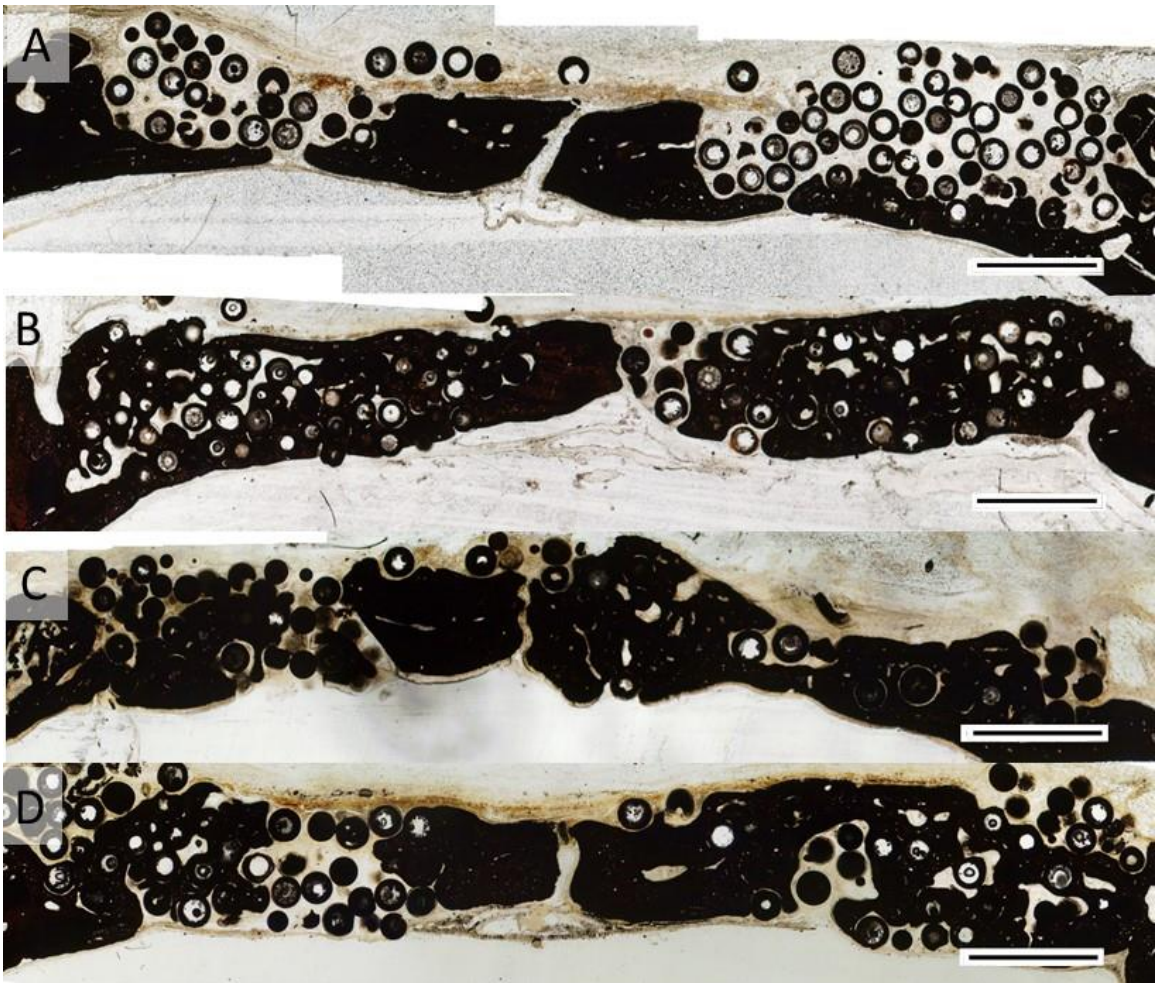

Figure 3.14. Von Kossa stained sections of rat calvarial defects implanted for 6 weeks with HA microspheres loaded with A) 0.5µg of BMP-2, B) 0.5µg of BMP-2 + 0.05µg of relaxin, C) 0.5µg of BMP-2 + 0.1µg of relaxin, D) 0.5µg of BMP-2 + 0.25µg of relaxin. Scale bar= 1mm.

4. DISCUSSION

HA microspheres have been shown to be a carrier for growth factors while being osteoconductive and bioactive [8]. The present study aimed to test the ability of relaxin to enhance the osteoinductive effect of BMP-2 and to lower the required dose of BMP-2 for safer clinical use. HA microspheres were tested for their control of the release of both BMP-2 and relaxin alone as well as in combination. *In vivo* studies were conducted to test the enhancement effect of relaxin and showed enhancement and an ability to lower the BMP-2 dose at certain amounts.

The results of BMP-2 release from the hollow HA microspheres were continuous and controlled but little was released over the measure time period (Figure 3.2B). BMP-2 and other similar growth factors have a high affinity to bind to hydroxyapatite [34]. This high affinity can result in an increase in adsorption but little release from the carrier. BMP-2 is believed to absorb on to HA with binding between functional groups on the BMP-2 protein and the calcium site of HA [35]. This can explain the low release percent of the initial amount (13%), assuming total absorption. The release profile in this study was carried out in a FBS:PBS system. A higher release rate has been observed *in vivo* when compared to *in vitro* because of a higher degradation rate of HA and the higher solubility of proteins *in vivo* [8]. The current gradual release profile informs us that the HA microspheres developed in this study can be used as a controlled release system. The dilemma between high affinity ad low release can be resolved by modifications of the microspheres.

Aggregation of the microspheres as well as the lack of media flow have influenced low release [8]. Eventually the unreleased BMP-2 would be released when the HA

microspheres degrade, however degradation of hydroxyapatite can take up to two years. Rohanizadeh and Chung tested different methods for incorporation of BMP-2 to HA and found the immersion technique used in this study had the lowest release percentage [35]. Use of either of the other two methods, incorporation during HA precipitation or incorporation during dicalcium phosphate dehydrate conversation, may lead to higher release. Testing *in vivo* release may more accurately depict responses for the bone regeneration of rat calvarial defects tested.

The results of relaxin release from HA microspheres were continuous but ended with a much lower release rate than BMP-2 release (Figure 3.2D). A lower affinity of relaxin to HA could be the reason for this lower release because the protein may not have adsorbed into the hollow HA core. The isoelectric point of proteins has an effect on adsorption on to hydroxyapatite [36, 37]. With an isoelectric point of 8.5, BMP-2 has a positive charge when in the neutral release buffer, consisting of PBS and FBS, and is attracted to hydroxyapatite, which has a negative charge. Relaxin, however, has an isoelectric point of 6.7 and therefore has a slight negative charge in the release buffer. Zhao *et al* measured the release of hemoglobin, another neutral protein, from hydroxyapatite [38]. They found that at a neutral pH of 7.2, release of hemoglobin was the lowest compared to a more acidic pH [38]. Testing the solution after loading may further determine whether the low release percent is due to low adsorption of relaxin or low release of the incorporated amount. The release rate of relaxin from HA microspheres can be improved before use *in vivo*. While the rate was continuous with a slight initial burst release, the overall amount release was much lower than the amount loaded. As the release

rate *in vivo* is much higher than release rate *in vitro*, we anticipate that the effect would be more substantial.

The release of BMP-2 and relaxin when loaded simultaneously onto HA microspheres can be seen in Figure 3.2. This was to verify that loading multiple proteins onto HA microspheres would not negatively impact the release of the individual proteins. In the case of BMP-2, addition of relaxin didn't not significantly lower the amount released. The addition also resulted in no initial burst release of BMP-2 into the release buffer. The co-release of BMP-2 and relaxin must lead to a more controlled release of BMP-2. This shows that loading additional proteins will not affect BMP-2 release and can be used in situations that require the release of multiple growth factors for sufficient tissue regeneration. For relaxin, the addition of BMP-2 significantly increased the amount of relaxin released. This shows that BMP-2 must have changed the ability of relaxin to bind to HA and increased the amount bound and subsequently released.

Figure 3.3 shows the dose response effect of BMP-2. As BMP-2 is a osteoinductive protein, an increase in the amount of BMP-2 can increase new bone formation. 1µg per defect produced the most new bone, with a significant increase from as prepared microspheres seen at 0.5µg per defect. This is demonstrated in Figure 3.8A. Figure 3.4 shows the dose response of relaxin. Relaxin is known as an enhancer to BMP-2 and does not have any osteoinductive properties on its own [26]. In the present study, relaxin did not produce any significant bone regeneration compared to as prepared microspheres (Figure 3.8B).

Figures 3.5 and 3.6 show the enhancement effect of relaxin to 0.25µg and 0.5µg of BMP-2, respectively and can also be seen in Figure 3.9. At an amount of 0.25µg of BMP-

2, no enhancement effect was seen at any measured amount of relaxin. At an amount of 0.05µg an enhancement effect can be seen on 0.5µg of BMP-2. Interestingly, at 0.5µg of BMP-2 no enhancement effect was seen at any other amounts of relaxin. Too much relaxin may have an inhibitory effect by over activation of similar growth factor pathways. Relaxin works through the TGF-β pathway which is closely related to the BMP pathway [25]. Activation of both these bone regeneration pathways may lead to a reduction of the enhancement effect. Additional studies can use even further reduced amounts of relaxin to determine the range for a significant enhancement effect. No enhancement effect can be seen with any amount of relaxin at 0.25µg of BMP-2. This may show that a sufficient amount of BMP-2 must be present for enhancement to occur. As mentioned above, the use of reduced amounts of relaxin may result in an enhancement effect at a lower BMP-2 amount.

The present study uses an amount of BMP-2 that is lower than the amount used in other studies with similar biomaterials [39]. This amount is also far below the safe clinical amount [7]. The overall goal of this study was to lower the previously published dose of BMP-2 with the addition of relaxin. Figure 3.10 shows these comparisons. With the addition of relaxin the amount of BMP-2 can be easily reduced by 50%. A 75% reduction can also be seen when using 0.05µg of relaxin. Figure 3.7 shows high magnification images of select groups. These high magnification images demonstrate that there is no histological difference between a defect loaded with 1µg of BMP-2 and a defect loaded with 0.5µg of BMP-2 in combination with 0.05µg of BMP-2 further verifying that relaxin

can lower BMP-2 doses. This reduction supports the hypothesis that relaxin has the ability to lower BMP-2 doses with its enhancement effect, especially seen in the combination of 0.5µg of BMP-2 with 0.05µg of relaxin.

5. CONCLUSIONS

Hollow hydroxyapatite microspheres can be used as a carrier for multiple proteins. *In vitro* release of BMP-2 and relaxin shows a slow and continuous release over the measured two-week span. No inhibition to release was observed when both proteins were loaded in combination. In the case of relaxin, more release was found in the combined group. When BMP-2 and relaxin are combined in animal studies, an enhancement effect occurs with 0.5µg of BMP-2 and 0.05µg of relaxin. When compared to a previously published dose (1µg per defect), a 50% reduction in BMP-2 amount when in combination with relaxin can achieve the same bone regeneration outcome. A 75% reduction in BMP-2 amount can produce comparable bone regeneration when combined with 0.05µg of relaxin. The reduction will lead to safer and cheaper biomaterials in the future.

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