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A collagen-hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates

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Abstract

An emerging paradigm in orthopedics is that a bone healing outcome is the product of the anabolic (bone-forming) and catabolic (bone resorbing) outcomes. Recently, surgical and tissue engineering strategies have emerged that combine recombinant human bone morphogenetic proteins (rhBMPs) and bisphosphonates (BPs) in order to maximize anabolism and minimize catabolism. Collagen-based scaffolds that are the current surgical standard can bind rhBMPs, but not bisphosphonates. We hypothesized that a biomimetic collagen-hydroxyapatite (CHA) scaffold would bind both agents and produce superior *in vivo* outcomes.

Consistent with this concept, *in vitro* elution studies utilizing rhBMP-2 ELISA assays and scintillation counting of ¹⁴C-radiolabeled zoledronic acid confirmed delayed release of both agents from the CHA scaffold. Next scaffolds were tested for their capacity to form ectopic bone after surgical implantation into the rat hind limb. Using CHA, a significant 6-fold increase in bone volume (BV) was seen in rhBMP-2/ZA groups compared to rhBMP-2 alone, confirming the ability of ZA to enhance rhBMP-2 bone formation. CHA scaffolds were found to be capable of generating mineralized tissue in the absence of rhBMP-2..

This study has implications for future clinical treatments of critical bone defects. It demonstrates the relative advantages of co-delivering anabolic and anti-catabolic agents using a multi-component scaffold system.

Keywords: zoledronic acid; bisphosphonate; rhBMP-2; bone morphogenetic protein; bone tissue engineering; collagen; hydroxyapatite; CHA

1. Introduction

Bone and fracture repair occurs naturally through a series of anabolic and catabolic processes ¹. However, in the cases of severe trauma or disease, surgical intervention is required. Bone grafts and bone graft substitutes are used in the surgical repair and reconstruction of critical defects. Currently the clinical “gold standard” approach is the use of autografts. This approach is not without complications, such as donor site morbidity, quantity of available tissue, and the need for a second surgery. The use of allografts can overcome these obstacles. However, there is a worldwide shortage of donors which, together with an associated risk of disease transmission, hinder allografts as an ideal treatment option ².

Bone tissue engineering aims to develop bone graft replacements that can repair bone defects without a need for allografts or autografts. Currently, collagen matrices loaded with anabolic agents are clinically utilized as bone graft substitutes. Recombinant human bone morphogenetic proteins (rhBMPs) are potent anabolic agents that are in clinical use for the treatment of non-union and critical sized bone defects. Treatment with rhBMPs facilitates the differentiation of mesenchymal progenitors into bone forming osteoblasts ³. This leads to robust induction of new bone formation. However, in non-load bearing defects or when used at high local doses, rhBMPs have been shown to both directly and indirectly induce osteoclast differentiation ^{4,5}. This is problematic as it can lead to the premature resorption of bone ⁵⁻⁷.

An emerging paradigm for orthopaedics is that bone formed from an anabolic stimulus can be maximized by use of an anti-catabolic agent ⁸⁻¹¹. Bisphosphonates (BPs) are anti-catabolics clinically used in the treatment of osteoporosis and metabolic bone diseases, and also have also been shown to minimize bone loss due to unloading or stress shielding ¹². BPs have a high affinity for the hydroxyapatite within bone mineral. Once bound, they potently inhibit osteoclast mediated resorption ^{13,14}. Only the osteoclasts are able to mobilize and internalize bisphosphonates bound with high affinity to the bone mineral. As the

osteoclasts resorb the bone, they internalize the BPs, leading to inhibition of osteoclast activity and/or osteoclast apoptosis¹⁵⁻¹⁸.

To date, studies utilizing combined anabolic and anti-catabolic treatments have typically relied on systemic BP delivery^{12,19}. However, there are a number of adverse affects associated with systemic dosing of bisphosphonates, including stomach ulceration (oral BPs) and flu-like symptoms (intravenous dosing)^{20,21}. In rare cases major complications have been reported, such as renal failure and osteonecrosis of the jaw, although these are typically linked to underlying kidney diseases or dental problems²². Local delivery has emerged as an appealing alternative to systemic delivery as it avoids these complications and can lower the absolute BP dose required. Furthermore, it simplifies treatment for the patient as both drug delivery and surgery are combined into one procedure rather than requiring post-surgical systemic/oral administration. Multiple studies have utilized polymeric-based (poly-L-lactide, PLLA; poly(lactic-*co*-glycolic) acid, PLGA) carriers to demonstrate synergistic benefits for co-delivering rhBMPs and BPs locally^{23,24}. However, these materials can be associated with problems, including the release of acidic degradation by-products that can alter the pH in surrounding tissue. In turn, this can cause adverse tissue and inflammatory reactions^{25,26}.

Collagen, as a naturally derived biomaterial, has many advantages over most synthetically derived polymers. It shows superior biocompatibility, biodegradability, interconnected porous architecture [16], and a capacity to bind rhBMPs. As such, it has been adopted as an rhBMP delivery system now in clinical use (rhBMP-2 - Medtronic INFUSE® and rhBMP-7 – Olympus OP-1® bone graft). However, while collagen provides structural stability to a number of endogenous tissues including bone, scaffolds comprising of collagen possess poor load bearing capabilities.

This study trialed a recently developed composite collagen-hydroxyapatite (CHA) scaffold that incorporates both collagen and hydroxyapatite in a porous scaffold matrix²⁷. These materials are the two major constituents of bone and a logical choice as the basis of a biomimetic scaffold capable of

supporting and promoting bone regeneration²⁸. Furthermore, the CHA scaffold combines the advantages conferred by the mechanical strength of ceramics with the biological advantages of collagen. The CHA scaffold has been developed using a patented fabrication process [20] and incorporates features used in the development of a range of collagen based scaffolds optimized in terms of composition^{26,29}, cross linking density^{30,31} and pore architecture³²⁻³⁵, for use in bone tissue engineering applications. The CHA scaffold has 99% porosity with higher pore interconnectivity than standard collagen, measured in terms of scaffold permeability ($0.4 \times 10^{-9} \text{ m}^4/\text{Ns}$ vs. $4.5 \times 10^{-9} \text{ m}^4/\text{Ns}$). The addition of hydroxyapatite confers improved mechanical strength over collagen alone (1.3 kPa vs. 0.5 kPa). This CHA scaffold has previously facilitated healing of a critical-sized rat calvarial defect without the addition of exogenous pro-osteogenic factors.³⁶

It was hypothesized that this biphasic collagen/HA scaffold would show superior binding of an rhBMP-2/BP combination. Collagen facilitates rhBMP-2 binding³⁷ and BPs have a high affinity for hydroxyapatite³⁸. It was further speculated that this combination of collagen, hydroxyapatite, rhBMP-2 and the bisphosphonate Zoledronic Acid (ZA) would achieve superior bone formation. The first aim of this study was to investigate the binding and retention of rhBMP-2 and ZA to the biphasic components of the scaffold in comparison to control collagen scaffolds. The second aim was to then test the efficacy of this system in an ectopic bone formation model in a rodent hind limb.

2. Materials and Methods

2.1. Pharmaceuticals

Recombinant human Bone Morphogenetic Protein-2 (rhBMP-2) from the INFUSE® Bone Graft Small Kit was purchased from Medtronic (Memphis, TN, USA). Zoledronic Acid (ZA) was purchased from AXXORA, LLC (San Diego, CA, USA). For *in vitro* studies, a stock solution of ¹⁴C-radiolabeled ZA (¹⁴C-ZA) with a specific activity of 7.027 MBq/mg was supplied by Novartis Pharma AG (Switzerland). This was diluted with unlabeled ZA to generate a 1mg/ml solution with a specific activity of 703 kBq/mg. AlexaFluor 647 was purchased from Life Technologies (Victoria, Australia).

2.2. Scaffold fabrication

The collagen-hydroxyapatite scaffold (CHA), was fabricated as previously described³⁶. In brief, collagen slurries were produced by the homogenization of fibrillar collagen (Collagen Matrix, Franklin Lakes, NJ, USA) within a 0.5 M acetic acid solution. Slurries were homogenized in a reaction vessel, cooled to 4°C by a WK1250 cooling system (Lauda, Westbury, NY, USA), using an overhead blender (IKA Works Inc., Wilmington, NC, USA). In parallel, hydroxyapatite (HA) particles with a mean diameter of 5 µm (Plasma Biototal Limited, North Derbyshire, UK) were suspended in a 0.5 M acetic acid solution. Collagen concentration was 0.1 g/mL and HA concentration was 0.2 g/mL. The final composite slurry was produced by lyophilization after being pipetted into a stainless steel pan (125 × 125 mm, grade 304 SS) and cooled to -40°C at a constant cooling rate of 0.9° C/ min. After freezing, ice crystals were removed by sublimation for 17 h at 0°C and 200mTorr^{32,39}. This produced scaffolds with a pore size of approx 100 µm. Dehydrothermal (DHT) cross linking treatment was carried out as previously described⁴⁰ under a vacuum of 0.05 bar at a temperature of 120°C for 24 hours. CHA scaffolds were sliced into 6mm x 3mm sections for both *in vitro* and *in vivo* analysis.

A porous collagen sponge was used as a control for *in vitro* investigations. Rectangular sponges (6mm x 3mm) were sliced from 4mm thick collagen sheets supplied from INFUSE® Bone Graft Small Kit (Medtronic, USA). Each scaffold was loaded with 10 µg rhBMP-2. Following manufacturer's instructions, rhBMP-2 solution (20 µL × 1 mg/mL) was dripped onto the collagen scaffold 20 minutes prior to surgical insertion.

2.3. Bisphosphonate binding to CHA and collagen scaffolds

Fluorescently labeled bisphosphonate (Pamidronate, PAM) was used to assess the binding of the BP within the CHA and collagen. PAM was fluorescently labeled with a commercially available AlexaFluor 647 (Life Technologies, Victoria, Australia) according to the manufacturer's protocol. The resultant fluorescently labeled BP was termed AlexaPam 647. PAM has a free amine group and can be readily labeled using protein labeling kits as opposed to the nitrogen containing ring-structure of the ZA side-chain. The addition of a dye moiety does not compromise the 'bone hook' involved with mineral avidity, allowing it to be used to investigate bisphosphonate binding interactions *in vitro*.

CHA scaffolds and collagen sponges were loaded with 2 µg AlexaPam 647 and washed for 24 h in PBS at 37°C. The scaffolds were imaged using Leica TCS SP5 confocal microscope (Leica Microsystems, NSW, Australia) both pre- and post-washing.

2.4. Bisphosphonate elution from CHA and collagen scaffolds

Release of bisphosphonate from both CHA and collagen scaffolds was measured using radiolabelled zoledronic acid (¹⁴C-ZA). Specimens loaded with either 1 µg ¹⁴C-ZA or 2 µg of ¹⁴C-ZA were prepared by the addition of 100 µl of ¹⁴C-ZA solution via pipette. Both CHA and collagen scaffolds were compared. The scaffolds were washed in PBS for 24 h at 37°C. ¹⁴C-ZA elution was measured with Tri-

Carb scintillation counter (Packard, MN, USA) using a scintillation cocktail (5mL Ultima Gold DIPN, PerkinElmer, Inc. MA, USA) and counted in 20 mL Pico Prias polyethylene vials (Perkin-Elmer, Australia).

2.5. RhBMP elution from CHA and collagen scaffolds

The release of rhBMP-2 from both CHA and collagen scaffolds was measured over 6 weeks. N=3 scaffolds were measured per group. Samples were loaded with 10 µg rhBMP-2 and were incubated at 37°C in Protein LoBind tubes (Eppendorf, UK) containing 2 mL of elution buffer (1% BSA/PBS) formulated to prevent adsorption to the vessel. At time points of 1 minute (0.01 h), 1 h, 1 day (24 h), 1 week (168 h), 3 weeks (504 h), and 6 weeks (1008 h) scaffolds were transferred to new tubes containing fresh elution buffer. Collected samples were stored at -20°C. The amount of rhBMP-2 drug released was quantified using a Qunatikine® BMP-2 Immunoassay (PDBP200, R&D Systems, Wiesbaden, Germany) as per the manufacturer's instructions.

2.6. Cell culture and cell viability

MC3T3-E1 murine pre-osteoblasts were cultured in α -MEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin (Invitrogen) and used to determine the ability of HA within CHA scaffolds to protect cells from cytotoxic ZA. MC3T3-E1 cells were seeded in 24-well plates at a density of 2×10^4 cells per well in 500 µl of standard media containing 50 µM Zoledronic acid (ZA, kindly supplied by Novartis Pharmaceuticals, NSW, Australia). In order to determine the ability of CHA to protect cell viability by binding and retaining BP in comparison to collagen scaffolds, 6 mm × 3 mm sections of both CHA and collagen scaffolds were placed within the wells.

Viability was measured at 4 day and 7 days post seeding. Cellular viability was assessed using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were incubated with the viability solution for 30 min at 37°C and read using a spectrophotometer at 490 nm. All samples were assayed in triplicate and the results are representative of n=6.

2.7. Animal care

Male Wistar rats (10-12 week, average weight 400-450 g) were housed in autoclavable polypropylene solid boxes with stainless steel lids and polycarbonate water bottles with food and mouse chow supplied *ad libitum*. Ethics approval was obtained for all procedures from the Westmead Hospital Animal Ethics Committee

2.8. Surgical model

Ectopic bone formation was assessed in a bilateral quadriceps muscle pouch via surgical implantation of the drug loaded CHA scaffolds (Table 1). The surgical model and dosing regimens were based on previously published methods where rhBMP-2 and ZA were delivered in a carboxymethylcellulose and polymer carrier^{41,42}. Agents were applied to scaffolds 20 min prior to implantation using a pipette.

In brief, anesthesia was induced and maintained using inhaled isoflurane. The operative sites was trimmed and prepared with povidone-iodine. Muscle pouches were created in the hind limb (in line with the femur) using a scalpel. The CHA scaffolds were inserted and the muscle and skin closed using a 5-0 Vicryl suture (Ethicon, Somerville, NJ, USA). Following surgery, animals were recovered on a heated pad and placed in recovery cages. Post-operative pain was managed with subcutaneously injected buprenorphine (0.05mg/kg) and dehydration was managed by saline injection, as required. Animals were

monitored daily and weighed weekly for the duration of the study. At 4 weeks post surgery, animals were euthanized via CO₂ asphyxiation, and the femurs and surrounding muscle were collected for subsequent analyses.

Group	Scaffold	Local Anabolic Agent	Local Anti-Catabolic Agent	Specimens
1	CHA	-	-	8
2	CHA	5 µg rhBMP-2	-	8
3	CHA	5 µg rhBMP-2	10 µg ZA	8
4	CHA	10 µg rhBMP-2	-	8
5	CHA	10 µg rhBMP-2	2 µg ZA	8
6	CHA	10 µg rhBMP-2	10 µg ZA	8

Table 1: Experimental design for the ectopic bone formation study

2.9. Radiographic analysis

The placement of the ectopic bone nodules relative to the femur was determined by radiography using a digital X-ray (XR) machine (Faxitron X-ray Corp, Wheeling, IL, USA) of whole hind limbs (27 kV, ×2 magnification). Ectopic bone samples and the surrounding soft tissue was further isolated and individual XR images captured (27 kV, 3× magnification). Samples were examined by micro-computed tomography (µCT) scanning using a SkyScan 1174 compact µCT scanner (SkyScan, Kontich, Belgium). The primary outcome measure for the study was total bone volume of the entire ectopic bone nodule. Samples were scanned in 70% ethanol at 14.7 µm pixel resolution, 0.5 mm aluminium filter, 50 kV X-ray tube voltage and 800 µA tube electric current. The images were reconstructed using NRecon, version 1.5.1.5 (SkyScan) and three dimensional assessments of the entire ectopic bone nodule were conducted

with CTAnalyser software, version 1.9.2.3 (SkyScan). The bone tissue was defined as calcified tissue detected by microCT using a predetermined global threshold set at 0.4 g/cm³ of calcium hydroxyapatite (HA). Secondary outcome measurements included bone tissue alone mineral density (bone TMD, g/cm³ of calcium hydroxyapatite, HA) of entire ectopic bone nodule. In addition, the trabecular-like region of the ectopic bone nodule was delineated from the denser cortical sheath. Additional outcome measurements of the trabecular measurements included trabecular thickness (Tb.Th, mm) and trabecular number (Tb.N, mm⁻¹). A global threshold representing bone (44-255) was defined following analysis of consecutive two-dimensional slices of bone samples on CTAnalyser software. Representative three-dimensional bone pellets were reconstructed with transaxial slices (50 slices) from the pellet's mid-section, using CTVol Realistic Visualization software version 2.1.0.0 (SkyScan). Representative samples from each group were selected as having a BV closest to the group's mean.

2.10. Tissue histology

For paraffin histology, samples were decalcified and embedded in paraffin blocks. 0.5 mm-thick sections were cut using a Leica RM 2155 Microtome (Leica Microsystems, Wetzlar, Germany). Sections were stained with Tartrate Resistant Acid Phosphatase (TRAP) for osteoclasts. Stained sections were scanned with ScanScope digital slide scanner (Aperio Technologies, Vista, CA, USA), and images were captured with ImageScope (Aperio Technologies). Representative images were selected and cropped using ScanScope and Photoshop (Adobe Technologies) software.

2.11. Statistical analysis

For the statistical analysis of *in vitro* elution assays, significance was determined by student's T-test. For the *in vivo* studies the formation of ectopic bone was not able to be assumed to follow a normalized

distribution. Thus non-parametric Kruskal Wallis and Mann Whitney U tests were performed. All analyses were performed using SPSS Statistics version 17 (SPSS, Chicago, IL, USA). Statistical significance was set at $\alpha < 0.05$.

3. Results

3.1. Bisphosphonate binding potential of CHA Scaffolds

To examine the binding affinity of BP to the HA particles within CHA scaffolds, the incorporation of AlexaPam 647 within the scaffold was assessed via fluorescent confocal microscopy. Control collagen sponge was used as a comparative. Both scaffold types were assessed immediately after loading with AlexaPam 647 and thorough washing. No florescent BP was observed to be bound to the collagen scaffold. In contrast, AlexaPam 647 bound to the HA particles within the struts of the CHA scaffold (Fig 1A).

Next this phenomenon was quantified by assaying the release of radiolabelled ^{14}C -ZA from both scaffold types (Fig 1B). Background controls were assayed using both no eluent and eluent from CHA scaffolds without ^{14}C -ZA loading and neither showed a significant reading. There was a significant difference between the two scaffold types in terms of total ^{14}C -ZA eluted ($P < 0.001$). The collagen scaffolds released $>90\%$ ^{14}C -ZA after 24 h. Only 7% of the ^{14}C -ZA was eluted from CHA scaffolds. Increasing the ^{14}C -ZA concentration from 1 μg to 2 μg within the scaffolds did not change the proportion of BP eluted from either scaffold type.

These results demonstrate the potential of the CHA scaffold to retain and slowly release BP in comparison to collagen alone.

3.2. Elution of rhBMP-2 from porous CHA and collagen scaffolds

In order to confirm the binding of rhBMP-2 to CHA scaffolds, an *in vitro* elution study was performed over 6 weeks with eluents quantified by ELISA. The release profile of control porous collagen scaffold was used as a comparison.

In both scaffold types the most rapid release of rhBMP-2 was observed within the first minute, showing an initial burst. This was followed by a sustained release of rhBMP-2 (Fig 2). The amount of rhBMP-2 released from CHA scaffolds was 45-50% of the amount of rhBMP-2 released from collagen, indicating a greater retention of rhBMP-2 within the CHA scaffolds. Although, the total release of rhBMP-2 from both scaffold types represented less than 30% of the total rhBMP-2 incorporated into the scaffold it is important to note that the scaffolds did not undergo the substantive biodegradation seen after surgical implantation.

3.3. HA within CHA scaffolds yields protection against the cytotoxic effects of ZA *in vitro*

A cell culture study was performed to determine whether the presence of CHA scaffolds containing HA could protect cells from the cytotoxic effects of high-dose BP. MC3T3 pre-osteoblasts were cultured in 50 μ M ZA, a dose that has been previously shown to be cytotoxic to cells⁴³. Consistent with prior reports, ZA treatment significantly decreased viable cell numbers in 4 days and no viable cells were detectable by day 7. However the presence of HA in the CHA scaffolds afforded substantive protection against these effects (Fig 3) with cells surviving at 7 days while in contrast, the Collagen ZA group failed to offer a cytotoxic-protective benefit against the effects of bisphosphonate treatment on cell viability at either time point, whereby no cell viability was detected by Day 7.

These data indicate that the presence of the CHA scaffolds is able to ameliorate the cytotoxic effects of unbound bisphosphonate on culture osteoprogenitors.

3.4. Local co-delivery of rhBMP-2 and bisphosphonate via porous CHA scaffolds

The capacity of rhBMP-2 and BPs to act synergistically when delivered by CHA scaffold was assessed in an *in vivo* ectopic bone formation model (Table 1). No complications occurred with surgical

implantation. At 4 weeks post-op, specimens were harvested and analyzed by X-ray, μ CT, and tissue histology. X-ray revealed ectopic bone formation in the hind limb in all the treatment groups, including some radiodense tissue in the saline-soaked CHA scaffold controls (Fig 4). Via radiographs, greater bone formation was apparent in the rhBMP-2 + ZA combination groups compared to rhBMP-2 alone.

The volume of bone was subsequently measured by μ CT (Fig 5). Net bone volume (BV, mm^3) significantly increased with local co-delivery of rhBMP-2 and ZA in comparison to the rhBMP-2 and saline control groups (Fig 5A). ZA increased BV by 6-fold rhBMP-2/ZA treatment groups in comparison to rhBMP-2 alone controls ($p < 0.01$). In treatment groups containing ZA, no difference in BV was observed between 5 μg and 10 μg doses of rhBMP-2. Prior to implantation, the hydroxyapatite within the CHA scaffold was not detected via μ CT scanning or X-ray. Therefore the BV noted within the CHA control group was an indication of the osteogenic properties of this scaffold. Furthermore, no significant additional benefit was seen with the addition of either a low or high dose of rhBMP-2 (5 μg or 10 μg); highlighting the capability of the CHA scaffold to produce *de novo* mineralized tissue in an ectopic site without the need for growth factors.

Bone tissue mineral density (BTMD) and both trabecular thickness (Tb.Th) and number (Tb.N) was investigated to determine the degree of bone formation as a result of the different treatment groups. In the combined rhBMP-2/ZA dosing regimens, 10 μg doses of rhBMP-2 led to significantly higher BTMD in comparison to 10 μg rhBMP-2 alone groups. The BTMD of CHA control scaffold was significantly lower in comparison to both the rhBMP-2 alone groups and the combined rhBMP-2/ZA groups. There was no difference within the 5 μg rhBMP-2 groups either with or without ZA (Fig 5B). Furthermore, Tb.Th increased significantly in the combined rhBMP-2/ZA groups in comparison to the rhBMP-2 alone controls. A significant increase in Tb.N was only observed in 10 μg rhBMP-2 + 10 μg ZA group compared to the 10 μg rhBMP-2 control. There was no significant difference between the other groups (Fig 5C & D).

As a secondary outcome measure, the microarchitecture of the ectopic bone nodules was examined by 3D μ CT reconstructions of transaxial mid-sections of bone nodule (Fig 6). Larger pellets with greater internal trabecular-like bone were observed to form as a result of combined rhBMP-2/ZA treatment compared to rhBMP-2 alone groups and CHA control scaffolds, which formed smaller, hollow nodules. Histological analysis comparing TRAP positive staining in all the treatment groups demonstrated a reduction in osteoclasts in the ZA co-treatment groups compared to the corresponding rhBMP-2 controls (Fig 7B-F). This is consistent with reduced bone volume rhBMP-2 control groups compared to the rhBMP-2/ZA groups, suggestive of reduced catabolism. No TRAP positive cells were observed in the saline-soaked CHA group (Fig 7A).

4. Discussion

An emerging paradigm in orthopedics is the concept of synergistically harnessing anabolic and anti-catabolic drug treatments for improved bone healing⁴⁴⁻⁴⁷. BPs alone inhibit bone resorption and do not promote bone formation. However, when combined with an anabolic agent such as rhBMPs, bone formation is increased significantly in comparison to BMPs alone¹². Historically, a majority of studies have used local rhBMPs delivered with systemic BPs, in accordance with current clinical practice. However, local delivery has increasing appeal as it can minimize the total BP dose and avoid adverse effects associated with systemic BP treatment. This study describes a combination rhBMP and BP delivered via a collagen-hydroxyapatite (CHA) scaffold. Critically, fluorescent AlexaPam 647 and ¹⁴C-ZA were found to bind to the CHA scaffold but not control collagen sponge (Fig 1) and both showed a similar sustained release profile for rhBMP-2 (Fig 2).

Currently porous collagen sponge remains the standard for orthopedic delivery of rhBMP-2 with rhBMP binding being cited as a major advantage. However, we hypothesized that collagen would show poor BP binding, and this was confirmed experimentally. The addition of 0.2g/ml hydroxyapatite in the CHA scaffolds was sufficient to bind BP and, with no difference in proportion bound between 1 µg and 2 µg ZA, this indicates the scaffolds were not saturated. The binding and sequestration of BP within the CHA scaffold could have multiple advantages. High local doses of BPs are potentially toxic and can impede healing or bone-graft fixation⁴⁸. The presence of a ceramic phase that can prevent the rapid uptake of BP by cells other than osteoclasts is hypothesized to ameliorate these negative effects. This concept is further supported by our data showing that the cytotoxicity of high dose ZA to cultured pre-osteoblasts can be counteracted by the presence of CHA scaffolds but not control collagen (Fig 3). This concept of preventing local BP toxicity using scaffolds containing ceramic microparticles is likely to be broadly translatable to a range of polymeric and natural scaffolds but the CHA scaffold also has the advantage of binding rhBMP-2.

The capacity of collagen scaffolds to bind rhBMP-2 has been previously reported^{49,50}. It was hypothesized that the inclusion of hydroxyapatite in the CHA scaffold would not significantly impair rhBMP-2 binding, as collagen remained a major component. ELISA assays of serial eluent samples demonstrate that the release rate of rhBMP-2 from CHA is slower than that of control collagen, although following a similar sustained release profile. While burst release is an important initiator of new bone formation, sustained delivery may reduce the initial inflammatory response⁵¹, and has been associated with increased bone formation in prior reports⁵². The combination of collagen and hydroxyapatite has been utilized in a number of previous studies^{53,54}, but many compositions demonstrate only limited porosity and osteoconductivity. The CHA scaffold is a highly porous collagen-hydroxyapatite scaffold that exhibits significantly improved bioactivity and *in vitro* mineralization over collagen alone³⁶. CHA has the benefit of the biocompatibility and rapid degradation associated with collagen combined with the more robust mechanical properties conferred by the HA component. Both the composition (HA for ZA binding and collagen for rhBMP-2 adsorption) and hydrophilic qualities (for absorption of rhBMP-2/ZA solution) of CHA ensures easy application of the drugs onto the scaffold.

To investigate the relative advantages of an rhBMP-2 vs. rhBMP-2/ZA combined treatment, a dosed CHA scaffold was implanted into the hind limbs of rats to induce ectopic bone formation. Doses of rhBMP-2 were chosen at a lower range (5 µg and 10 µg) than other synthetic scaffold systems^{10,11} due to the favorable release profile of rhBMP-2 from collagen. Moreover, rhBMPs are costly to produce and one translational benefit of combined therapies is the potential to reduce the amount of rhBMP required to produce a comparable bone formation outcome. A significant increase in both BV and Tb.Th (Fig 5) was observed in the rhBMP-2/ZA treatment groups in comparison to their rhBMP-2 alone controls. This highlights the considerable increase in net bone that can be achieved by co-delivery of an anti-resorptive agent, which has been borne out by the prior art^{11,23,24,48,55-57}. 3D µCT reconstructions confirmed larger bone nodules with greater internal structure in the samples containing ZA (Fig 6). Mechanistically, the

anti-resorptive effects of ZA were confirmed by histology, and revealed decreased osteoclasts in the presence of ZA (Fig 7). This decrease in remodelling is desirable within an unloaded model, but future work will be required to determine dosing that produces bone with good material properties and eventual remodelling.

One noteworthy observation was the formation of mineralized tissue in all treatment groups, including saline-soaked CHA scaffolds. This was further confirmed using μ CT. While the CHA scaffolds contain hydroxyapatite that would produce a basal level of radiopacity, saline-soaked CHA was not visible by X-ray. Further, saline-soaked CHA analyzed by μ CT and a comparison to implanted specimens showed a net and significant increase in bone volume. The potential of CHA scaffolds to promote mineralization and heal bone voids was observed previously within a calvarial defect ¹. However this study demonstrates for the first time the capacity of this material to promote matrix mineralization in an ectopic and non-osseous site. The significantly greater BMTD levels compared to the rhBMP-2 groups as well as lack of TRAP+ cells suggests that this may be the result of dense mineral depositions initially forming along the struts of the scaffold. This capacity for mineralization independent of rhBMP-2 may be suggestive of with increased osteoconduction and/or osteoinduction in the context of an osseous defect, although this will need to be confirmed experimentally.

5. Conclusion

This study demonstrates the advantages of co-delivery of anabolic and anti-catabolic agents using CHA, a scaffold system comprising collagen and hydroxyapatite. CHA demonstrates a capacity for both rhBMP-2 and BP binding and delivery, which may make it superior for combination treatments compared to commercially available pure collagen scaffolds. Notably, the CHA scaffolds demonstrate a capacity for mineralization in the absence of additional osteogenic factors. This approach can be seen as a template

for developing multi-component scaffolds to allow for improved delivery of multiple pharmaceutical or biological agents.

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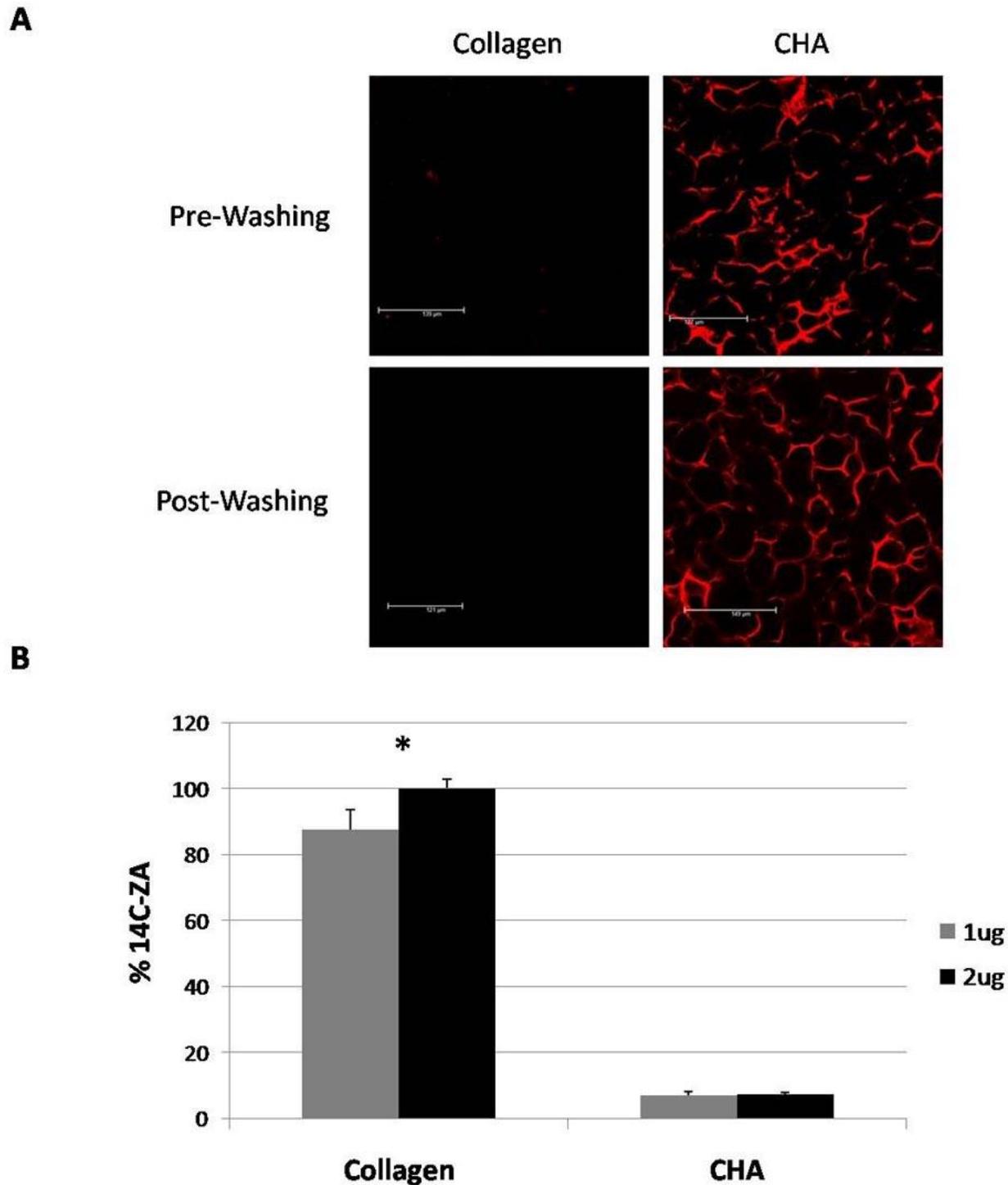


Figure 1: (A) Representative images of the fluorescently labeled BP (AlexaPam 647 – red in colour) within both scaffold types. No AlexaPam 647 is visible in the collagen scaffolds pre- or post-washing. Within CHA scaffolds the struts are labelled red both pre- and post-washing due to the retention of AlexaPam 647 by the hydroxyapatite. (B) Quantification of ^{14}C -ZA elution from porous collagen and CHA scaffolds post-washing. * $P < 0.01$ in comparison to CHA elution of both $1\ \mu\text{g}$ and $2\ \mu\text{g}$ ^{14}C -ZA

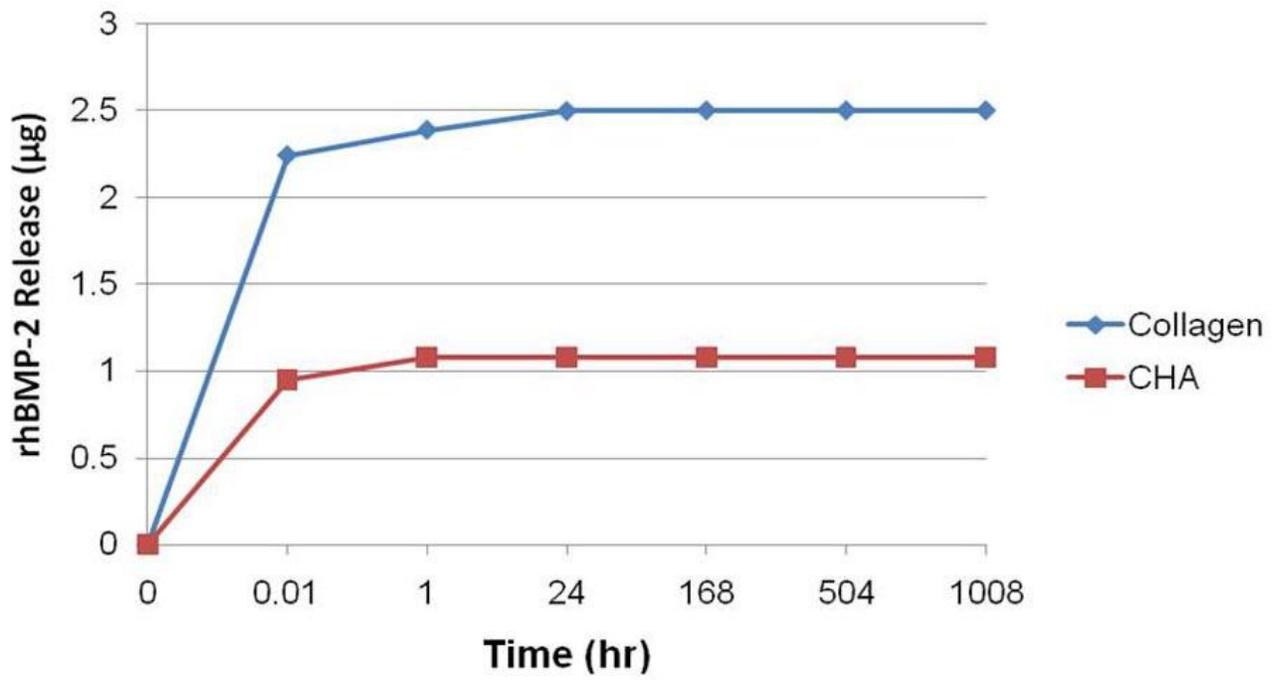


Figure 2: *In vitro* rhBMP-2 elution characteristics over 6 weeks from porous collagen and CHA scaffolds

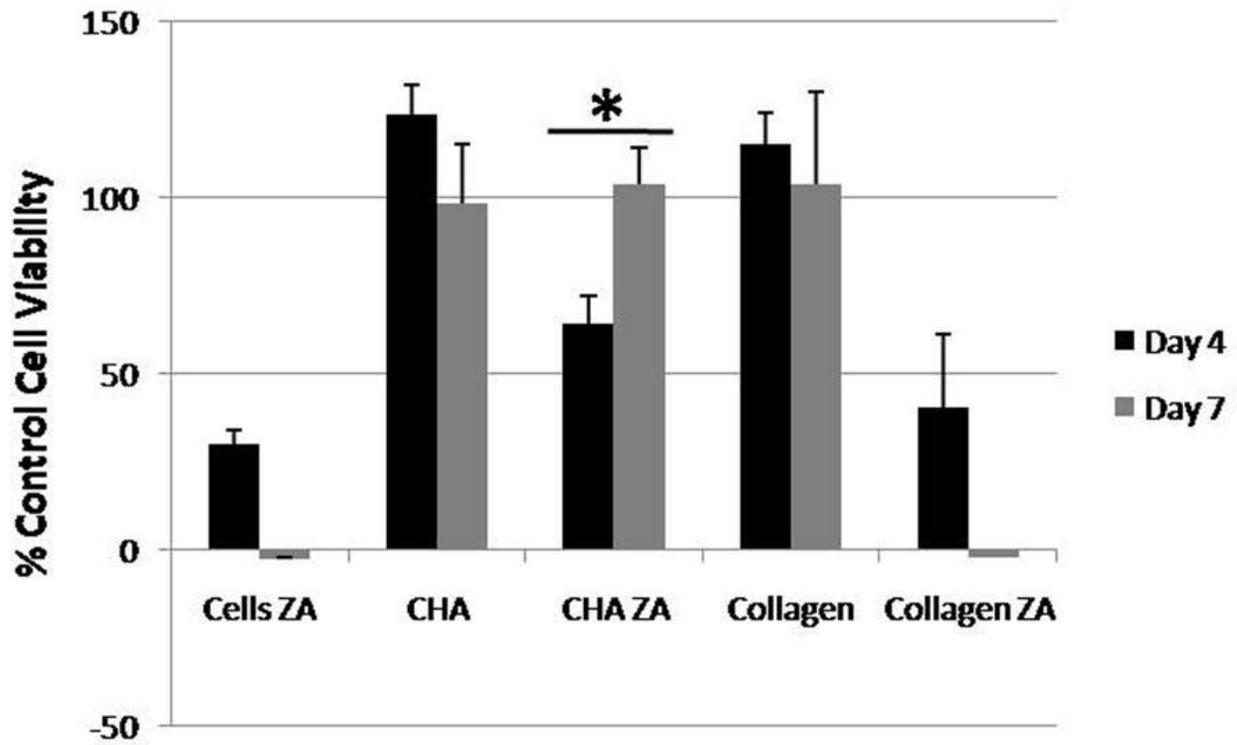


Figure 3: MC3T3 cell viability in the presence of collagen and CHA with and without ZA. CHA has a protective effect against the cytotoxic effects of ZA on cell viability in comparison to collagen. *P < 0.05 in relation to Collagen ZA at Day 4 and Day 7

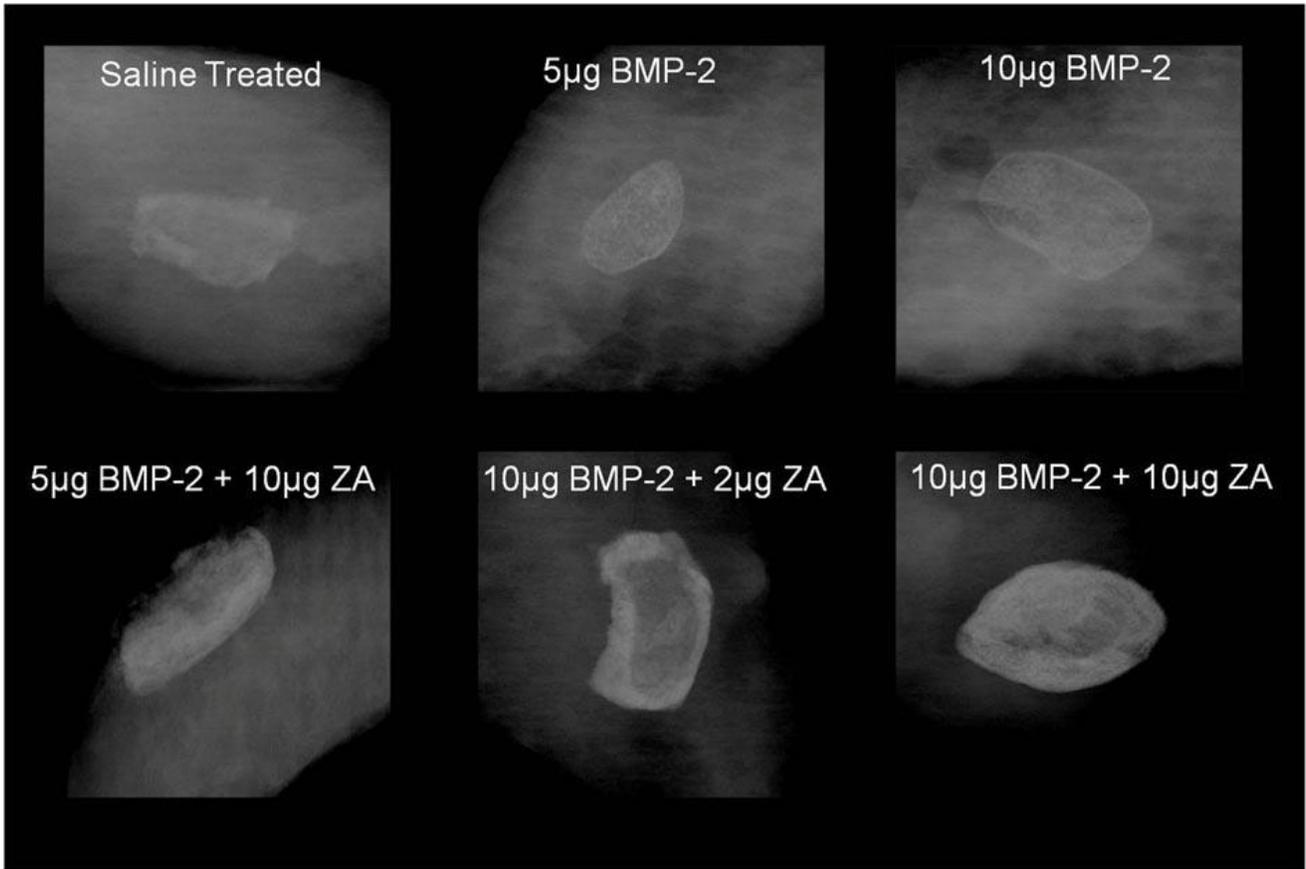


Figure 4: Representative XR images of pellets within all treatment groups at 4 weeks post-implantation demonstrating bone formation

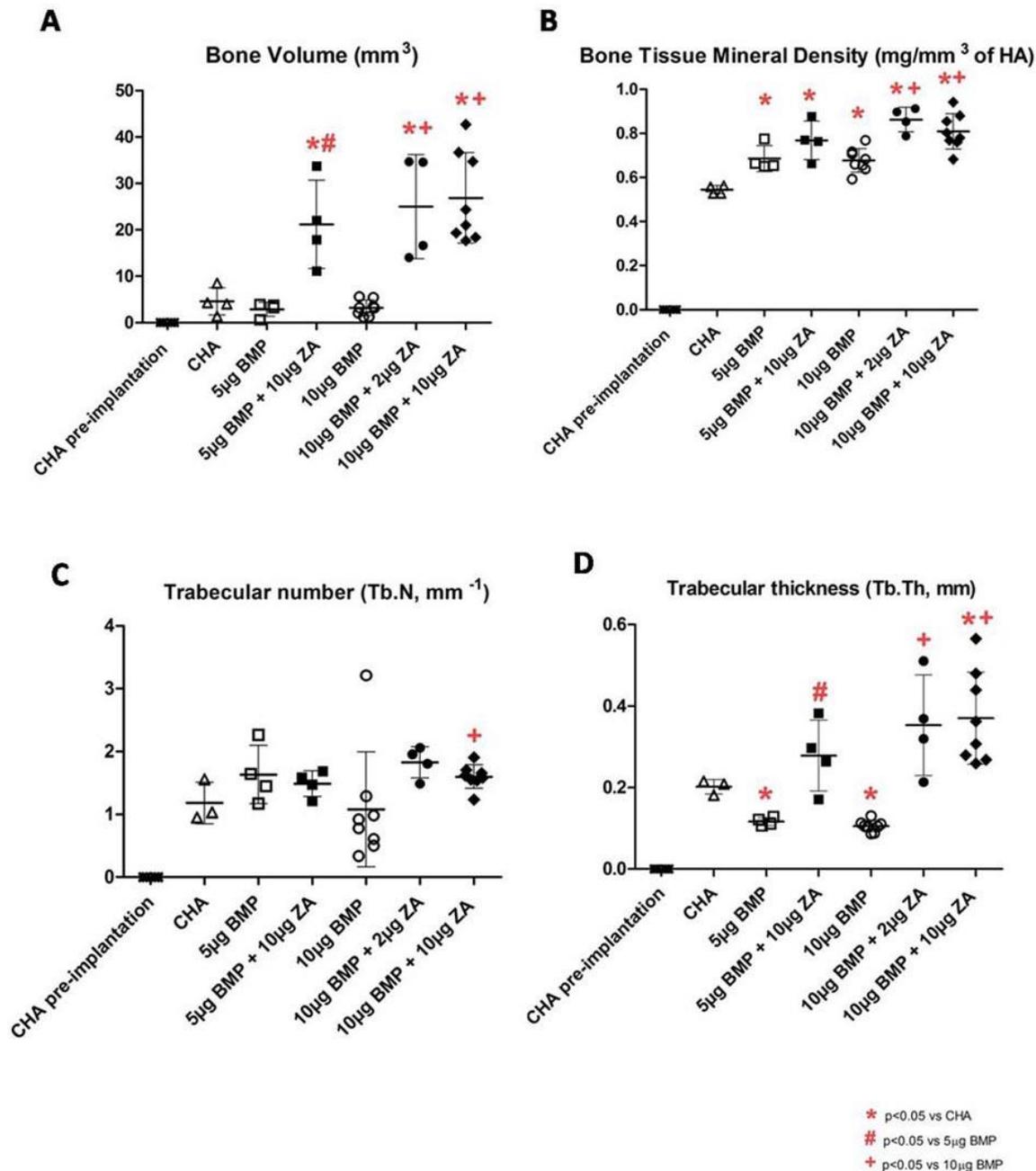


Figure 5: MicroCT analysis of ectopic bone nodules at 4 weeks post-scaffold implantation. Bone volume (BV, mm³) significantly increased in the combined rhBMP-2/ZA treatment groups compared to their respective rhBMP-2 controls (A). Bone tissue mineral density (BTMD, mg/mm³) significantly increased with the addition of rhBMP-2. This was further increased in the 10 µg rhBMP-2/ZA groups compared to respective 10 µg rhBMP-2 control group (B). Trabecular number (Tb. N, mm⁻¹) increased with the highest doses of rhBMP-2/ZA. There was no difference seen between the other groups (C). The addition of ZA increased trabecular thickness (Tb.Th, mm) in comparison to rhBMP-2 alone treatment groups (D). Error bars represent standard deviation.

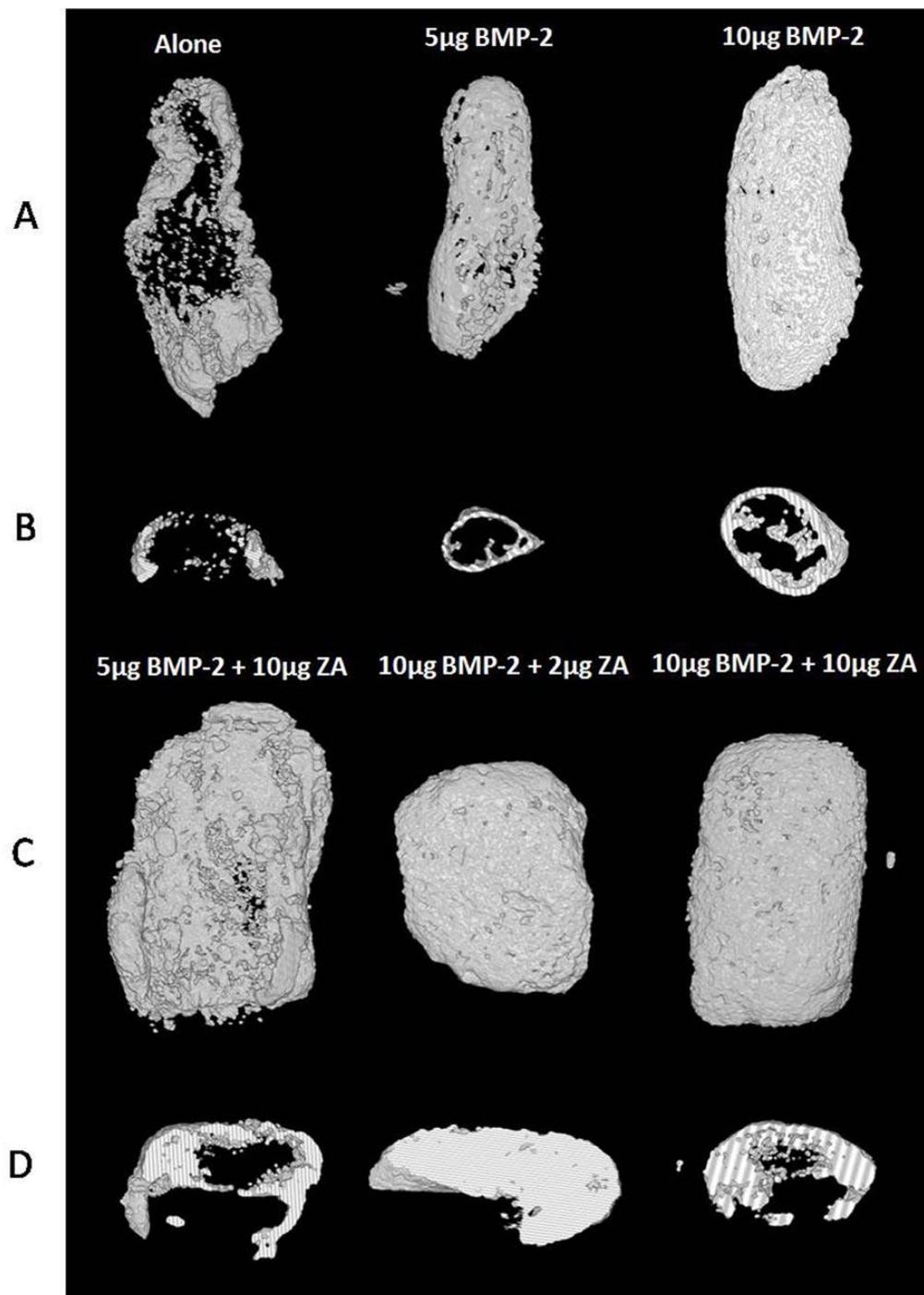


Figure 6: (A & B) Representative MicroCT reconstructions, (C & D) corresponding transaxial slices (stack of 50 slices) of microCT images of bone nodules resulting from CHA scaffold loaded with saline/rhBMP-2/rhBMP-2 + ZA treatments

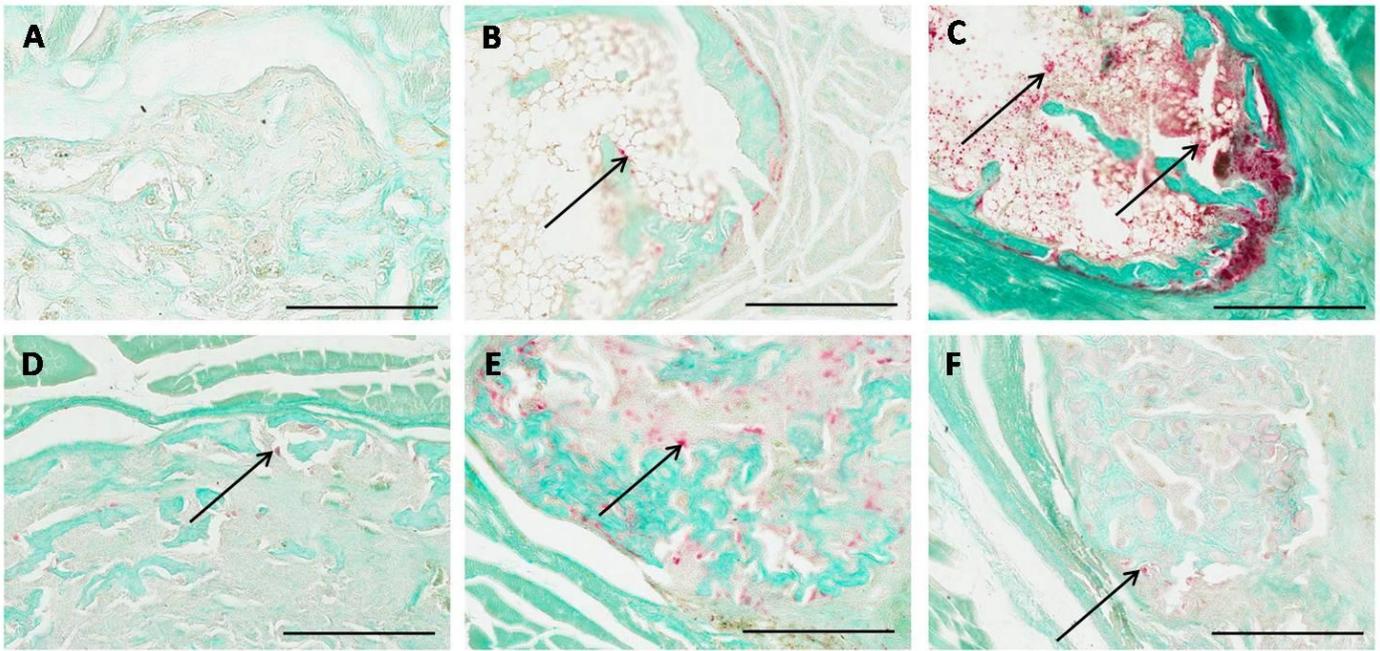


Figure 7: Representative Tartrate Resistant Acid Phosphatase (TRAP)-stained histological sections of osteoclasts (stained in red) in trabecular-like structure of ectopic bone formed following 4 weeks intramuscular implantation. Arrows indicate stained osteoclasts. (A) CHA alone, (B) 5 µg rhBMP-2, (C) 10 µg rhBMP-2, (D) 5 µg BMP +10 µg ZA, (E) 10 µg BMP + 2 µg ZA, (F) 10 µg BMP + 10 µg ZA. Scale bars = 500 µm