# COMPARATIVE STUDY OF HETEROTOPIC BONE INDUCTION USING PORCINE BONE MORPHOGENETIC PROTEINS DELIVERED INTO THE RODENT SUBCUTANEOUS SPACE WITH ALLOGENEIC AND XENOGENEIC COLLAGEN CARRIERS

by

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A research report submitted to the Faculty of Health Sciences, School of Dentistry, University of Pretoria, in partial fulfilment of the requirements for the degree of Master of Dentistry in the branch of Periodontics and Oral Medicine.

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# DECLARATION

I, Govindrau Udaibhan Mohangi, declare that this research report is my own work and has not previously been submitted for any other degree at this or any other university.

_____                    _____

Govindrau Udaibhan Mohangi                          Date

## DEDICATION

To my wife, Kesh for her invaluable encouragement during my studies and her unwavering support during the preparation of this research report.

To my daughters, Tashta and Tahlia, for their patience and understanding when the research report had to take preference.

# ACKNOWLEDGEMENTS

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# ABSTRACT

Demineralised bone matrix (DBM) is an allograft material widely used in orthopaedics and dentistry for the repair of bone defects and as a bulking agent for autogenous bone grafts. The DBM contains bone morphogenetic proteins (BMPs), a distinct family of morphogens which form part of the larger transforming growth factor-س (TGF-ں) super-family and are responsible for promoting osteogenesis, cellular differentiation, proliferation and maturation. BMPs induce and regulate bone formation during embryogenesis and in postnatal life participate in normal bone homeostasis and in the healing of bone defects, e.g. traumatic or infective defects.

Data are presented that demonstrate that allogeneic DBM can be fortified with xenogeneically sourced BMP complex to improve DBM performance *in vivo*. Rat DBM (rDBM) was fortified with BMP complex purified from porcine diaphyseal bone. Porcine BMP (pBMP) complex was standardised for its content of BMP-2, by using enzyme-linked immunosorbent assay.

Implantation of 25 mg rat allogeneic DBM fortified with 0, 3, 6 and 12 mg BMP complex per gram of DBM resulted in dose dependant up-regulation of bone formation on day 21, as scored histologically and against the activity of alkaline phosphatase, an enzyme marker specific for bone formation. Allogeneic rat insoluble collagenous bone matrix (rICBM), and inactivated xenogeneic porcine ICBM (enzymatically treated porcine ICBM or pICBM), loaded with the same doses of pBMP complex, were capable of inducing new bone formation, but with lower osteogenesis scores when compared to DBM on day 21.

The results taken from the study over 21 days adds to our knowledge of the activity of naturally sourced BMPs with collagenous delivery systems in medium term heterotopic bioassays. This information may also assist in developing tissue banked DBM that is fortified by the adsorption of xenogeneically sourced BMP complex, thereby improving the performance of human-sourced DBM.

# INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta (TGF-ß) super-family, a large family of growth factors whose numbers increase as newer members are discovered (Ripamonti and Renton, 2006; Wikesjö *et al*., 2007). BMPs are made up of highly conserved amino acid sequences and show similarity across species and mammals share significant sequence homologies in their BMPs (Sampath and Reddi, 1983). Their actions have been described in development of many tissues other than bone, such as heart, gut, kidney and teeth (Reddi, 1992; Nakashima and Reddi, 2003; Matthews, 2005).

Marshall Urist demonstrated the ability of demineralised bone matrix to induce ectopic bone formation when implanted in intramuscular sites in rabbits, rats, mice and guinea pigs (Urist, 1965). He was aware that the process of bone induction was complex but was unable to provide further details (Urist, 1965). He later referred to the active agents as bone morphogenetic proteins (Urist and Strates, 1971).

The sequence of events leading to induced bone formation include the attachment of mesenchymal cells to a substrate, proliferation of the stem cells, differentiation of these cells and the formation of cartilage and bone (Luyten *et al*., 1989; Huang, 2008). The entire process is reminiscent of embryonic bone formation (Ripamonti *et al*., 1995).

At least 40 BMPs have been identified (Wikesjö *et al.*, 2007; Ripamonti and Renton, 2006). BMP-1 has no osteogenic activity (Reddi, 2001), does not have the C-terminal-like sequence of the TGF-ß family (Matthews, 2005) and is thus not considered a member of the TGF-ß super-family. It is a procollagen C-proteinase playing a role in the proteolytic processing of mature collagen (Kessler *et al.*, 1996; Li *et al.*, 1996).

BMPs are the only known growth factors, or morphogens, that can transform connective tissue cells into osteoprogenitor cells that mature into osteoblasts capable of forming new bone either by means of intramembranous or endochondral ossification (Rengachary, 2002).. Other growth factors (mitogens) can only induce multiplication of cells (Rengachary, 2002).

BMPs involved in bone formation can be broadly grouped into 3 subfamilies, based on amino acid sequence homology (Rengachary, 2002):

- BMP-2 and BMP-4
- BMP-5, BMP-6 and BMP-7 (also known as Osteogenic Protein 1 (OP-1))
- BMP-3 (also known as Osteogenin)

Sampath and Reddi (1981) reported that the extraction of BMPs with chaotropic agents such as guanidinium hydrochloride results in the loss of the bone inductive property of DBM. The unpurified BMPs are referred to as a BMP complex, because they have not been purified to homogeneity. The biologic activity of this inactivated DBM can, however, be restored by the reconstitution

of the inactive collagenous residue with the soluble BMP extract (Sampath and Reddi, 1981).

In recent years human BMPs have been shown to play major roles in bone regeneration. BMPs have been purified, sequenced, cloned and recombinantly produced from many animals (Reddi, 1992; Sampath and Reddi, 1981).

BMPs have been demonstrated to have significant therapeutic potential in periodontal regeneration (Ripamonti *et al.,* 1994) and in bone regeneration in fractures (Johnson, 1992), and in defects in long bones and in calvarial defects (Duneas *et al.,* 1998; Ripamonti *et al.,* 1998; Urist, 1965).

The scarcity of human bone in tissue banks, limits its therapeutic use. The amount of BMPs obtained from bone is in the range of a few micrograms per ton of bone (Luyten *et al.*, 1989). A more readily available source of BMPs is required. The pig is an ideal source of tissue because of its genetic similarity to humans, and because it is possible to breed large numbers of disease free animals. The long term safety and efficacy of xenogeneically sourced BMPs must, however, be carefully evaluated prior to their introduction as routine adjuncts to bone regenerative procedures.

This study is designed to assess the efficacy of porcine-derived BMPs in generating bone formation in ectopic sites in the rat.

# LITERATURE REVIEW

In his classic study, Urist (1965) demonstrated that bone formation could be induced following implantation of DBM into the muscles of various animals. This key discovery initiated a search for the bone-inducing substance within the bone matrix. Subsequent studies revealed that the low molecular weight proteins responsible for the induction of bone could be extracted from the DBM and these were called bone morphogenetic proteins (BMPs) (Urist and Strates, 1971).

To date, more than 40 BMPs have been identified (Ripamonti and Renton, 2006) as members of the TGF-ß super-family. BMP-1 is a proteinase and not a morphogenetic protein (Li *et al.,* 1996). BMPs are highly conserved over generations and there is great similarity across different species, the BMPs of some species being identical (Sykaras, 2003; Ripamonti, 1997; Reddi, 1994; Rengachary, 2002; Duneas *et al.*, 1998).

BMPs are dimeric molecules with the two chains held together by one disulphide bond, each monomer consisting of about 120 amino acids with seven conserved cysteine residues (Reddi, 1998; Nakashima and Reddi, 2003).

BMPs are involved not only in bone formation, but they have several functions that have been described in embryonic patterning and organogenesis including that of teeth, kidney, lung and gut (Reddi, 1998; Nakashima, 2005; Nakashima and Reddi 2003; Ripamonti and Renton, 2006).

Extracellular matrix (ECM) has been shown to be crucial in adhesion, growth and differentiation of cells during embryogenesis and post-natal repair and regeneration of bone. ECM reactions elicited by BMPs responsible for bone formation are reminiscent of the cascade of biochemical and morphogenetic events that take place during embryonic bone development (Ripamonti and Reddi, 1992).

BMPs act via two different serine-threonine kinase receptors in the cell membrane causing a cascade of signalling events either stimulating or inhibiting morphogenesis (Sykaras and Opperman, 2003). The BMPs can bring about a net cellular effect that is either synergistic, or negative or additive (Ripamonti and Renton, 2006).

Intracellular control of BMP signalling is by the family of proteins called Smads (human homologue**S** of **M**others **A**gainst **D**ecapentaplegia) (Lagna *et al.,* 1996; Ripamonti and Renton, 2006), which can work in either an inhibitory way (Smad 6 and 7) or a stimulatory way (Smad 1 and 5) (Sykaras and Opperman, 2003). The Smads function by translocating into the nucleus and activating genes which ultimately signal the start of morphogenesis (Heldin *et al.*, 1997; Wu *et al.,* 2007).

BMPs have pleiotropic effects (Ripamonti *et al.,* 2006). Pleiotropy is the phenomenon whereby a single gene has multiple effects in numerous tissues. Amino acid sequence variations in the C-terminal domain confer specialised and pleiotropic activities on each isoform. These variations are the molecular basis of the structure and of the range of activities of each morphogenetic

protein. These pleiotropic properties are made more striking by the distinct signalling pathways of the individual Smads after receptor activation (Ripamonti and Renton, 2006).

BMPs are extracted from DBM by crushing dehydrated diaphyseal bone and then sieving the bone particles to collect a particle size of 74-420 $\mu$m in size. The bone particles are then demineralised in 0.5 N HCl until all minerals are removed. The residue is washed in ethanol and ethyl ether, dried and lyophilised. The BMPs are extracted from the DBM by chaotropic agents. The soluble extracted BMPs, are centrifuged and the supernatant dialysed and purified, but not to homogenous purity (Reddi and Huggins, 1972; Sampath and Reddi, 1981; Sampath and Reddi, 1987; Wang *et al.,* 1990; Sampath *et al.,* 1992 Ripamonti *et al.*, 1994; Duneas 1998). The residue is ICBM.

Cloning has allowed for the recombinant production of larger amounts of BMPs making them more readily available. The main BMPs that have been studied in bone regeneration include; BMP-2 (Wozney *et al.*, 1988), BMP-3 (Ripamonti and Reddi, 1992), BMP- 4, BMP-5 (Celeste *et al.*, 1990) and BMP-7 (Ozkaynak *et al.*, 1991; Reddi, 1998; Hsiong and Mooney, 2006).

To have any effect on the ECM, the highly soluble BMPs have to be reconstituted on a suitable carrier (Sampath and Reddi 1981). The carrier has to prevent premature diffusion of BMPs away from the site of surgical implantation, to protect the BMPs from non-specific proteolysis, to act as a provisional substrate, and to serve as a space maintainer later to be replaced by new bone as it forms (Sampath and Reddi, 1983). Through manipulation of

the pharmacokinetics of the carrier, the efficacy of BMPs can be optimised (Ripamonti, 1993).

Reddi (2001) suggested that further progress in clinical application of the BMPs depends on improvement and development of carriers with ideal release kinetics for the delivery of the BMPs. Although human collagen is an ideal delivery system, it has limited availability.
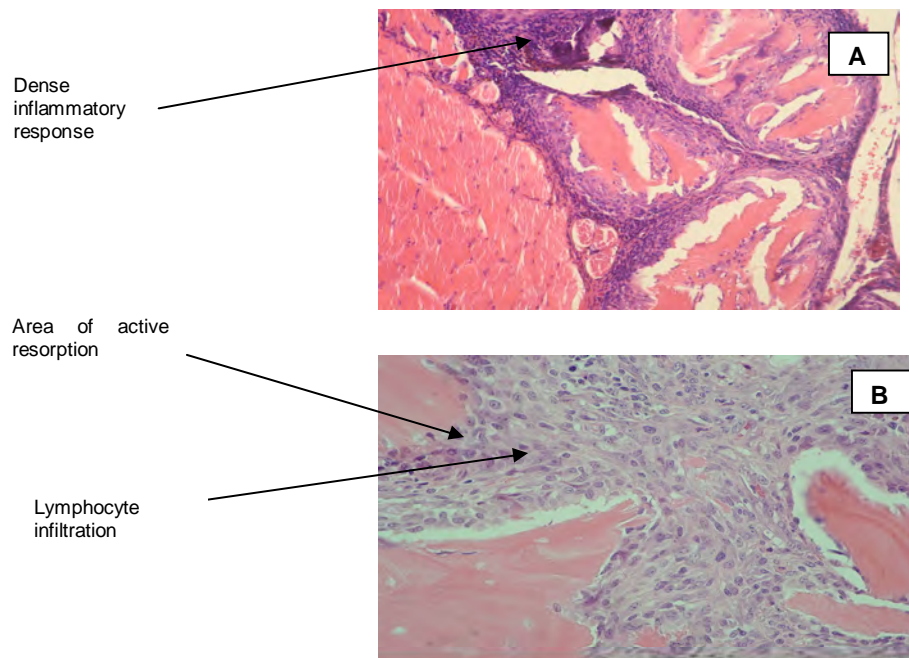


**Figure 1  Photomicrograph showing an inflammatory response to untreated BMP carrier, pICBM in rat model (courtesy Altis Biologics, South Africa) A=x10; B=x40**

Xenogeneic collagen carriers are generally immunogenic and fail to induce comparable bone formation when compared to allogeneic DBM, sometimes even causing inhibition of osteogenesis (Sampath and Reddi, 1983). As demonstrated in Figure 1 (A is at 10 x magnification; B is at 40 x magnification), non-manipulated porcine bone collagen loaded with 3mg of porcine BMP

complex indicates a lack of new bone formation, and presents an associated inflammatory response. Note the purple haematoxylin stained lymphocyte infiltration, and the resorption of the red eosin stained particles of implanted bone matrix collagen (see Figure 1). The treatment of xenogeneic collagen with peptidases and organic acids renders it biocompatible (personal communication, N Duneas, Altis Biologics, South Africa).

Sampath and Reddi (1983) reported that there is homology of the BMPs extracted from human, monkey, cow and rat DBM. The BMPs from these species were reconstituted with rDBM and bio-assayed in rats and all showed bone inductive activity. Subcutaneous implantation of human, monkey or cow DBM into the rat, however, showed that bovine DBM alone had a weak capacity to induce bone formation (Sampath and Reddi, 1983). This suggested that optimum bone induction is achieved if the implanted DBM originates from the same species. Later studies have shown that it is the carrier that elicits an immune mediated inhibition of osteogenesis (Reddi, 2005). If DBM can be altered so that it does not induce an immune response when implanted as a carrier, the potential exists for its use in bone induction in other species.

The osteoinductive properties of DBM are essentially obtained from its BMP content. There is a need for quantitative assessment of the osteoinductive activity of bone matrix from different donors and after different processing methods in order to assess the potential clinical effectiveness of demineralised bone grafts (Zhang *et al.,* 1997). The osteoinductive potential of DBM is not an all or none effect , but rather depends on the quantity and quality of the bone

matrix. There is a dose-response effect of the DBM that can be supplemented with BMPs (Zhang *et al.*, 1997). A biphasic response to BMPs was observed in studies both *in vivo* and *in vitro,* which indicated that a higher concentration of BMPs does not necessarily lead to more bone formation (Zhang *et al.,* 1997; Sykaras *et al.,* 2003).

Tissue alkaline phosphatase (ALP) is a major indicator of osteogenesis. *In vitro* studies have shown that BMP-7 enhances the specific activity of ALP in osteoblast-rich cultures alone and is dose dependent (Sampath *et al.,* 1992). Unlike BMPs, TGF-ß does not increase the specific activity of ALP in osteoblast-enriched cultures and in fact at higher concentrations reduces the enzymatic activity (Sampath, *et al.,* 1992). TGF-ß is a multifunctional autocrine regulator of bone formation. It down-regulates ALP and is a powerful bone growth stimulant at the level of pre-osteoblasts (Sykaras, 2003) but it does not induce ectopic bone formation (Reddi, 1994; Cheifetz *et al.*, 1996).

The destructive inflammatory process of periodontal disease presents a complex challenge to treatment in that not only alveolar bone must be regenerated but also other components of the periodontium, namely the periodontal ligament and cementum. The ideal of periodontal treatment is to generate a new connective attachment with functionally oriented periodontal ligament fibres inserted into new cementum and alveolar bone (Boyne, 1973; Bartold *et al.*, 2000).

The control mechanisms of periodontal tissue regeneration are not entirely understood, or fully documented. The discovery and cloning of BMPs (Wozney,

1992; Reddi, 1992) has opened up the exciting possibility of developing novel biomaterials that could stimulate the regeneration of the periodontal tissues, including the cementum, periodontal ligament and associated alveolar bone to prevent tooth loss. Ripamonti and Reddi (1992) reported on the role of BMP-3 and related BMPs in craniofacial and periodontal bone repair.

In a review by Ripamonti and Reddi (1994), attention was also drawn to the composite of BMPs and a complementary substrate as a possibility for future periodontal therapeutic application. This has been demonstrated in studies where BMPs were delivered into surgically created furcation defects leading to regeneration of the periodontal attachment apparatus (Ripamonti and Reddi, 1994).

Probably the first human clinical study involving BMPs is that of Bowers *et al.,* (1991) in which BMP-3 and DBM as a composite graft material was found to enhance periodontal regeneration in periodontal defects when compared to other treatment modalities.

A study on the delivery mode and efficacy of a calcium phosphate coating enriched with BMP-2 on dental implants revealed improved bone healing, and an enhanced and accelerated bone augmentation (Liu *et al.,* 2007).

Most research regarding BMPs in rat bioassays have been performed at 12 days, when the induction cascade and ALP activity are maximal (see Graph 1, courtesy of Altis Biologics, South Africa). Little is known of the fate of ossicles induced by implanted BMPs in the medium term.
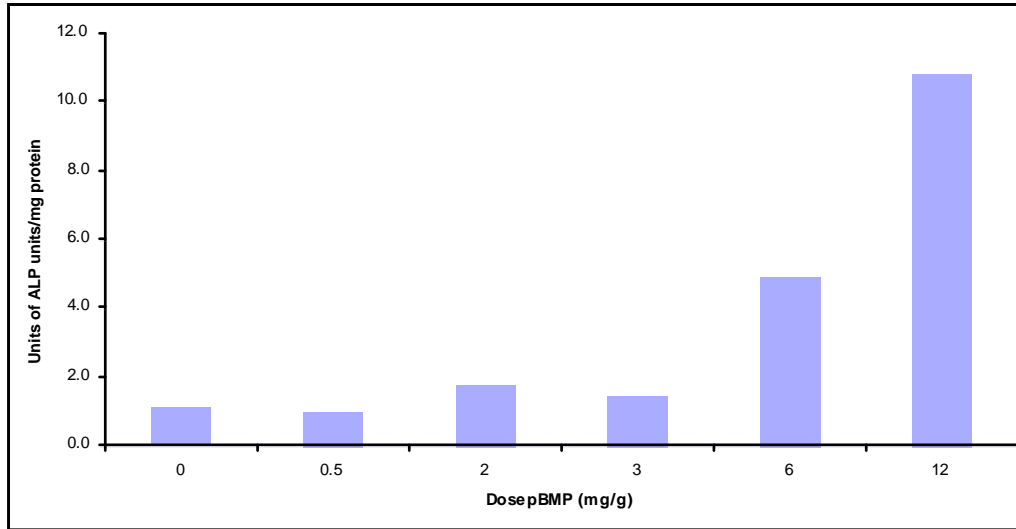
**Graph 1  Showing increasing ALP activity with increasing doses of pBMP, at 12 days**

There is a growing interest in utilisation of bone substitutes alone, or in combination with osteogenic proteins for regeneration of periodontal or craniofacial defects e.g. as a result of trauma or infection. The successful use of xenogeneic bone will eliminate morbidity when harvesting autogenous bone.

In the nonhuman primate model, BMPs were used to study the temporal sequence of tissue morphogenesis and the induction of bone in large calvarial defects of adult baboons (Ripamonti *et al.*, 1996) where complete regeneration was seen by day 90 and architectural restoration by 1 year.

Baboons have a similar bone turnover and bone physiology to man (Schnitzler *et al.,* 1993), and are thus an ideal model to study periodontal regeneration of the alveolar bone, periodontal ligament and cementum. Before one can proceed to develop periodontal therapeutics based on BMPs and related morphogens,

one would have to demonstrate its potential efficacy and safety in animal models first. It is a prerequisite that BMPs and its effects are tested on suitable animal models before contemplating its possible therapeutic application in man.

The need to find alternative sources of BMPs is highlighted by the fact that only a few micrograms of BMPs are obtained from a tonne of bone (Luyten *et al.*, 1989; Reddi, 2005). The use of bovine bone as a possible source of BMPs was prompted due to its abundant availability from abattoirs.

Naturally-sourced human BMPs were used in osteoinduction studies for the successful repair of bony defects in patients with tibial and femoral non-healing fractures, which had previously failed surgical grafting (Johnson *et al.*, 1992).

Tissue from pigs and humans have extensive similarities. Donor xenogeneic tissue from pigs have been safely used in humans for many years. Pigs are sources of insulin, heart valves, skin, collagen and other surgical materials (Clark *et al.*, 2007).

Boyan *et al.,* (2000) studied the osteopromotive activity of an enamel matrix derivative (EMD), which was derived from embryologically developing enamel of porcine origin and its successful use in humans is based on the high level of homology of porcine and human enamel proteins. EMD was developed as a clinical treatment to promote periodontal regeneration. It is not osteoinductive, but has osteoconductive properties in a threshold concentration.

Osteopromotive proteins support wound healing and tissue regeneration but do not alone initiate *de novo* tissue formation. It was found to enhance the osteoinductive ability of the graft material when added to freeze dried bone.

A study is required to determine if pBMPs can induce bone formation at heterotopic sites at longer time periods. This research was focused on obtaining histological and ALP data at 21 days of tissue generated at heterotopic sites in the rat by the implantation of pBMPs delivered at different doses using rDBM and rat and porcine insoluble collagenous matrix carriers.

# AIMS AND OBJECTIVES

This is a comparative study of heterotopic bone induction using pBMPs delivered into the rodent subcutaneous connective tissues and muscle with allogeneic and xenogeneic collagen carriers. The positive control is rDBM, a proven osteoinductive biomaterial at heterotopic sites in the rat.

The different combinations of carrier and BMP concentrations were:

A. Rat ICBM (rICBM) containing doses of 0, 3, 6 and 12 mg/gram of pBMP complex.

B. Porcine enzymatically modified ICBM (pICBM) containing doses of 0, 3, 6 and 12 mg/gram of pBMPs.

C. rDBM containing doses of 0 (positive control), 3, 6 and 12 mg/gram of pBMPs.

# RESEARCH HYPOTHESIS

Null hypothesis:

There is no histological or biochemical difference between the three systems when assessed in terms of bone induction at the end of a three week period.

# MATERIALS AND METHODS

The protocol for this study was approved by the Research Committee, School of Dentistry, Faculty of Health Sciences, University of Pretoria (Addendum 1), and by the Animal Ethics Committee, Tshwane University of Technology, Pretoria, South Africa (Study Number A55/05/06) (Addendum 2). This study complies with ISO standard 10993-6, and which includes experimental times of 21 days; which specifies the test methods to be used for the assessment of local effects of an implant material on living tissue, both macroscopically and microscopically after subcutaneous implantation. ISO standard 10993-6, describes the tests to be conducted for registration of a new medical device. (International Standard: ISO 10993-6, Biological evaluation of medical devices-Part 6, Tests for local effects after implantation, First Edition, 1994-07-15).

BMPs were extracted from porcine bone using a method previously described (Sampath and Reddi, 1983). The BMPs were not purified to homogeneity and will be referred to as pBMP complex. The pBMPs were combined with one of the delivery systems: rICBM, enzymatically treated pICBM and rDBM. 100mg of each delivery system was divided into four aliquots. To three aliquots, pBMP in doses of 3, 6, 12 mg per gram of the delivery system was added with the fourth aliquot being used as a control for the delivery system. The aliquots and pBMPs were reconstituted on the day of the implantation using two volumes of sterile water (Addendum 5).

Twelve male Wistar rats, between 28 to 32 days old, weighing between 100 and 200 g each, were obtained from National Health Laboratory Services, Cape Town. The rats were housed, two per cage, at a research laboratory at Tshwane University of Technology, where food and water were provided on-demand, the ambient temperature was controlled between 18 to 23 °C and humidity of 45 to 60 %. The animals were prepared for the study by dorsal shaving the day before commencement of the study.

The animals were sedated by a veterinary surgeon, Dr D Goosen of the research laboratory at the Tshwane University of Technology using ether in a bell jar. The implantation sites were swabbed with an ethanol wipe and the aliquots injected into the subcutaneous tissues using a wide bore needle and a 1 ml insulin syringe (Addendum 5). The sites were selected in a predetermined sequence following the Latin Square design as shown in Figure 2.

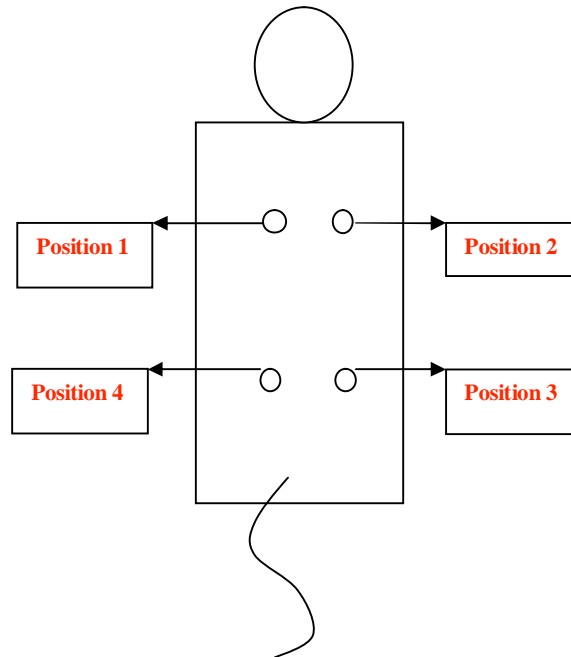|            | Animal 1 | Animal 2 | Animal 3 | Animal 4 |
|------------|----------|----------|----------|----------|
| Position 1 | 0        | 12       | 6        | 3        |
| Position 2 | 3        | 0        | 12       | 6        |
| Position 3 | 6        | 3        | 0        | 12       |
| Position 4 | 12       | 6        | 3        | 0        |



**Figure 2  Latin Square representation of the dorsal subcutaneous implantation sites in the rat**

In Group A there were four rats. The first rat received implants at the sites labelled 1 to 4 as in Figure 2

Site 1:     rICBM alone as control

Site 2:     3 mg/g pBMP and rICBM

Site 3:      6 mg/g pBMP and rICBM

Site 4:      12 mg/g pBMP and rICBM

Rat 2 of Group A received exactly the same doses of pBMP and carrier as Rat 1, but the sites were rotated according to the Latin square as described above.

Site 1:  12 mg/g pBMP and rICBM

Site 2:  rICBM alone as control

Site 3:      3 mg/g pBMP and rICBM

Site 4:      6 mg/g pBMP and rICBM

Rat 3 of group A received implants in a similar way with rotation of the implant sites as previously described:

Site 1:      6 mg/g pBMP and rICBM

Site 2:  12 mg/g pBMP and rICBM

Site 3:  rICBM alone as control

Site 4:      3 mg/g pBMP and rICBM

Rat 4 of Group A received implants in a similar way as previously described, with rotation of the implant sites as previously described to complete the Latin Square rotation:

Site 1:     3 mg/g pBMP and rICBM

Site 2:     6 mg/g pBMP and rICBM

Site 3:  12 mg/g pBMP and rICBM

Site 4:  rICBM alone as control

In a similar way, the four rats of groups B and C were implanted with pICBM with pBMP and rDBM with pBMP respectively, the carrier in Group B being pICBM and that of Group C being rDBM. The carriers, in groups A, B and C, when implanted alone, without pBMP content acted as control for each animal.

The rats were killed at 21 days after implantation using carbon dioxide asphyxiation, and then dissected to harvest the implant sites by firstly separating the skin from the underlying tissues and then removing the implant site with some of the surrounding tissues making sure that the tissue envelope was not damaged (Addendum 5).

Each implant sample was then bisected using a scalpel. One part was snap frozen to - 80 °C to preserve the alkaline phosphatase activity for later measurement in the biochemical evaluation of the implant sites. The other part of the implant sample was fixed in 10 % buffered formalin and later processed

for light microscopic histological examination at Vetpath Laboratories, Onderstepoort, Pretoria.

Four implant samples which were identified as cranial left, cranial right, caudal right and caudal left were submitted for histological evaluation for each rat and for each of the delivery systems tested.

The sections of undemineralised tissue were cut at a thickness of 5 μm mounted on microscope slides and stained using both haematoxylin and eosin and Goldner's trichrome stains, then microscopically evaluated using a Leica light microscope / camera system at different magnifications. The sections were evaluated for:

- Vascularisation: the formation of new blood vessels in tissues. This is of great importance in new bone formation to sustain the new bone formed.

- Inflammation (Indicator of Immunogenicity): the presence of mono-nuclear inflammatory cells (lymphocytes and plasma cells) as well as polymorphonuclear leukocytes (neutrophils). The inflammation was graded from none to substantial.

- Osteogenesis: The formation of new bone tissue.

- Osteoblasts: precursor cells to new bone deposition. They are often observed as a basophilic cells lying adjacent to the implanted material.

- Osteoclasts: indicator of bone resorption: responsible for bone resorption and are observed as large, multi-nucleated cells. They are often found in the inflammatory infiltrate adjacent to the implants (xenogeneic bone).

Evidence of these factors were evaluated and graded by the resident histopathologist at Vet-Path Laboratories, Onderstepoort, Pretoria. These grades were then coded with the scores 0 to 3, on the basis of the impression of the histopathologist (see Figure 3), and the data recorded in Microsoft Office Excel, 2003, running on a computer with a Pentium 4 processor, (for statistical analysis, see Addendum 3).

**Figure 3  Grading scale for the histological measurement of the parameters**

| Grade | Score |
|-------|-------|
| None | 0 |
| Some | 1 |
| Moderate | 2 |
| Substantial | 3 |

Other criterion evaluated was implant mineralization.

Haematoxylin and eosin (H&E) staining and Goldner s trichrome, blue, green and red (differentiates mineralized from unmineralised components of the tissue specimen) stains were used to evaluate the slides. Digital photomicrographs of all histological sections were taken using a Leica microscope system.

The results were analysed statistically by the specialist statistician Dr Olorunju, of the Medical Research Centre, Pretoria, South Africa, (see results in Addendum 4). The software used included STATA 10- Statistical Analysis. Data were captured using Microsoft Excel, and data were converted into the STATA format using Xcopy.

Analysis of variance-ANOVA compared the significance of the different delivery systems at various doses and sites. A non parametric comparison, the Kruskal-Wallis test, was used to verify the results of ANOVA.

# RESULTS AND DISCUSSION

The three delivery systems at all doses of BMPs demonstrated degrees of inflammation (Graph 2). Those for pICBM were substantially higher than either rICBM or rDBM.

**Comparison of inflammation scores**
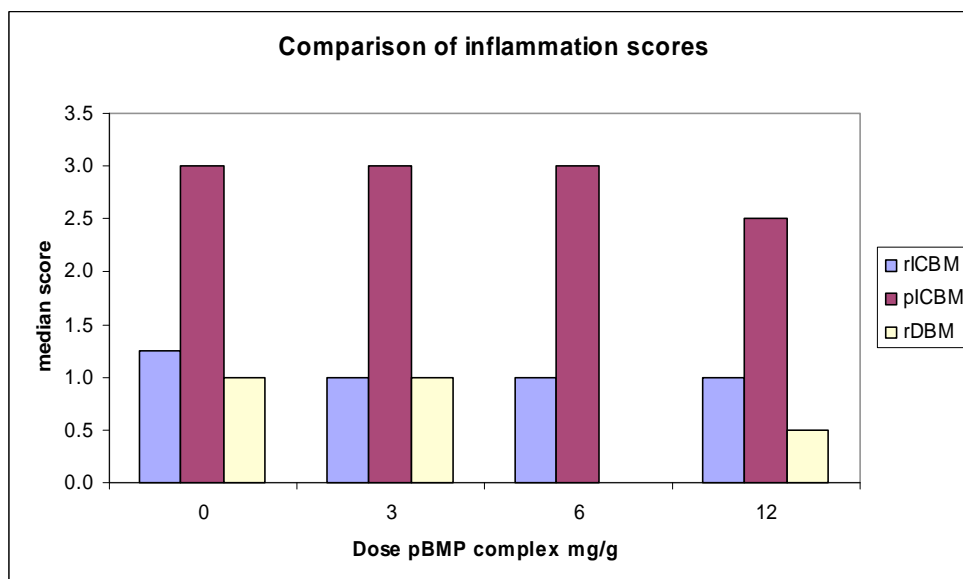


**Graph 2  Inflammation scores to three carriers at different pBMP doses**

The presence of inflammation indicates an antigenic response to the xenogeneic porcine carrier. It would appear that despite manipulation of pICBM using proteases and organic swelling agents, inflammatory responses still persist. It is striking that a higher dose (12 mg) of BMP complex resulted in a reduced inflammatory response (see Figure 4), and the reduction in inflammation occurring from 6mg to 12mg pBMP with pICBM (see Graph 2 and Graph 4B, page 30). In Figure 4 areas of osteogenesis (black arrows) can be

observed in addition to increased chronic inflammatory response as seen by infiltrating purple-staining lymphocytes (white arrow, 4B).

This data corroborates finding by this laboratory at 12 days (see
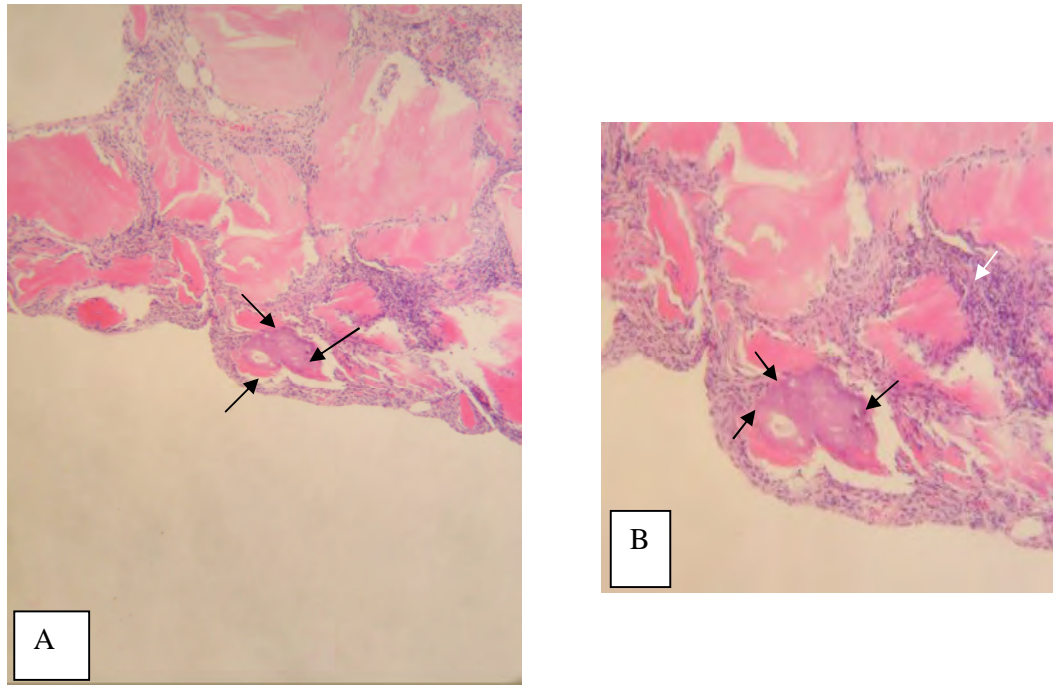


**Figure 4** **Photomicrograph of histological section (H&E stain 10x magnification). 12 mg pBMP complex per gram pICBM, A. Area of interest enlarged in B**

Graph 1, page 11). The data point to the possibility that BMP at higher doses may induce bone formation, and thereby reduce inflammation i.e. in the xenogeneic matrix, the morphogenesis cascade appears to lead to bone induction and reduced inflammation (Graph 2 and Graph 3).

**Comparison of osteogenesis scores**
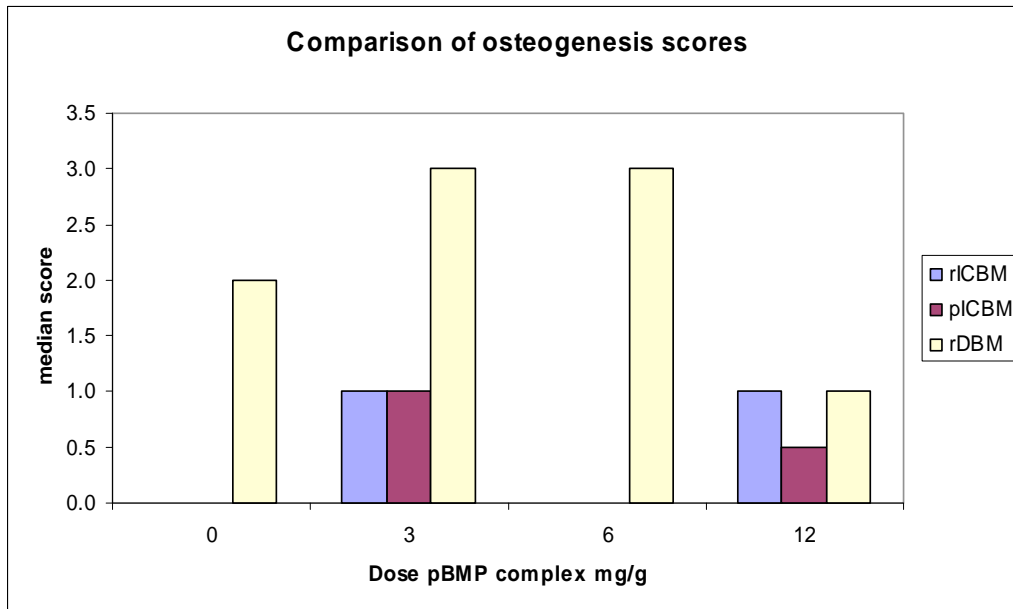
**Graph 3  Comparison of Osteogenesis scores**

At the 6 mg/g dose of pBMP complex, the rDBM delivery system reached the minimum inflammation score of nil (Graph 2) when compared to other doses and groups. A rebound of minimal inflammation appears at 12 mg/g pBMP complex. It would appear that this allogeneic system was the least antigenic of the three systems. Graph 3 demonstrates the superior osteogenesis scores when using the rDBM delivery system. Note that pICBM performs the same as rICBM at 3mg pBMP suggesting biocompatibility of the xenogeneic delivery system despite increased inflammation seen in Graph 2. When considering the pICBM delivery system, the trend for osteoclasts reflected the trends for inflammation scores (dark blue in Graph 4, group B).
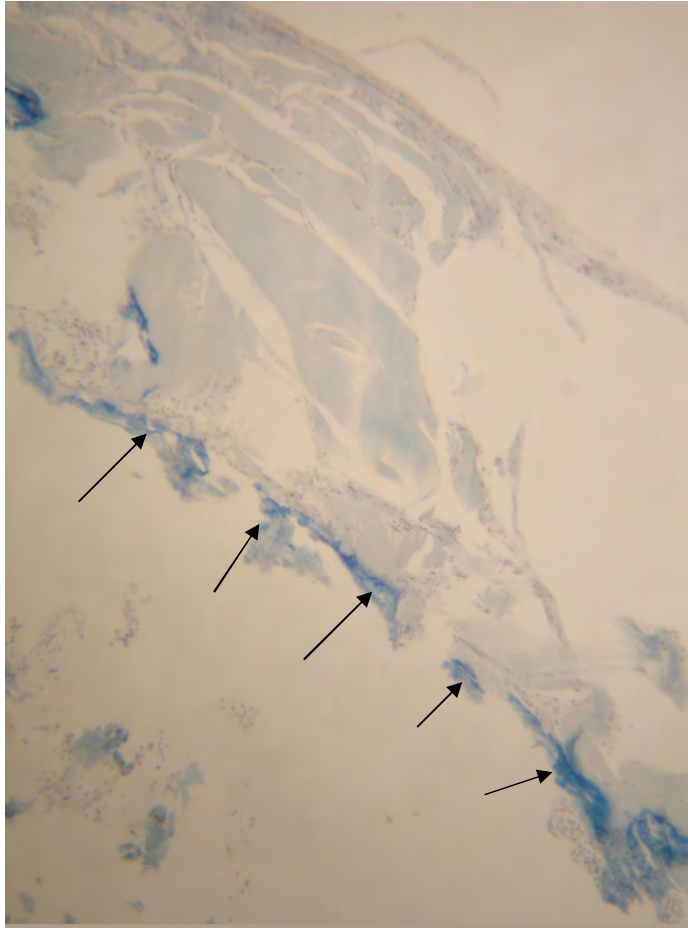
**Figure 5**　**Photomicrograph of histological section-Goldner's trichrome blue, (10x magnification); 6mg pBMP complex per gram rDBM**

Figure 5 also shows a mineralisation front in dark blue, generated by new bone grafting onto the implanted rDBM loaded with 6mg pBMP per gram (indicated by arrows).
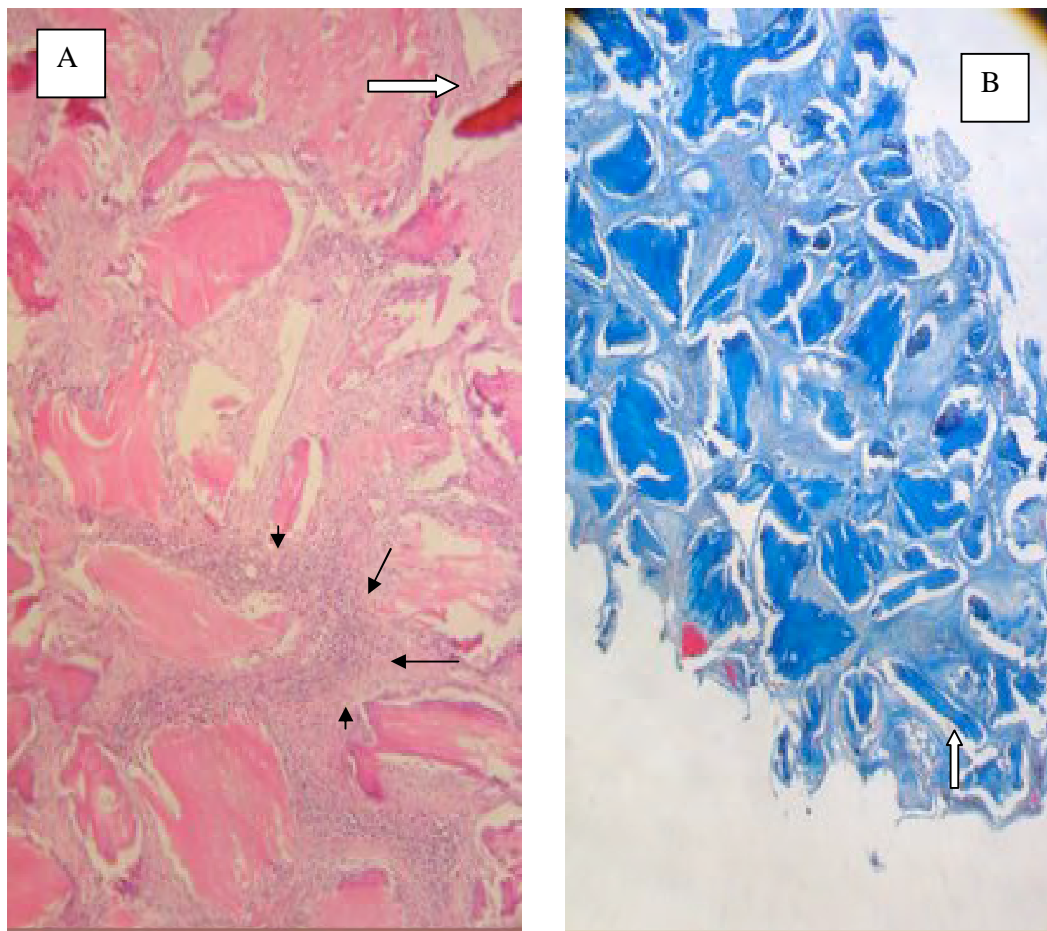
**Figure 6  Photomicrograph of histological section (H&E stain), 10x magnification in Panel A.: no pBMP complex added to pICBM carrier as control; Panel B: Photomicrograph of histological section Goldner's trichrome – blue stain**

In Figure 6, dissolution of the matrix due to resorptive activity (black arrows) is seen, particles remain separated from each other, and there is a lack of bridging bone (white arrows; Panel A and B). The implant is therefore inactive in terms of osteogeneic properties.

In Figure 7, collagenous matrix (ICBM) particles are rarely bridged by new bone formation, and a moderate inflammatory response can be noted with sparse foci of osteogenesis (black arrows).
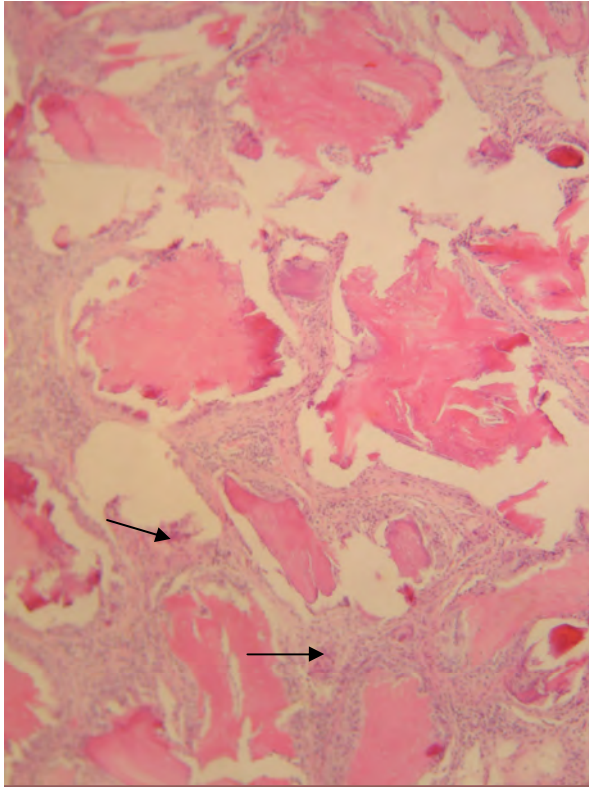


**Figure 7   Photomicrograph of histological section (H&E 10x magnification) 3 mg pBMP complex per gram pICBM**

It can be noted (Graph 4B) that the simultaneous reduction of both inflammation and osteoclasts between 6 and 12 mg/g dose from a score of 3.0 down to 2.5 osteoclastic activity and inflammation were closely correlated, presumably due to the resorption processes taking place induced by the implanted xenogeneic collagen.

**A**

**rICBM**

**B**

**pICBM**

**C**

**rDBM**
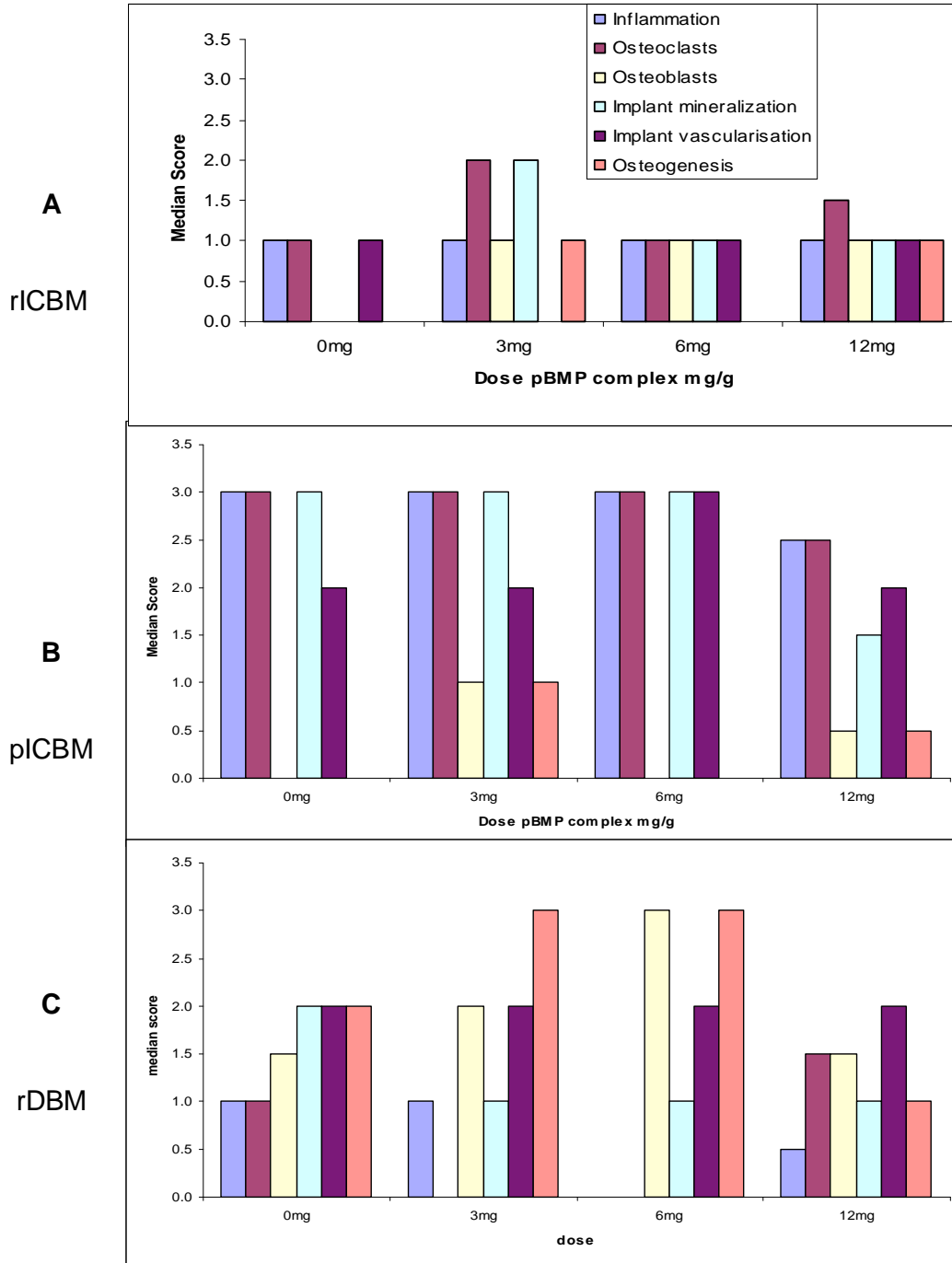


**Graph 4  Composite graphs - scores with different dose pBMP using three delivery systems**

In all three biomaterials tested, there were dose dependent increases in osteoblast numbers (Graph 4, groups A, B and C), confirming the osteogeneic effects of pBMPs. rDBM presented with the highest osteoblast scores, presumably due to co-operation of its endogenous BMPs with exogenously applied pBMP complex. It can be appreciated that even with control rDBM as seen in Figure 8, induction of osteogenesis is evident, black arrows, and minimal inflammation, white arrow, (also see Graph 2).
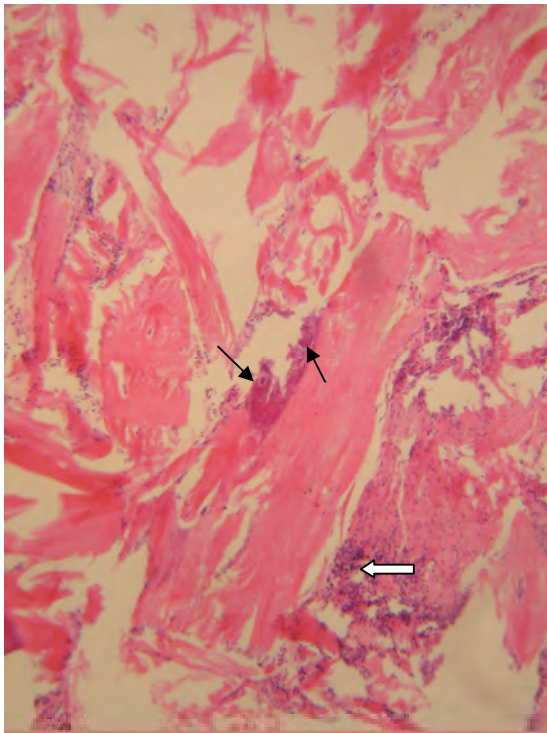


**Figure 8   Photomicrograph of histological section (H&E stain 10 x magnification) showing osteogenic region (black arrow), demonstrating the inductive potential of control rDBM and mild inflammation (white arrow)**

It follows that rDBM that was supplemented with exogenous BMPs was capable of higher biologic activity, as seen in Figure 9; black arrows indicate areas of osteogenic activity adjacent to implanted material, 3mg pBMP in rDBM.



**Figure 9 Photomicrograph of histological section (H&E 10 x magnification) 3-mg/g pBMP in rDBM**
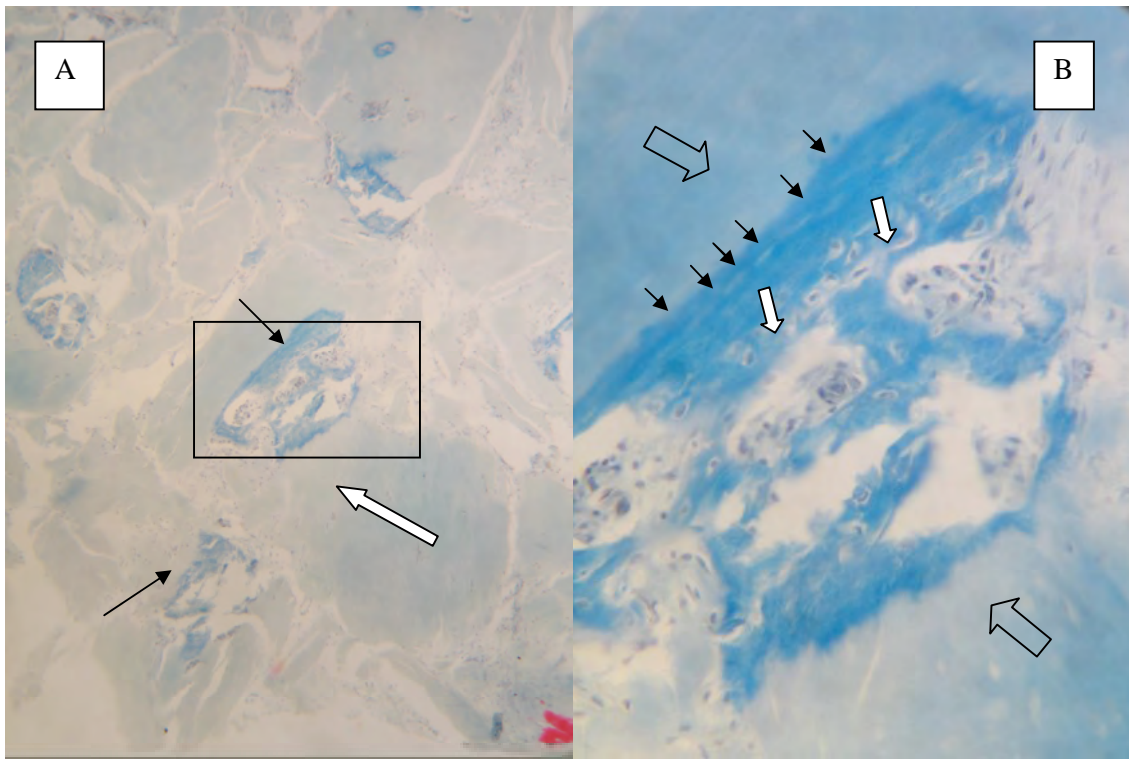
**Figure 10 Photomicrograph of histological section: (Goldner's trichrome stain - blue). Panel (A) low power magnification (10x) of 3 mg pBMP /gram rDBM delivery system. Boxed area in panel A magnified 40x shown in Panel (B)**
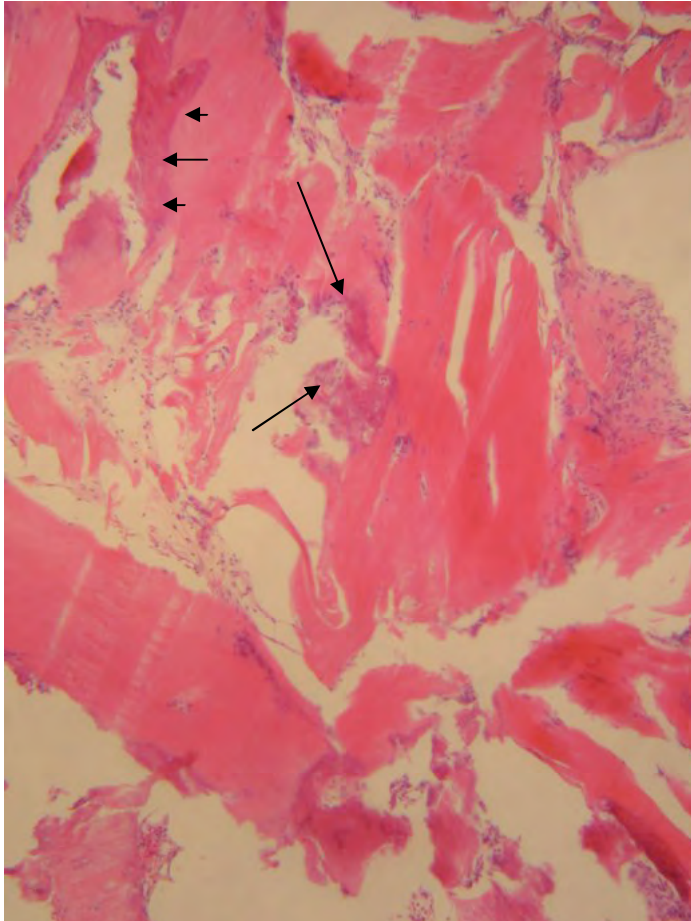
Figure 10 shows histological section (Goldner s trichrome stain - blue) in panel A. The low power magnification illustrates islands of mineralization with doses of 3 mg pBMP complex per gram rDBM delivery system. New mineralization is evidenced as a mineralization front arising in new bone (white arrow) grafting onto the implanted non-mineralized collagenous matrix (open arrow). Boxed area in panel A, magnified 40x in (B), showing mineralized new bone, bridging between implant particles (open arrow). The mineralization front is clearly observable against the implanted insoluble collagenous bone matrix (small

black arrows). Newly deposited osteoid is visible (lighter blue) with embedded new osteocytes (small white arrows).
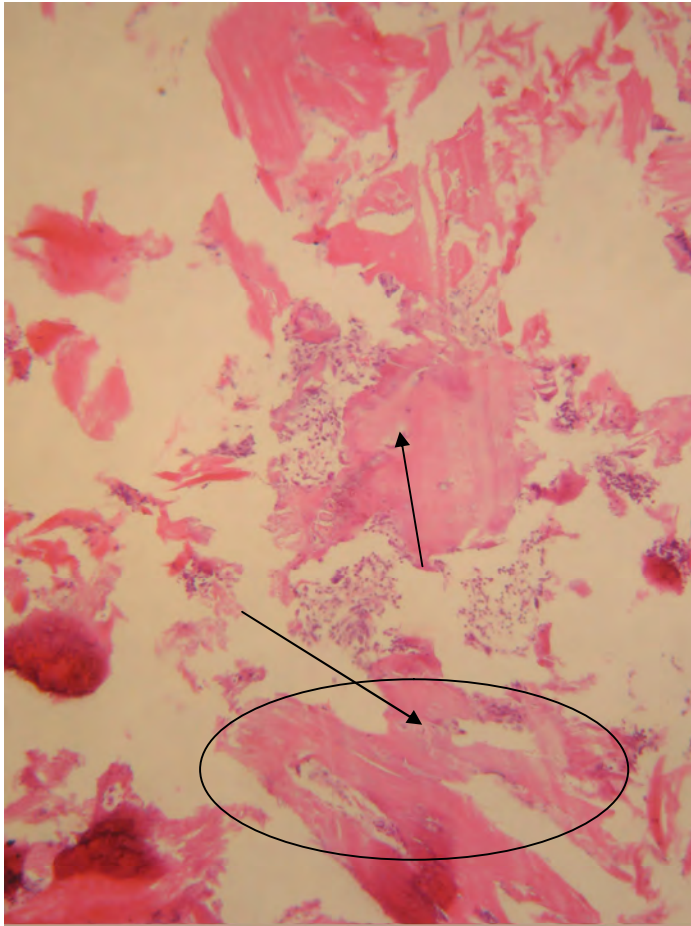


**Figure 11  Photomicrograph of histological section (H&E 10 x magnification). 12 mg pBMP complex per gram rDBM**

Arrows in Figure 11 indicate large areas of new bone formation with 12 mg pBMP complex per gram rDBM, with new woven bone in circle. Note in Figure 11, extensive bone remodeling and woven morphology of the newly generated ossicles using 12 mg pBMP complex per gram rDBM.

In Figure 12, section stained with Goldner s trichrome (green), newly formed osteoblasts (black arrows) lining osteoid seams (white arrow) are seen.
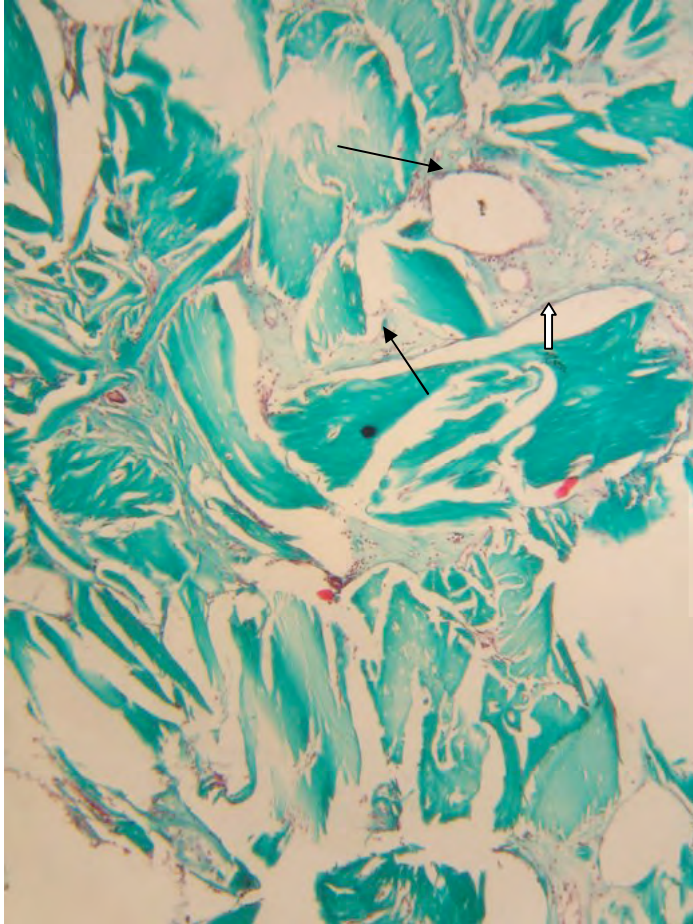
**Figure 12  Photomicrograph of histological section stained with Goldner's trichrome green; 10x magnification; using 12 mg pBMP complex per gram rDBM**

**Figure 13 Photomicrograph of histological section; (Goldner's trichrome stain, 40x magnification). 12 mg pBMP complex per gram rDBM**

In Figure 13, section stained with Goldner s trichrome (blue), newly formed osteoblasts (black arrows) lining osteoid seams (open arrow) are seen.

Large islands of cartilage can be seen in Figure 14, section stained blue with Goldner s trichrome, where an implant of rICBM containing 6 mg of pBMP complex had been placed, indicating osteoblastic activity (see Graph 4A). Implanted particles of rICBM are now fused into a solid mass of new bone. A large island of cartilage is apparent (open arrow).
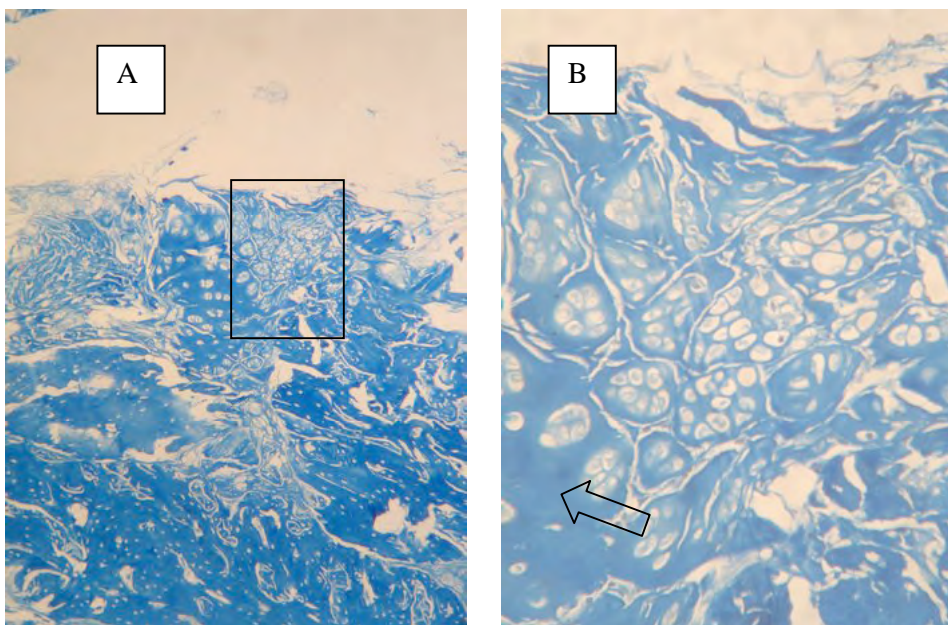


**Figure 14 Photomicrograph of histological section. Goldner's trichrome stain – blue, 6mg pBMP in rICBM. Panel A (10x magnification); Panel B (boxed area from A - 40x magnification)**

New woven bone is apparent (solid arrow) in juxtaposition to new cartilage (open arrow) in Figure 15, in response to 6mg pBMP associated with rICBM, indicating osteoblastic activity (see Graph 4A).



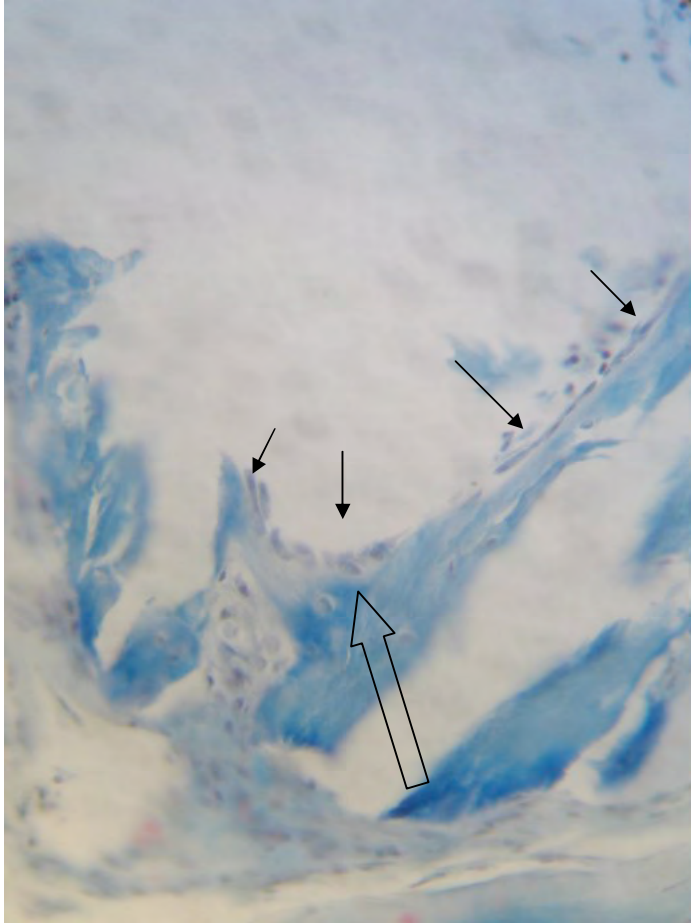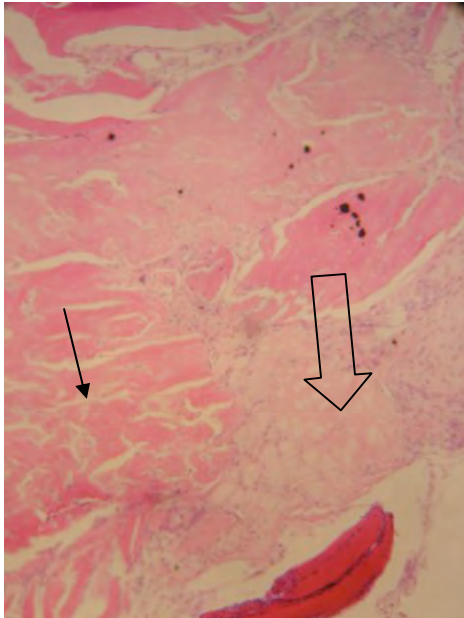**Figure 15  Photomicrograph of histological section (H&E stain, 10x magnification); 6 mg pBMP complex per gram rICBM**

In Figure 16, individual isolated rICBM implant particles containing 12 mg pBMP complex per gram rICBM, are no longer encountered. Instead, the implanted rICBM has been incorporated into a larger mass of woven bone.
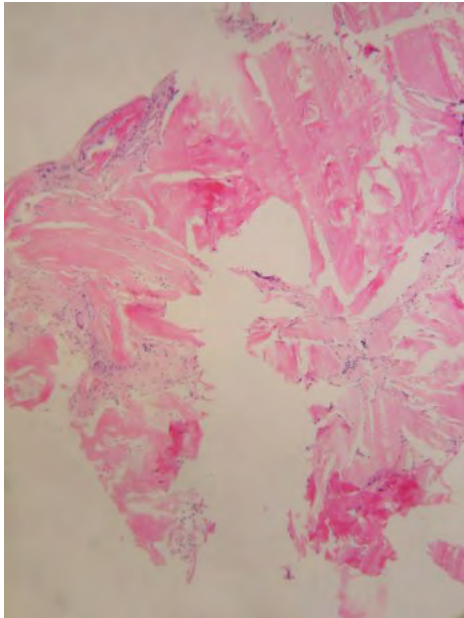


**Figure 16  Photomicrograph of histological section (H&E stain, 10x magnification); 12 mg pBMP per gram rICBM**

At 21 days, for the rDBM delivery system the osteoblastic activity peaked when using 6mg pBMP and then declined at the dose of pBMP (12 mg), giving a biphasic response (Graph 3). This may be interpreted similarly to findings by Zhang *et al.,* (1997), who reported a biphasic curve for *in vivo* and *in vitro* assays using BMPs. They attributed these paradoxical findings to possible interference by inhibitory agents other than BMPs which may be active at higher DBM concentrations (Zhang *et al.,* 1997). The ectopic location of the newly formed ossicle may cause it to be prone to resorption reaction. The

osteogenesis cascade, induced by DBM, also follows a wave of activity which then subsides, peaking in ALP activity on day 8-10 (Zhang *et al.,* 1997). This may explain the reduced ALP activity at the highest dose (Graph 5).

**Comparison of ALP units activity - rICBM, pICBM and rDBM**
**21 day study**

| | 0 | 3 | 6 | 12 |
|---|---|---|---|---|
| ■ Rat ICBM | 0.5 | 0.5 | 0.2 | 0.3 |
| ■ Porcine ICBM | 0.4 | 0.5 | 0.6 | 0.5 |
| □ Rat DBM | 0.8 | 0.9 | 1.1 | 1.0 |

dose pBMP on delivery device mg/g

(Y-axis: ALP activity in units/mg protein)
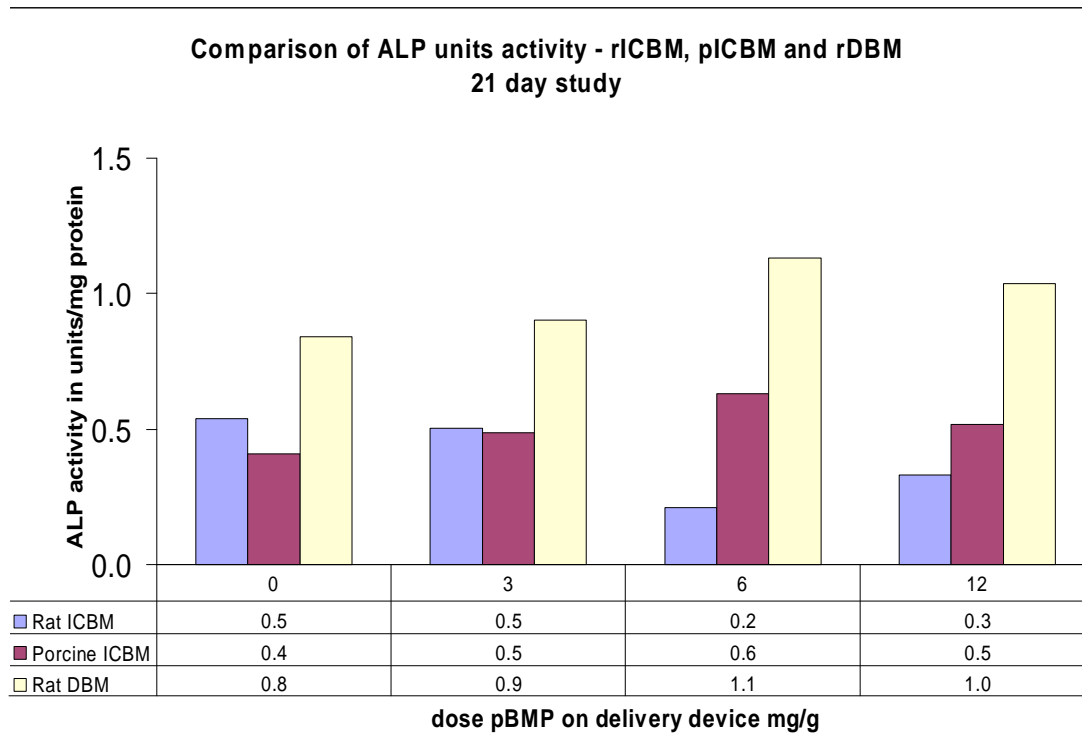
**Graph 5  Comparison of ALP unit activity using three delivery systems at different doses pBMP**

The high dose of 12mg pBMP may speed up initial bone formation, but then a subsequent bone resorption may follow, so that at day 21, the parameters for osteogenesis at the highest dose are in fact lower than the parameters for the lower dose. It is also possible that TGF-ß, a growth factor present in DBM, a multifunctional autocrine regulator of bone formation, down-regulates ALP and is a powerful bone growth stimulant at the level of pre-osteoblasts, confirming

the findings in this research (Sykaras 2003). TGF-ß1 did not increase the specific activity of ALP and in fact reduced the enzyme activity at higher concentrations, similar to other reports (Sampath and Maliakal *et al.,* 1992). A biphasic curve was observed *in vivo* and *in vitro* assays, which indicated a dose-associated response, the reasons for which remain unknown (Sampath and Maliakal *et al.,* 1992). The following parameters are possibly linked to the biphasic phenomenon: inflammation, factors that affected induction of bone growth at the site of implantation including micromotion of implants, tissue stresses, too much or too little BMPs, reduced vascularity leading to altered metabolism, presence of inhibitory agents which may become active at higher DBM concentrations of BMPs, and activity of TGF-ß (Zhang *et al.,* 1997, Sykaras *et al.,* 2003).

Osteogenesis score was always higher in the pBMP implants versus the controls (0 mg) pBMPs for all delivery systems. Not unexpectedly, the DBM group scored the highest values (Graph 3), presumably due to the higher overall amounts of active pBMPs contributed by the endogenous pool naturally present in the matrix. Yet again, the 12 mg dose for rDBM showed a substantial decline when compared to the lower pBMP complex doses at 21 days, possibly again reflecting an acceleration of initial bone formation, followed by earlier heterotopic resorption.

There seemed to be little correlation between implant mineralisation as scored histologically and dose of pBMP (Graph 4, B). pICBM system demonstrated highest mineralization scores at 0, 3 and 6 mg, and declined at 12 mg (Graph 4,

B). It must be borne in mind that certain foreign biomaterials may cause spontaneous mineralisation without involvement of bone formation, as in the case of dystrophic calcification, and the xenogeneic delivery system lends support to this possibility. The analytical assay of calcium using techniques such as flame photometry would be a more quantitative assessment of calcium content, and may shed more light into the mineralisation process of implants containing various doses of pBMPs.

There was greater ALP activity with the rDBM carrier and an optimum level of activity found at the dose of 6 mg pBMP. Histological findings also showed greatest evidence of osteogenesis using rDBM with 6 mg pBMP, however, there was evidence of bone formation at different doses with other carriers.

Comparison of all the parameters by site indicated that there were no significant differences. Using non-parametric method of comparing the systems indicates that significant differences between systems were evident for all the parameters. This suggests that differences exist in ALP activity between rICBM, pICBM and rDBM for each of the parameters considered. There is, however, evidence to suggest that the individual systems appear to be influenced by the various doses. Kruskal-Wallis analysis of all the parameters by system, indicated that there were significant differences between systems and doses. The results indicate a confirmation that the systems differed significantly from each other in terms of bone induction at three weeks, disproving the null hypothesis.

# CONCLUSION

It is well recognised from the days of Hippocrates, that of the various tissues that make up the human body, bone has the highest potential for regeneration. It is recognised from relatively recent studies that the major players in bone regeneration are BMPs. Although our understanding of molecular pathways of bone regeneration have improved exponentially, the translation of this knowledge into tissue engineering strategies remain in its infancy.

This research indicates that further refinement of xenogeneic carriers may provide ideal biomimetic scaffolds; and abundant alternative sources of BMPs will make treatment of craniofacial and periodontal defects economically viable.

Retention of transmissible viruses e.g. human immunodeficiency virus and hepatitis B virus in human ICBM used in allografts will limit its clinical use.

It has been shown in this study that xenogeneic collagenous carriers fortified with pBMPs are capable of promoting bone regeneration, following the principle that morphogens active in embryonic development can be exploited and re-deployed for the initiation of postnatal morphogenesis and regeneration. The ideal dose of BMP for this purpose is still not defined. In this study the optimal dose was found to be 6 mg pBMP per gram of carrier. Further studies are needed to examine the zones that are antigenic on xenogeneic collagen carriers. Techniques to manipulate these zones require testing to create a biocompatible and readily obtainable viable carrier for BMPs.

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# ADDENDUM 1 - NOTIFICATION BY THE DEAN

2006/05/25
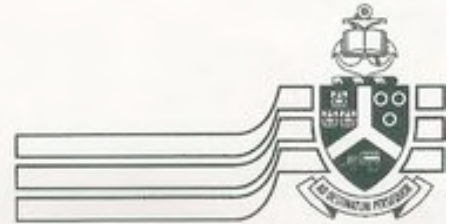
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http://www.up.ac.za

Faculty of Health Sciences
School of Dentistry

Dr GI Mohangi
Periodontics & Oral Medicine

## PROTOCOL APPROVAL

We would like to inform you that your Protocol and Research project, as recommended by the Research Committee, has been approved by the Dean.

**Title:** **Comparative study of heteropic bone induction using porcine bone morphogenetic proteins delivered into the rodent subcutaneous space with allogeneic and xenogeneic collagen carriers**

Good luck with your studies!

**PROF PJ VAN WYK**
**CHAIRMAN: RESEARCH COMMITTEE**

# ADDENDUM 2   APPROVAL OF ANIMAL ETHICS COMMITTEE

**Tshwane University of Technology**
*We empower people*

**Animal Ethics Committee**

14 June 2006

20/06/06

Ref#: AEC/2006/06/001
Name: LA-BIO Research
Student #: Not applicable.

Dr D Goosen
C/o Mr Deon Brown
LA-BIO Research
P O Box 251
Wierdapark
Pretoria
0149

*for LBR*

*Deon Brown*

Dear Dr Goosen

EVALUATION OF LA-BIO RESEARCH PROPOSAL BY TUT ANIMAL ETHICS COMMITTEE

Thank you for submitting the following proposal via e-mail for Ethical approval:

1. **Study number:**   A55/05/06
   **Title:**   "Comparative study of heterotopic bone induction using porcine bone morphogenetic proteins delivered into the rodent subcutaneous space with allogeneic and xenogeneic collagen carriers"

   **Ethics Committee Ref #:**   AEC/2006/06/001

The Animal Ethics Committee of Tshwane University of Technology reviewed the research proposals from LA-BIO Research via e-mail on 14 June 2006 and approval is hereby **granted**.

Yours sincerely,

D DU TOIT (Prof)
Chairperson: Ethics Committee
(Ref#AEC=2006=06=001=A550506)

*We empower people*

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## ADDENDUM 3 - DATA

| Animal no | A11C | A11C | A11C | A11C | A12U | A12U | A12U | A12U | A23C | A23C | A23C | A23C | A24U | A24U | A24U | A24U |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Histo ref 10604 | A1 | B1 | C1 | D1 | A2 | B2 | C2 | D2 | A3 | B3 | C3 | D3 | A4 | B4 | C4 | D4 |
| Delivery system | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM | R-ICBM |
| Site | Cran L | Cran R | Caud R | Caud L | Cran L | Cran R | Caud R | Caud L | Cran L | Cran R | Caud R | Caud L | Cran L | Cran R | Caud R | Caud L |
| Dose | 0mg | 3mg | 6mg | 12mg | 12mg | 0mg | 3mg | 6mg | 6mg | 12mg | 0mg | 3mg | 3mg | 6mg | 12mg | 0mg |
| Inflammation | 1 | 1 | 1 | 0 | 2 | 1 | 0 | 2 | 0 | 2 | 2 | 1 | 2 | 2 | 1 | 1 |
| Osteoclasts | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 3 | 2 | 2 | 2 |
| Osteoblasts | 0 | 1 | 1 | 0 | 2 | 0 | 2 | 2 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| Implant mineralization | 0 | 2 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Implant vascularisation | 1 | 0 | 1 | 1 | 1 | 1 | 2 | 2 | 3 | 2 | 1 | 2 | 1 | 2 | 1 | 1 |
| Osteogenesis | 0 | 1 | 0 | 0 | 2 | 0 | 1 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |

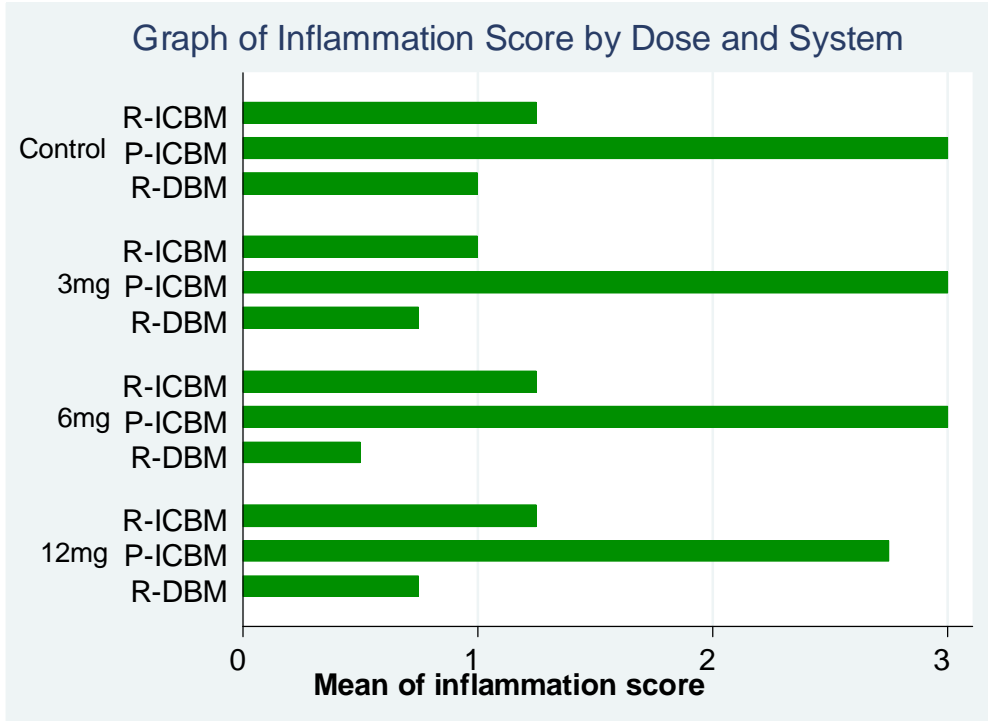1. Vet Path: Histopathological findings: subcutaneous implantation study: project A55/06/05: ref 2006-10604

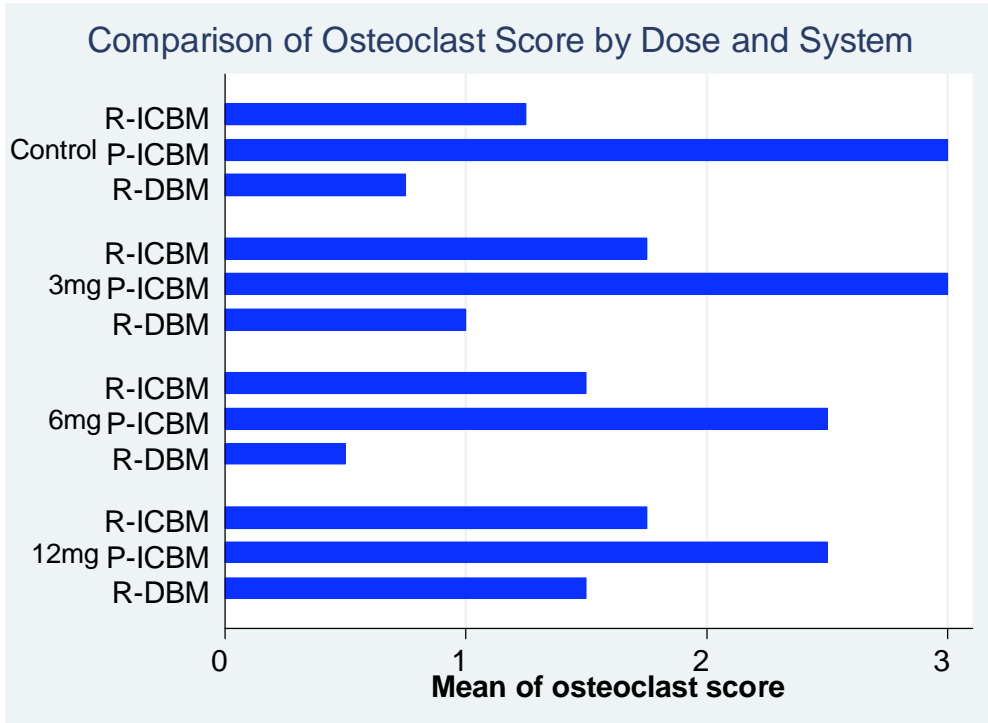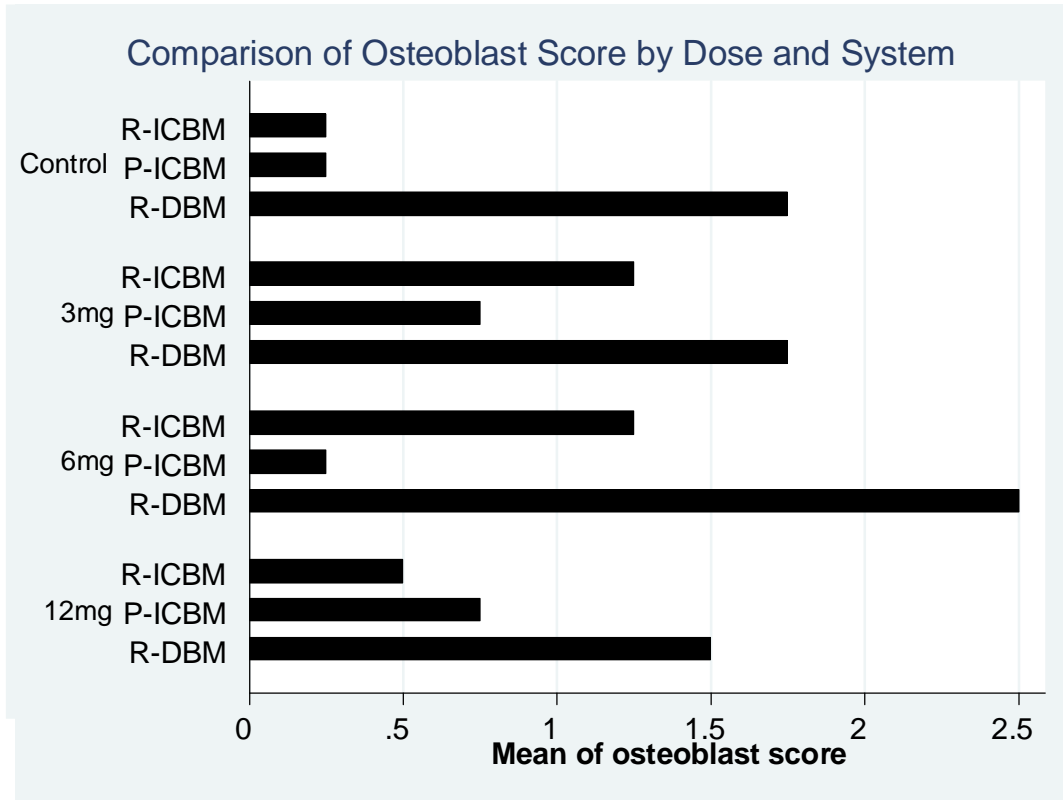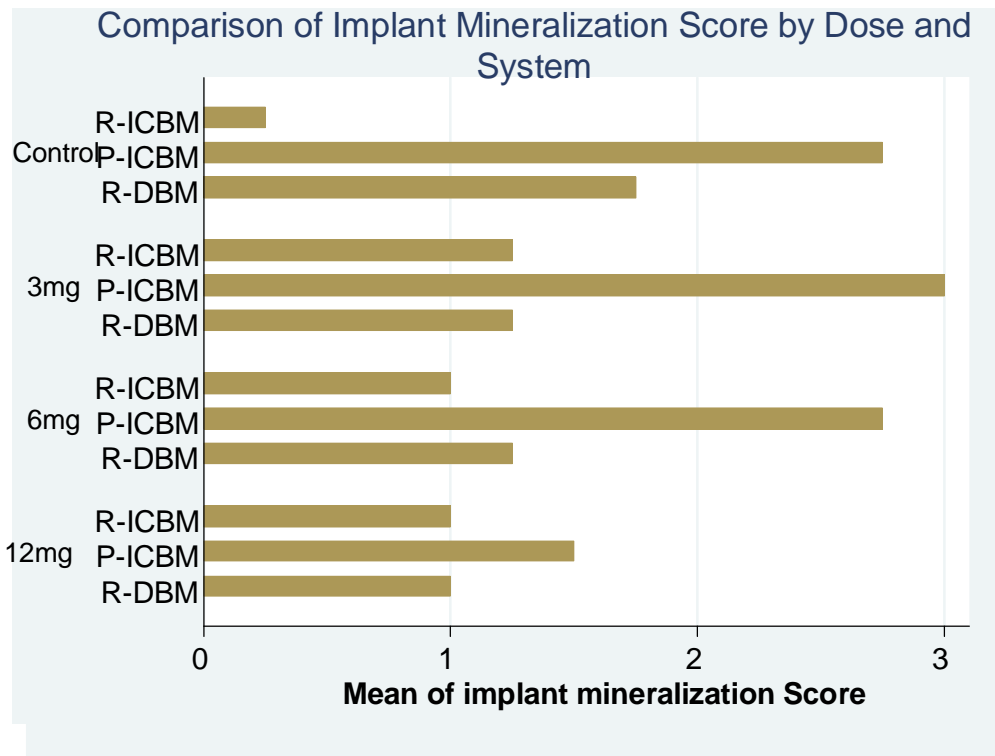| Animal no | A35C | A35C | A35C | A35C | A36U | A36U | A36U | A36U | A47C | A47C | A47C | A47C | A48U | A48U | A48U | A48U |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Histo ref 10604 | A5 | B5 | C5 | D5 | A6 | B6 | C6 | D6 | A7 | B7 | C7 | D7 | A8 | B8 | C8 | D8 |
| Del system | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM | P-ICBM |
| Site | Cran L | Cran R | Caud R | Caud L | Cran L | Cran R | Caud R | Caud L | Cran L | Cran R | Caud R | Caud L | Cran L | Cran R | Caud R | Caud L |
| Dose | 0mg | 3mg | 6mg | 12mg | 12mg | 0mg | 3mg | 6mg | 6mg | 12mg | 0mg | 3mg | 3mg | 6mg | 12mg | 0mg |
| Inflammation | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Osteoclasts | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 |
| Osteoblasts | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| Implant mineralization | 2 | 3 | 3 | 1 | 2 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 2 | 1 | 3 |
| Implant vascularisation | 2 | 2 | 3 | 2 | 2 | 1 | 2 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 2 | 2 |
| Osteogenesis | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 |

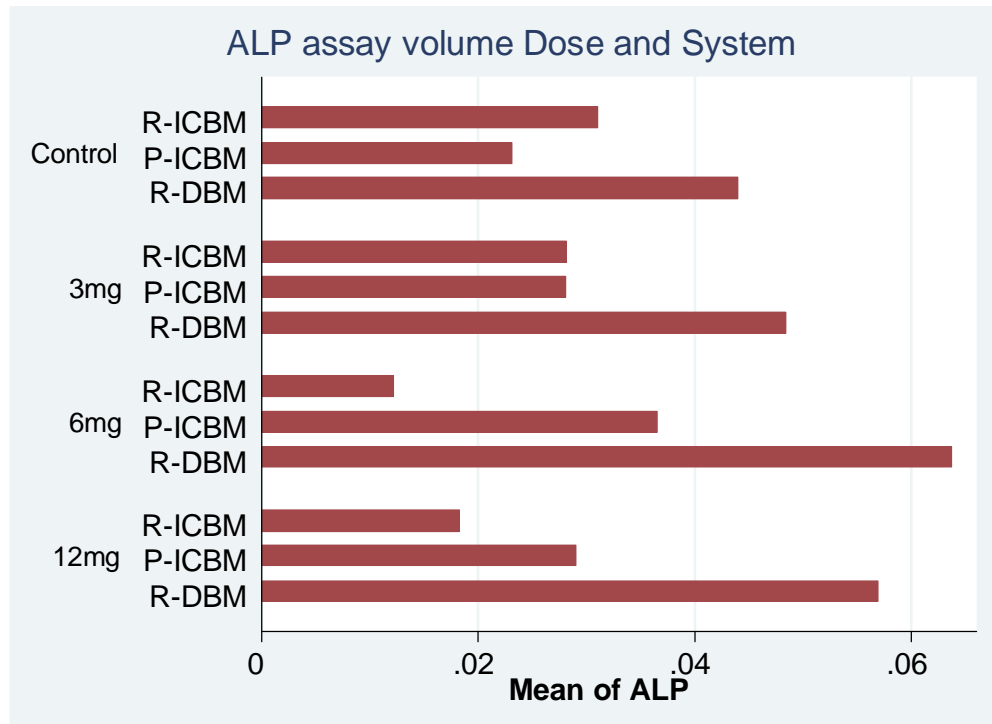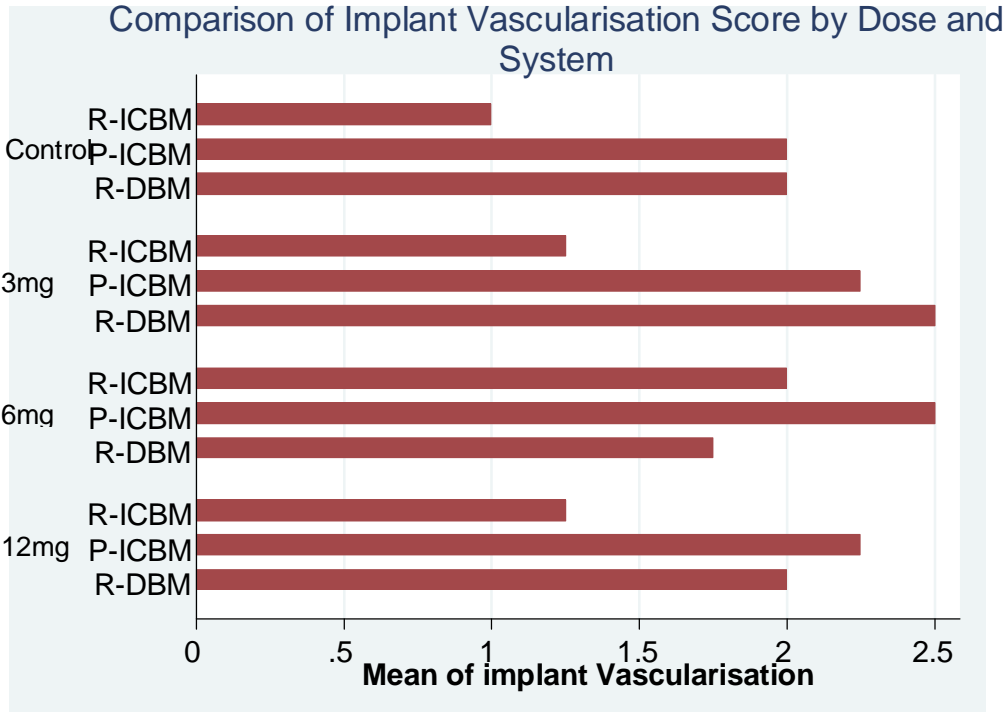2. Vet Path: Histopathological findings: subcutaneous implantation study: project A55/06/05: ref 2006-10604

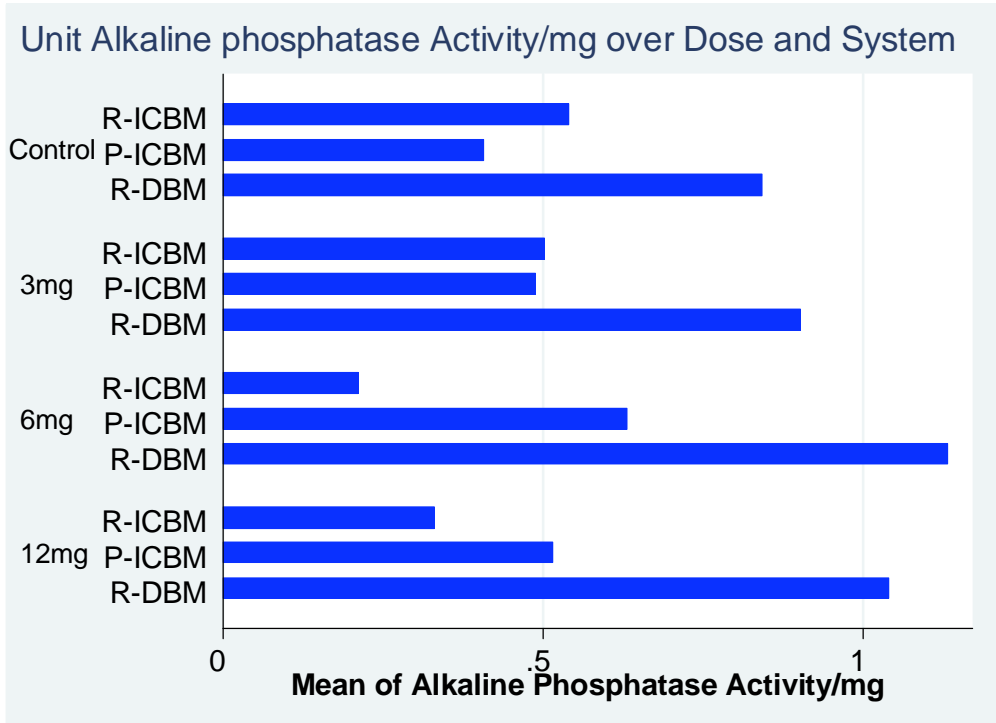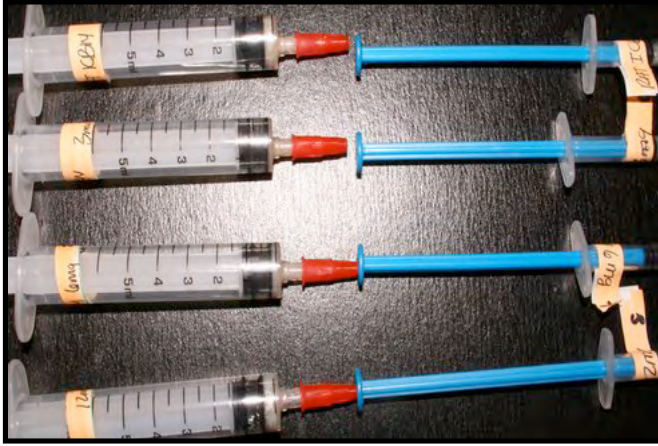**ADDENDUM 4**   GRAPHIC REPRESENTATION OF RESULTS



Graph of Inflammation Score by Dose and System

Mean of inflammation score

Comparison of Osteoclast Score by Dose and System

Mean of osteoclast score

Comparison of Osteoblast Score by Dose and System

## Comparison of Implant Mineralization Score by Dose and System



**Mean of implant mineralization Score**

Comparison of Implant Vascularisation Score by Dose and System



ALP assay volume Dose and System

Unit Alkaline phosphatase Activity/mg over Dose and System

# ADDENDUM 5 - METHODS



PREPARATION OF IMPLANT DEVICES: 0, 3, 6, 12mg/g pBMP in 1 carrier device as shown above, the same for the other 2 carriers were prepared
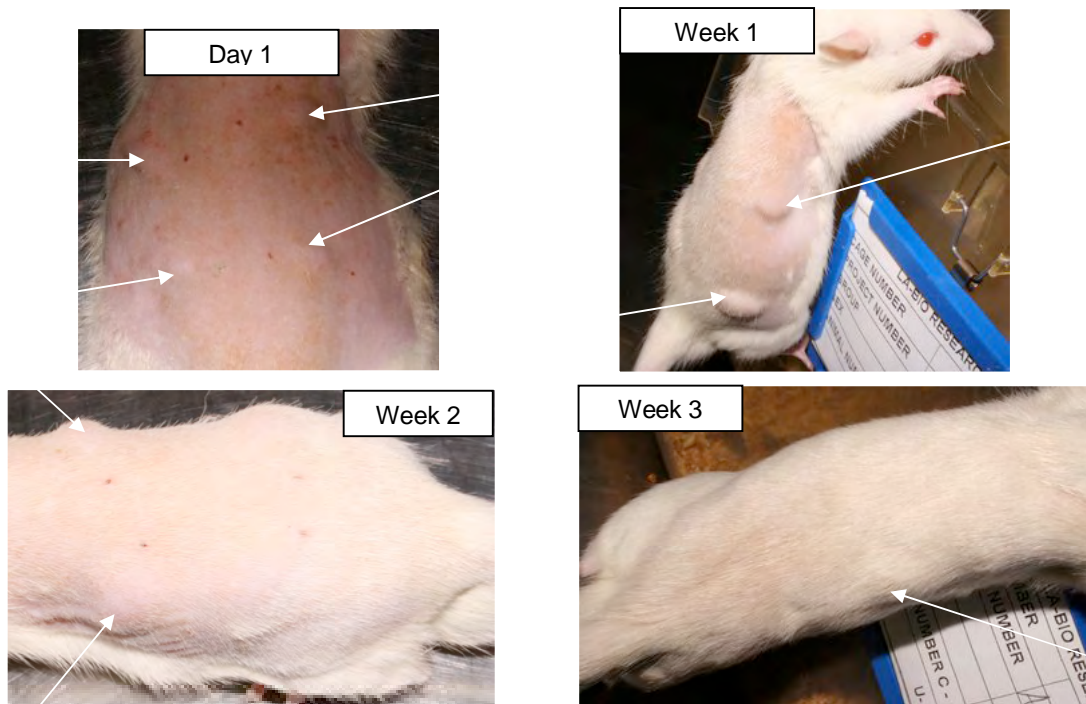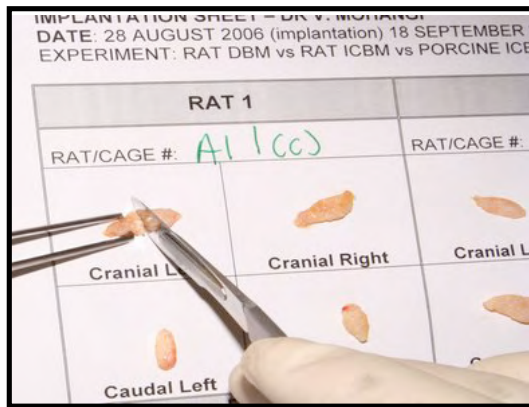


From day 1- implantation to day 21, arrows indicating sites of implantation at different time periods

DAY 21 Harvesting of implant sites (top left: dissection of rat dorsally showing 4 implanted material encapsulated; top right: tissue samples placed on data sheet; bottom left: sectioning of sample of one site, half for histology and the remainder for ALP study; bottom right: sectioned samples half in 10% formalin for histology and other in test-tube for ALP study)