Transforming growth factor-β_3 and recombinant human bone morphogenetic protein-7 for the regeneration of segmental mandibular defects in *Papio ursinus.*

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A research report submitted to the School of Oral Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Dentistry in the branch Maxillofacial and Oral Surgery.

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DECLARATION

I, Nika Vafaei, declare that this report is my own work and has not been submitted before for any degree or examination at this or any other institution.

Nika Vafaei

10 day of November 2014

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ABSTRACT

The reconstruction of osseous mandibular defects remains a significant challenge. The use of autologous bone for mandibular reconstruction is associated with numerous limitations, and alternatives to autologous bone would provide significant benefits for patients. The aim of this study was to evaluate and compare binary application of recombinant human bone morphogenetic protein 7 (rhBMP-7) and recombinant human transforming growth factor β_3 (rhTGF-β_3) to *solo* application of recombinant human bone morphogenetic protein 7 (rhBMP-7) in full-thickness mandibular defects in the non-human primate *Papio ursinus*. In four baboons, a 2.5cm segmental defect was created in the mandible and stabilized with a 2.7mm titanium reconstruction plate. Two defects were implanted with rhBMP-7 *solo*, and the other two with binary application rhBMP-7 and rhTGF-β_3 at a ratio of 20:1. All four baboons were euthanazed at 180 days post implantation. All four specimens were radiographed prior to sectioning. Tissue processing and histomorphometry were done on the undecalcified sections prepared from the harvested mandible specimens. In all defects bone regeneration re-established bony continuity at six months. The mean area of the regenerate was 336 ± 107.5 mm^2 (range 229-444.7) in the *solo* specimens, and 312 ± 63.5mm^2 (range 249-376.6) in the binary specimens. Radiographic examination confirmed complete bone healing in all defects but variable restitution of defect volume. The regenerated bone had a trabecular pattern consistent with mature mandibular bone and the defect interfaces were indiscernible. Due to the small sample size no performance advantage could be identified between the two treatment groups. These results confirm that successful bone regeneration by tissue induction in surgically created mandibular defects can be achieved with osteogenic proteins of the transforming growth factor-β superfamily.

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1 INTRODUCTION

1.1 Mandibular defects

The reconstruction of osseous mandibular defects remains a significant challenge for reconstructive surgeons. The complex anatomy of the mandible and the harsh environment in which a reconstruction is needed magnify the complexities of the task.[1] The goal of mandibular reconstruction is to restore mandibular continuity and morphology to allow functional rehabilitation with osseointegrated dental implants. Functional loading of the repaired mandible demands that the regenerated bone is of sufficient height, width and strength to withstand the forces of mastication to which it is exposed. Autogenous bone grafts (either free or pedicled) are the most effective choice for mandibular reconstruction.[2, 3] Free grafts can be either cortical, cancellous or corticocancellous blocks, and can be obtained from local, regional or distant sites depending on the size of the defect to be reconstructed. The most used donor site for bone grafting is the anterior and posterior ilium, which provides large volumes of corticocancellous bone. Costochondral grafts are required to provide for temporomandibular joint replacement. Less commonly used alternatives include cranial and tibial grafts.[3] Microvascular free flaps (either from the fibula, iliac crest, scapula or radius) have the advantage of having their own blood supply independent of the local tissue bed.[3] Although autogenous bone graft provides an effective reconstructive option, its use is associated with numerous draw backs including donor site morbidity, costs of extended operative time, and difficulty of adapting the graft to appropriate shape and contour. In addition, in young patients, insufficient bone at the donor sites may preclude successful reconstruction.[3]

It has therefore been the hope of reconstructive surgeons to regenerate bone without harvesting autogenous bone, by using homologous (allograft) and heterogenous grafts (xenograft). None of these materials have equalled the outcomes of autogenous grafts.[3] In light of these challenges focus has shifted to investigate alternatives for effective bone tissue regeneration to rehabilitate mandibular and other bony defects. Improved understanding of the biology of bone grafting, and biotechnological advancements have provided reconstructive alternatives to autologous bone for mandibular reconstruction.[1]

1.2 The induction of bone formation

Bone formation is initiated prenatally and continues throughout development. This process is referred to as osteogenesis and involves the synthesis of new bone matrix by cells called osteoblasts.[4] Osteogenesis may occur either by intramembranous or endochondral ossification. In the former, bone is formed in primitive mesenchyme. In endochondral ossification, bone formation occurs over a precursor of cartilage. In post natal life, this process is recapitulated during fracture healing.[4] The cells involved in bone formation and remodelling are osteoblasts, osteoclasts and osteocytes. Numerous growth factors play important roles during embryonic formation of bone: bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), growth and differentiation factors (GDF) and cartilage derived morphogenetic proteins (CDMP).[4]

Marshall Urist's seminal work confirmed that demineralized bone matrix when implanted in rabbits, rats, mice, guinea pigs and humans, induced formation of vital cellular bone.[5] He surmised that this induction was due to the activity of new morphogens which he termed bone morphogenetic protein (BMP).[5, 6] Sampath and Reddi demonstrated that the putative osteoinductive proteins could be dissociatively extracted from the demineralized bone matrix using chaotropic agents.[7, 8] Demineralized bone matrix subjected to dissociative extraction, yields two components: a protein extract, and an insoluble collagenous matrix or residue.[7, 8] After chaotropic extraction, the osteoinductive capacity of the resultant insoluble collagenous bone matrix (ICBM) is lost.[7, 8] However, reconstitution of the extracted soluble signals with the residual inactive collagenous matrix resulted in the restoration of osteoinductive activity.[7, 8] This bone-inductive potential is not species specific and osteogenic proteins from different species induce bone formation in another species when reconstituted with their homologous insoluble collagenous bone matrix.[7, 8]

The isolation of putative osteogenic tissues within the bone matrix proved to be a challenge given the small quantity of proteins bound to the extracellular matrix of bone.[9] The process of purification requires solubilisation of bone inductive fractions from the bone matrix. Increasingly refined purification schemes were instrumental to purify to homogeneity naturally-derived BMPs.[10] BMPs could be purified in sufficient quantity and purity to obtain amino acid sequence information.[10] From this information, full-length complementary DNA clones were isolated encoding the human equivalent of several recombinant human BMPs (rhBMP).[10]

1.3 The osteogenic proteins of the transforming growth factor-β supergene family

The transforming growth factor- β (TGF-β) supergene family is a large family of structurally related cell regulatory proteins that are pleiotropic peptides that control cellular proliferation, differentiation and a plethora of other functions in different cell types.[11-16] BMPs are members of the TGF-β supergene family that also include the three mammalian TGF-β isoforms, the amphibian TGF-β_5 isoform, GDF and CDMPs.[11-16] BMPs are critical morphogens in bone induction and regeneration. There are at least 20 BMPs which have been identified, characterized and cloned.[3, 14-17] The induction of bone formation is conferred by these osteogenic molecular signals collectively referred to as BMPs or osteogenic proteins (OPs). The BMPs are involved in the formation of bone and cartilage during embryonic development, and are also known to be involved in postnatal osteogenesis. Moreover, BMPs are involved in axial growth, hard tissue development and repair, tooth morphogenesis, and neural development.[18] Many members of the TGF-β superfamily possess the unique ability to induce bone formation not only orthotopically, but also heterotopically, recapitulating embryonic development.[2-4, 18]

BMP-2 and 7 have been produced by recombinant DNA technology using mammalian cells.[10, 16] Cloned rhBMPs have been shown to singly induce bone formation after implantation in heterotopic sites in murine models. rhBMPs can induce in a dose dependant fashion mesenchymal cell chemotaxis and infiltration, differentiation of the cells into chondrocytes, chondrolysis, formation of bone with bone marrow elements, and ultimately normal remodeling of the bone.[10] At different concentrations of the rhBMPs, various amounts of cartilage and bone

can be formed, with the cartilage always being replaced by bone marrow after vascular invasion. The larger the dose of rhBMPs, the earlier osteoinduction occurs, and the more significant the amount of bone formed in preclinical studies in a range of animal models.[19-21]

Thus they can be referred to as differentiation and/or inducing factors. Ultimately, as differentiation factors they cause the host to induce bone formation which functions and remodels in a way appropriate for the environment into which it takes place. Despite the fact that heterotopic implantation of single rhBMPs induce bone differentiation, there is a cascade of events at molecular and cellular levels which involves the expression of different BMPs and other growth factors in a particular spatial and temporal sequence culminating in the formation of bone tissue and bone marrow organs.[11, 18]

1.4 Delivery system

Bone tissue engineering requires a scaffold to initiate and spatially regulate the process of osteogenesis.[11, 18] The use of biomimetic matrices controlling the expression of the soluble molecular signals provides one of the three key components for bone regeneration. The use of insoluble collagenous bone matrix (ICBM) and other collagen-based materials provide a substratum for the functional expression of BMPs. This carrier is the inactive and insoluble residue obtained after dissociative extraction of the bone matrix. It is the reconstitution of the soluble signal (BMPs) with this insoluble signal or substratum of the bone matrix that

demonstrated the critical role of the carrier for the induction of bone formation.[7] Inactive ICBM has also been shown to play a role in cell recruitment, attachment and proliferation of mesenchymal cells. The organic collagenous matrices however, have operative limitations which include no structural support, immunogenic response, and potential transmission of viral antigens. These drawbacks have motivated scientists to search for alternative substrata to deliver the biological activity of rhBMPs.[22, 23]

1.5 Synergy

Synergy is the combination of two products to produce a result greater than the sum of their individual effects. Serendipitously, Ripamonti *et al.* in 1997, discovered that TGF-β_1 and BMP-7 synergize to rapidly promote the induction of bone formation.[24, 25] Binary application of TGF-β_1 and BMP-7 induce a several fold increase in bone volume as compared to *solo* use of either protein heterotopically.[24, 25] It was shown that significant angiogenesis characterizes the synergistic induction of bone formation which thus enables a faster induction of bone formation. It has been hypothesized that TGF-β_1 may regulate the expression of different BMPs, acting upstream of the BMPs and may induce the induction of bone by expressing selected BMP gene products eventually resulting in the induction of bone formation.[24, 25] This phenomenon may be exploited to improve clinical performance in human patients.

1.6 Preclinical and clinical application of BMPs and TGF-β_3

Tissue engineering is defined as the construction of new tissues by regeneration for replacement based on principles of developmental and molecular biology.[26] Bone regeneration in preclinical and clinical contexts requires a number of key components which includes an insoluble signal or substratum, an inductive molecular signal, and responding host cells capable of differentiating into osteoblasts.[12-14] It is this fundamental understanding of bone induction that has become the basis of hard tissue regeneration.

The theoretical potential of BMPs to be deployed therapeutically has been tested in several animal models. Critical size bone defects were created in the mandible of animal models into which rhBMP-2 and rhBMP-7 were implanted.[19-21] The application of recombinant or natively sourced BMPs have been found to successfully restore critical sized defects in rats, pigs, rabbits, dogs, sheep, monkeys, and baboons.[19-21] Combined use of rhBMP-2 and rhTGF-β_1 induce bone formation in heterotopic sites in mice.[27] Comparative histomorphometry of iliac crest biopsy specimens from humans and *Papio ursinus* demonstrate a remarkable degree of similarity between the two.[28] This data indicates that the adult baboon is ideally suited for comparative bone physiology and repair with relevance to humans.[28] Preclinical studies in the non-human primate *Papio ursinus*, have shown that a single application of the rhBMP-7 results in complete regeneration of both craniofacial and periodontal defects. rhTGF-β_3 combined with allogeneic ICBM induces regeneration of segmental mandibular defects as evaluated 30 days post implantation in *Papio ursinus*, and induces periodontal tissue regeneration. [18, 23, 25, 29-31]

Exploitation of bone induction was first attempted in humans with allogeneic demineralized bone matrix to reconstruct congenital craniofacial defects and mandibular defects. These trials determined success based on radiographic findings of bone formation with no histological evidence confirming the induction of bone formation.[32, 33]

Reports of the use of BMPs in the craniofacial region are appearing on a regular basis. BMPs have been used in sinus lifting, mandibular osteotomies and reconstruction.[34-38] Bone induction by rhBMP-2 in sinus augmentation was found to be an effective inductor of bone formation but required massive doses and incurred significant costs.[26, 31, 39, 40]

The first reported attempt to reconstruct a defect of the maxillofacial skeleton with naturally derived BMPs was done by Moghadam *et al.* in 2001. Their positive assessment of the outcome is not supported by critical assessment of the radiographic evidence which did not reveal ossification in the defect even nine months after implantation of BMPs.[41] The first series of patients to provide histological evidence of bone induction in mandibular segmental defects in humans treated with naturally derived BMPs was done by Ferretti and Ripamonti.[1] Thirteen patients were enrolled in the trial, comparing autogenous iliac crest graft in seven patients, to six patients receiving an osteogenic device human demineralized bone matrix as a carrier for highly purified naturally-derived bovine BMPs. Histological analysis showed that the osteogenic devices induced bone in only two of six patients treated, which provided valuable insights for the use of BMPs in human mandibular defects, necessitating the need for further clinical research.[1]

Warnke *et al*. attempted the growth of a custom bone transplant in the *latissimus dorsi* of a human patient using rhBMP-7, for transplantation into a mandibular defect. Seven months after transplantation, the graft became exposed and secondarily infected leading to its failure.[42, 43] Radiographic examination of the mandible did not support the conclusion of successful and clinically significant bone induction. Despite this failure the authors felt it was a technique that warranted further investigation. Heliotis *et al.* also prefabricated a hydroxyapatite/BMP-7 implant in a vascularized pedicled bone flap in the human chest for hemimandible reconstruction. Despite histological evidence of osteogenesis within the hydroxyapatite carrier, transplantation of the pedicled bone flap to the mandibular defect was not successful.[44]

Other studies conducted to investigate the use of BMPs in human subjects with mandibular continuity defects have found sparse areas of bone production and poor radiographic correlation to support the claim of osseous healing.[41, 46-49] Herford reported his experiences of mandibular reconstruction with rhBMP-2 in an absorbable collagen sponge carrier.[47] Once again the paper concluded that the technique worked in all cases and advocated its use in maxillofacial skeletal defects, however critical review of the radiographs accompanying his publication cannot support this conclusion (Figure 1).

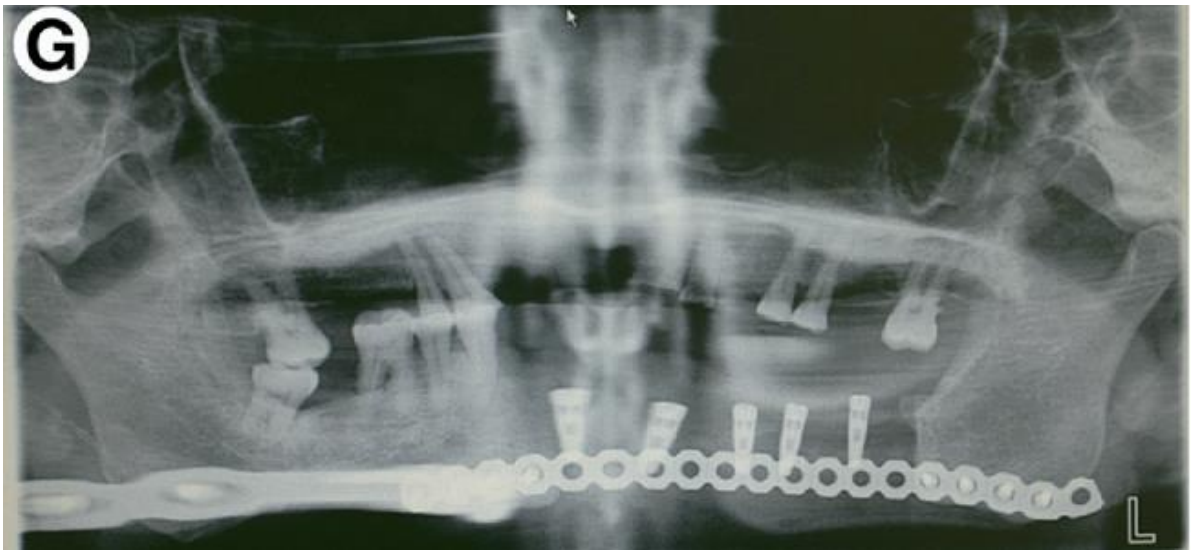

Figure 1: Panoramic radiograph post implantation with BMP-2 in mandibular defect. Objective assessment shows the defect bone density and architecture is of inferior quality to that of the adjacent mandible. [47]

BMP-2 enjoyed a surge in popularity for spinal fusion. This enthusiasm was based on a series of publications from several authors detailing the clinical success associated with the use of BMP-2. Subsequently, Eugene Carragee published a scathing review of the data associated with the above mentioned trials to conclude that in fact the use of BMP-2 for spinal fusion was associated with numerous complications.[50]

Translating the successful preclinical outcomes from non-human primates into clinical context has proven to be a formidable task. In marked contrast to preclinical data, supraphysiological doses of a single recombinant gene product are required to induce clinically unacceptable amounts of bone formation by induction, thus incurring high costs while failing to achieve the

outcomes of autogenous bone grafts.[26, 39, 40] It is clear that the promise of therapeutic bone tissue engineering remains elusive.[50]

2 AIM

The aim of this study is to evaluate and compare binary application of rhBMP-7 and rhTGF-β_3, to the single application of rhBMP-7 in full-thickness mandibular defects in the non-human primate *Papio ursinus*. This investigation will assess whether clinically significant osteoinduction by rhBMP-7 singly and in combination with rhTGF-β_3, can be obtained in surgically created mandibular defects. Success in this binary application could lead to the clinical application of rhBMPs and rhTGF-β_3 in human subjects for tissue regeneration and may provide an alternative therapeutic option for human bone tissue engineering.

3 MATERIALS AND METHODS

3.1 Experimental animal selection

Ethics approval was obtained from the Animal Ethics Committee, University of the Witwatersrand, Johannesburg, clearance code AESC 2006715. Four clinically healthy adult Chacma baboons (*Papio ursinus*) with normal hematological and biochemical profiles [51] and skeletal maturity confirmed by radiograph evidence of closure of the distal epiphyseal plates of radius and ulna were selected from the primate colony of the University of Witwatersrand, Johannesburg.[13, 30] Non-human primates were housed at the Central Animal Services of the University, Medical School in compliance with the National Code for Animal Use in Research and Education in South Africa.[52]

3.2 Morphogens

3.2.1 rhBMP-7

Mature rhBMP-7 is a gylcosolated 36kDa homodimer of 139 amino acid residue chains. rhBMP-7 prepared as previously described by Sampath *et al*., was kindly supplied by Stryker Biotech (Hopkinton, Massachusetts, U.S.A.).[53] It was supplied in 12 sterile vials each containing 1g of bovine insoluble collagenous bone matrix preloaded with 2.5mg rhBMP-7.

3.2.2 **Transforming Growth Factor-β_3**

Mature recombinant rhTFG-β_3, a gylcosolated 25kDa homodimer with a C-terminal domain of 112 amino acids with nine cysteine residues, was obtained from Novartis Pharma AG (Basel, Switzerland) and supplied as a stock solution of rhTGF-β_3 9.37mg per mL of diluent. This was further diluted with 5mmol acetic acid to obtain a concentration of 1µg rhTGF-β_3 per µl of diluent.

3.3 **Preparation of implants**

Twelve vials each containing 2.5mg rhBMP-7 and 1g bovine insoluble collagenous bone matrix, were provided for implantation. To six of the vials was added 125µl of the diluted rhTGF-β_3 solution for a final dose of 125µg of rhTGF-β_3 per vial to obtain a 20:1 ratio rhBMP-7: rhTGF-β_3. This has been the ratio with the highest biological activity when implanted in heterotopic sites of *Papio ursinus*.[22] The other six vials were used for implantation of rhBMP-7 *solo*.

3.4 Surgery

3.4.1 General anaesthesia

Baboons were operated under general anaesthesia induced with a mixture of Ketamine (100mg/ml, Bayer, India (Pty) Ltd) at a dose volume of 5mg/kg, and Midazolam (5mg/ml, Roche Prod. (Pty) Ltd) at a dose of 0.25mg/kg given intramuscularly. Oro-tracheal intubation was done to provide inhalation of Isoflurane (Forane 1.5% 250mL, Piramal Critical Care, Inc, Bethlehem U.S.A.) and oxygen for maintenance. Buprenorphine Hydrochloride (Temgesic 0.3mg/ml, RB Pharmaceutics Ltd, United Kingdom) at a dose of 0.01 - 0.03mg/kg and Meloxicam (Mobic, 10mg/ml, Ingelham Pharm. (Pty) Ltd) at a dose of 0.2-0.3mg/kg were given subcutaneously for analgesia. All baboons were given an intramuscular dose of oxytetracycline (Phenix, Peni LA, Phenix S.A. (Pty) Ltd 1ml/10kg) intraoperatively. Doxapram Hydrochloride (Dopram 20mg/ml, Amdipharm Mercury Ltd. UK) was used as reversal agent at a dose of 0.5-1mg/kg intravenously in all cases. Postoperatively Carpofen (Novox 100mg/tab, Impax Labs, U.S.A.) was given at a dose of 4mg/kg *per os*, crushed in banana, as an anti-inflammatory.

3.4.2 Extractions

All baboons had extractions of teeth on the right side of the mandible and maxilla from the right canines posteriorly. Eight weeks thereafter, the baboons were returned to theatre and the operative sites inspected for complete healing of the oral wound in order to avoid contamination when implanting the osteogenic devices.

3.4.3 **Preparation of mandible defect**

The skin over the right hemimandible was shaved and the right hemimandible exposed via an extra oral submandibular approach. A 2.7mm titanium reconstruction plate (Biomet Microfixation, Jacksonville U.S.A.) was then pre-bent against the exposed mandible to ensure accurate contouring of the plate and maintenance of occlusion post-resection. A 2.5cm defect was marked in the right body of the mandible using a bone saw and the pre-bent plate secured with titanium screws to proximal and distal segments of the mandible for immobilization.The screws were then removed, the defect completed and the plate replaced (Figure 2).

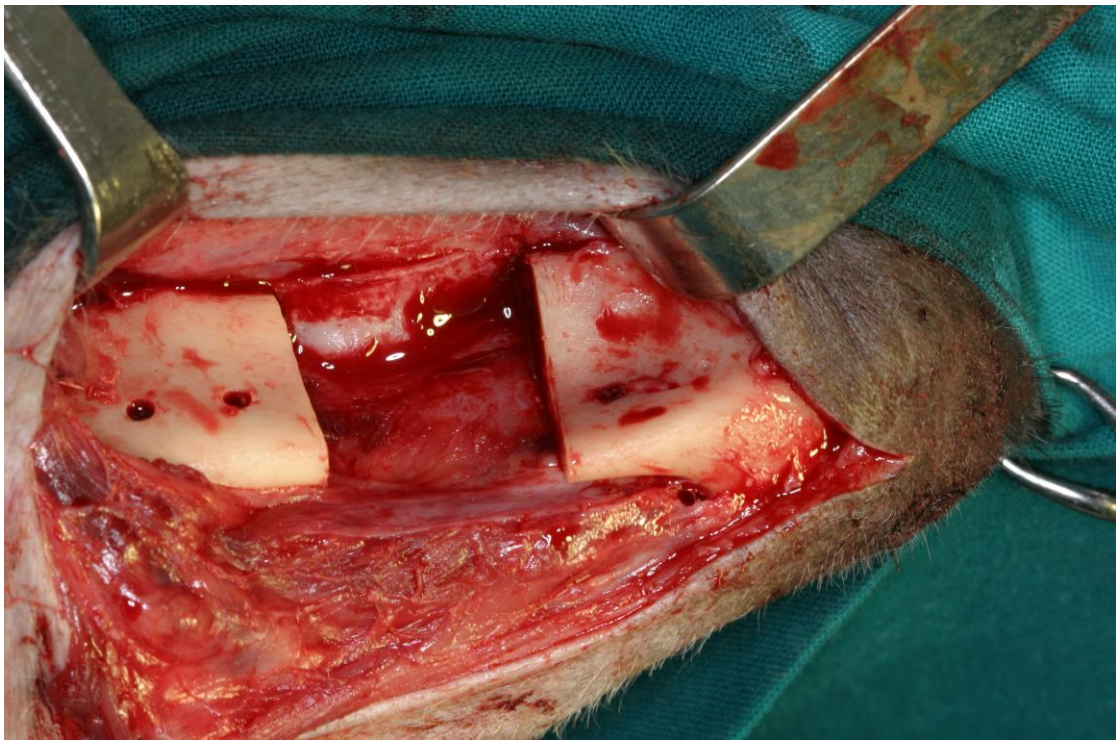

Figure 2: Lateral view of right mandible showing surgically created defect with screw holes on either side.

3.4.4 **Implantation protocol of device into mandible defect**

The osteoinductive device was moistened with sterile saline to form a paste. The paste was inserted into the defect (Figure 3) and primary closure of soft tissues ensured that the implant was maintained within the defect. Two of the baboons each received three vials of prepared device for a total of 3g of ICBM and 7.5mg rhBMP-7. The other two baboons received three vials each equivalent to 3g delivery and a total dose of 7.5mg rhBMP-7 and 375µg rhTGF-β_3 (ratio 20:1). Postoperatively the baboons were fed an all soft fruit and vegetable diet with Mealie-meal based porridge fortified with protein, vitamin and mineral powder for six weeks, thereafter returning to normal solid foods. The baboons were monitored daily for their ability to self-care and feed, and surgical wounds were examined for signs of infection.

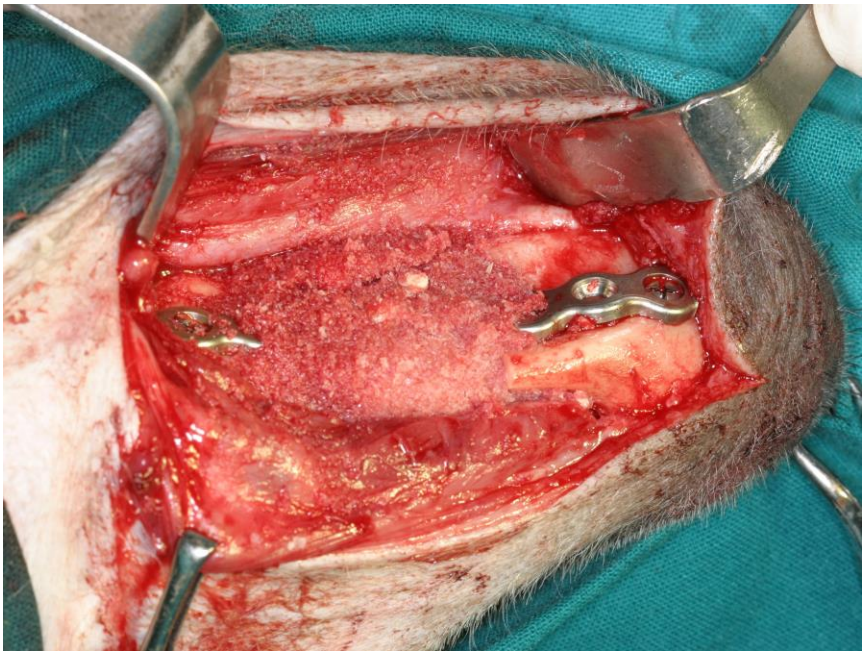

Figure 3: Defect implanted with saline moistened with osteoinductive device

3.4.5 **Euthanasia and Tissue harvest**

The baboons were anaesthetized 180 days post implantation, and the right and left carotid arteries were exposed via a midline incision in the neck. The carotids were transected and cannulated to allow perfusion of the mandible with 1 litre of saline in each carotid. The animals were thus euthanazed with Nembutal sodium (Pentobarbital 50mg/mL, Lundbeck Inc. Deerfield, IL U.S.A.) following which further perfusion of the carotids was done with 1 litre of formalin in each carotid. The hemi-mandibles were resected and skeletonised for radiographic imaging and histological processing. The defect and 1cm of normal bone was included in the final specimen.

3.5 **Analytical procedures**

3.5.1 Histomorphometry

Undecalcified tissues were processed according to the method of Donath and Breuner with minor modifications.[53] Briefly the tissues were processed in ascending grades of ethanol in an automatic tissue processor (Tissue-Tek, V.I.P.; Miles Inc, Elkhart, USA), under pressure vacuum cycles. Samples were infiltrated with ascending concentration of Technovit 7200 VLC (Heraeus Kulzer GmbH, Wehrheim, Germany) and embedded in a fresh solution of the same resin. Undecalcified sections were then ground and polished to 10μm and stained with a modified Goldner's trichrome. All sample preparation was performed using the EXAKT precision cutting and grinding system (EXAKT Apparatebau, Nordestedt, Hamburg, Germany).

Digital images were acquired using the Olympus BX16 microscope with built-in camera. Quantification of regenerated tissue was carried out using Stream Essentials software. Stream Essentials consists of four separate units including Olympus UPS, Microscope AX70, free standing control unit, a camera and associated software (Shinjuku Monolith, 3-1 Nishi-Shinjuku 2-chrome, Tokyo, Japan). Stream Essentials can perform various functions which include acquiring an image through the microscope camera, performing measurements on that image, and exporting data to create a spreadsheet for analysis. Stream essentials software identifies bone based on the colour taken up by the program, distinction cannot be made between osteoid and mineralised tissue both of which take up the program similarly, therefore the amount of new bone formation includes both osteoid and mineralised tissue.

Two sagittal sections were selected from each specimen for analysis. Each section included the entire defect and the interface with 1cm of intact mandible. A magnification factor of 10x was used for all specimens. The outline of the regenerated bone was selected to calculate the amount of regenerated bone formed within this outline (referred to as region of interest one (ROI 1)) (Figure 4). The original defect area was created by a quadrilateral whose sides are formed by the two interfaces and the superior and inferior borders by constructed lines joining the superior and the inferior ends of the interface lines. This was denoted as region of interest two (ROI 2). The total area of new bone was measured in mm^2 and the percentage within each ROI was calculated. The regenerate as a percentage of ROI 2 reflects the percentage of the original defect area filled with mineralized bone, whereas the ROI 1 reflects the density of mineralized bone within the regenerate. All data captured was recorded in Microsoft Office Excel 2007 spreadsheet.

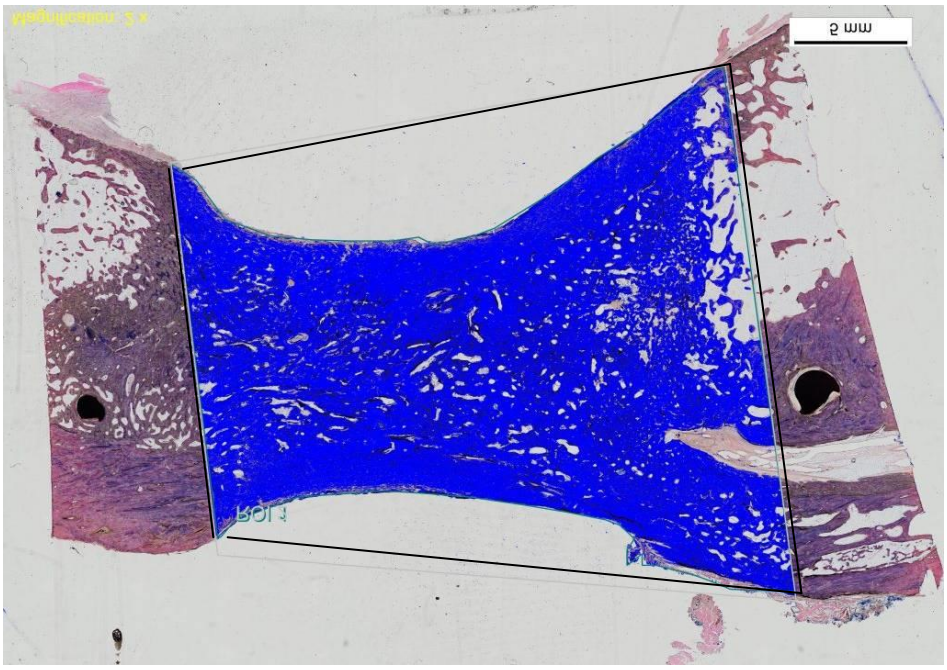

Figure 4: Photomicrograph of sagittal section through defect. Outlined in green is region of interest one (area of regenerate) and outlined in black is region of interest two (area of defect).

3.6.2 **Radiographic assessment**

Although subjective, with critical examination, radiographs serve as an important tool to determine clinically significant osteoinduction, remodelling, and integration. It is more reliable than the histological examination. Clinically significant osteoinduction refers to the concept that the quality of regenerated bone by osteoinductive agents is adequate to be identified radiographically as normal bone (in radiodensity and trabecular pattern) and that the morphology of the reconstructed segment resembles that of the former skeletal segment.[54] Although objective quantification of radiodensity and trabecular pattern on routine radiography is impossible it does provide an unequivocal overview of osseous regeneration in a defect.

All four resected specimens were radiographed prior to sectioning. Specimens were radiographed from a lateral and occlusal view at kV 53 and mA 0.09. Radiographs were taken to assess the radiodensity of the regenerated bone and the trabecular pattern. Moreover, the size and morphology of the regenerate will be assessed as will the visibility of the osseous interfaces (as a guide to remodelling). With this evaluation the density of bone regenerate in the defect can be compared to the adjacent normal mandible.

3.6.3 **Statistical analysis**

Due to the limited number of animals as well as of each treatment modality, statistical analysis was not performed.

4 RESULTS

4.1 Macroscopic examination of resected mandibles

All four defects were filled with highly mineralized bone re-establishing continuity of the mandible. In specimen 005 implanted with rhBMP-7 *solo* (Figures 5a & b), there is no macroscopically evidence of the erstwhile defect, thus restitution of integrity has been achieved. In specimen 805 implanted with rhBMP-7 *solo*, regeneration was excessive and morphologically irregular along the inferior border (Figures 6a & b). In the other two specimens (combined application of rhBMP-7 and rhTFG-β_3 at a ratio 20:1) there was uniform bone formation in all dimensions of the defect, blending imperceptibly to the adjacent margins of normal bone (Figures 7 & 8). Specimen 816 which was implanted with rhBMP-7 and rhTFG-β_3 had the lowest vertical height of new bone formed however the bucco-lingual dimension was equal to that of the adjacent bone (Figures 7a & b). Specimen 811 also implanted with rhBMP-7 and rhTFG-β_3 is shown in Figures 8a & b. This specimen shows complete restitution of morphology with bone covering the reconstruction plate. A sagittal section through the mandibular (Specimen 005) reveals the density of the bone regenerate within the defect and the remodelling of the regenerate to normal architecture (Figure 9).

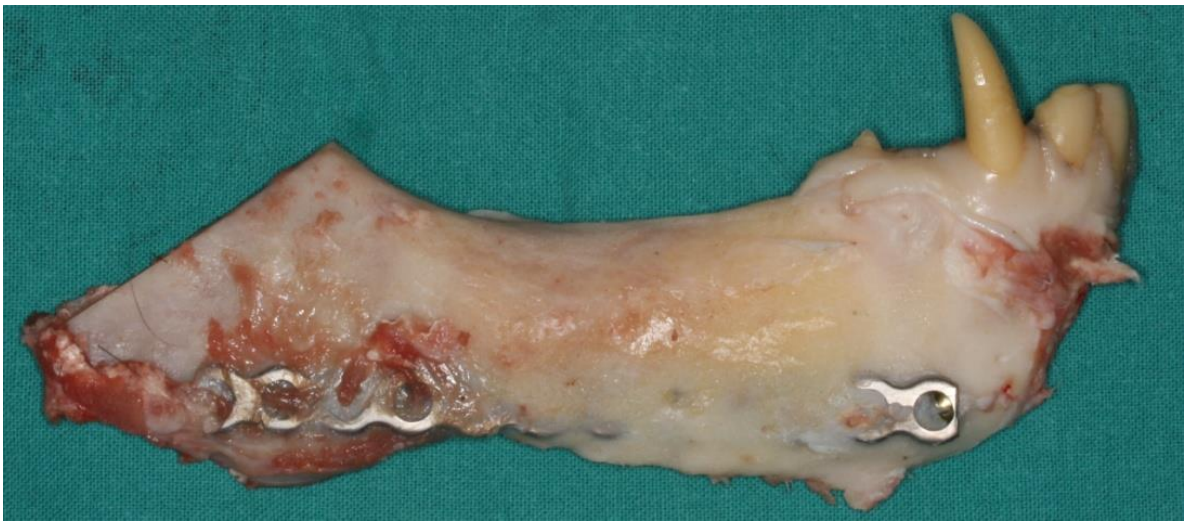

Figure 5a: Specimen 005 – rhBMP-7 *solo*. Lateral view of resected right hemimandible with reconstruction plate covered by bone regenerate with defect margins not apparent. *Restitutio ad integrum* of the mandibular defect.

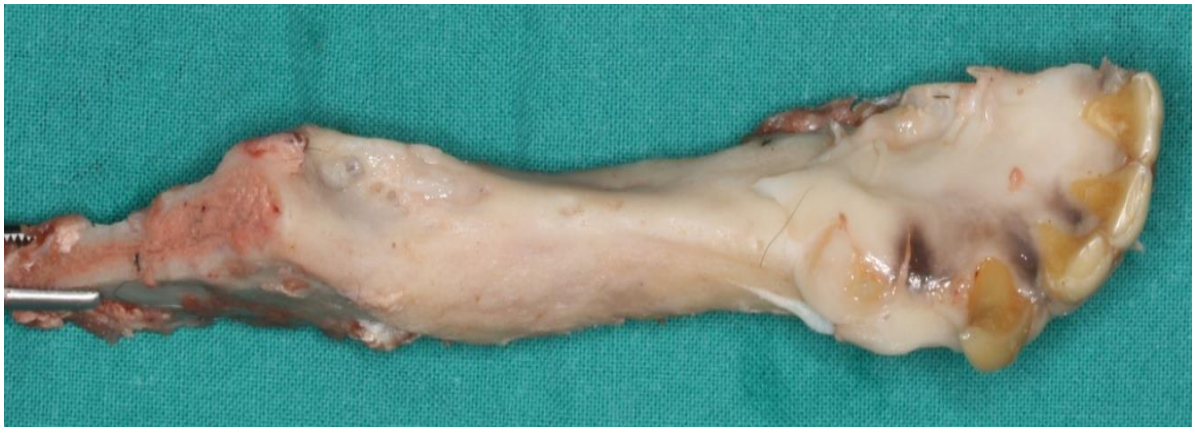

Figure 5b: Specimen 005 – rhBMP-7 *solo*. Occlusal view of right hemimandible showing restitution of bucco-lingual width in defect region restoring morphology of mandible.

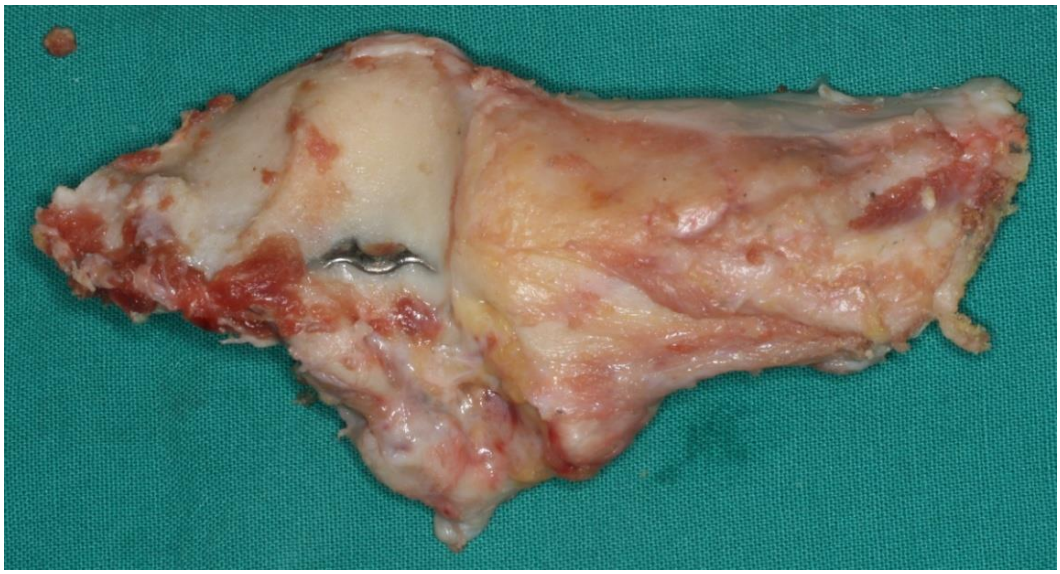

Figure 6a: Specimen 805A – rhBMP-7 *solo*. Lateral view of right hemimandible with reconstruction plate almost completely covered by bone regenerate. Regenerate overgrowth beyond the lower border of the mandible.

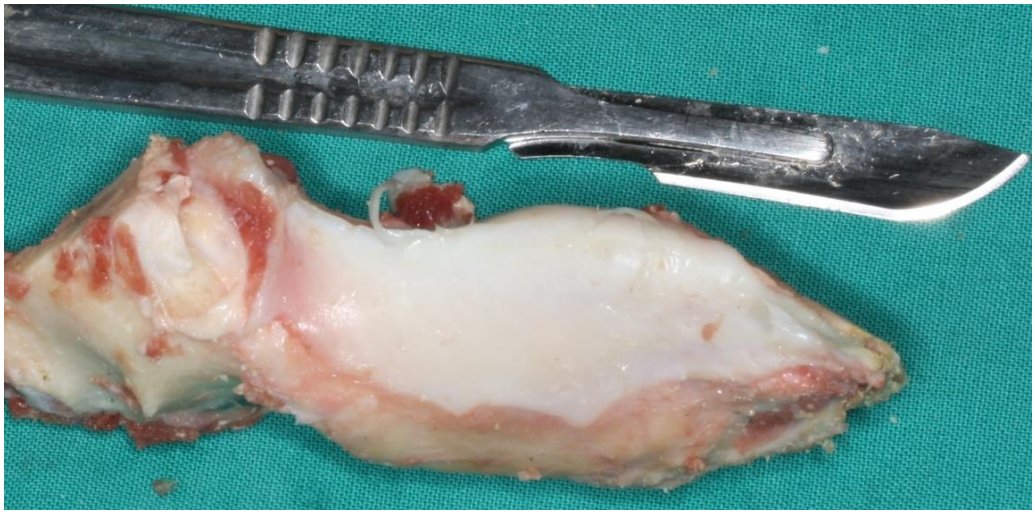

Figure 6b: Specimen 805A – rhBMP-7 *solo*. Inferior view showing bucco-lingual dimensions exceeding adjacent native mandible dimension.

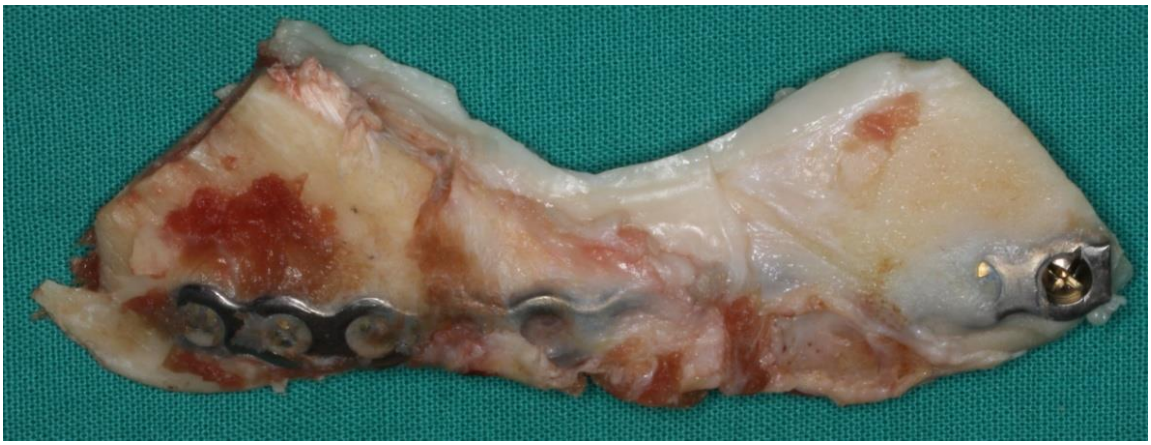

Figure 7a: Specimen 816 – rhBMP-7 + rhTGFβ_3. Lateral view of resected right hemimandible with reconstruction plate covered by bone regenerate. Decreased alveolar height in area of regenerate.

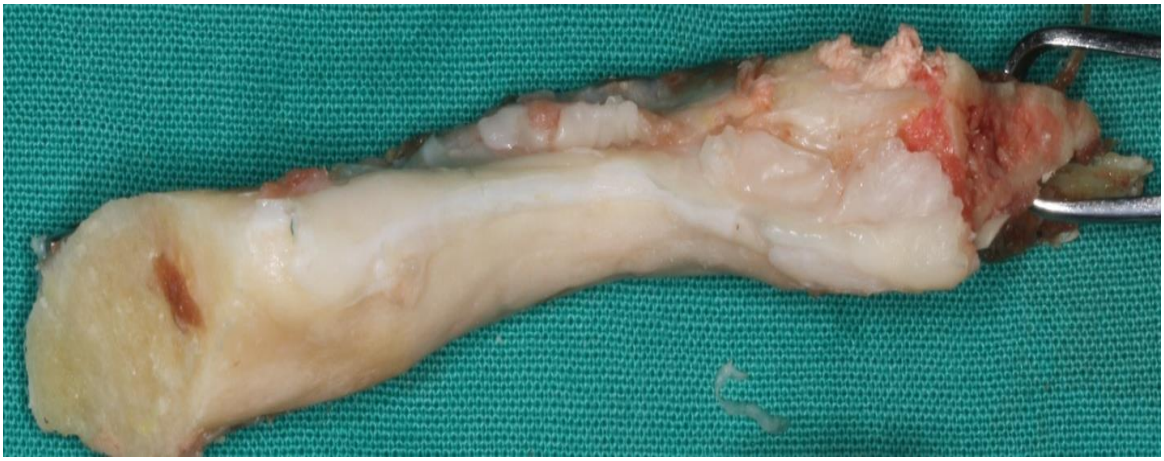

Figure 7b: Specimen 816 – rhBMP-7 + rhTGFβ_3. Occlusal view showing bucco-lingual dimension similar to the adjacent native mandible.

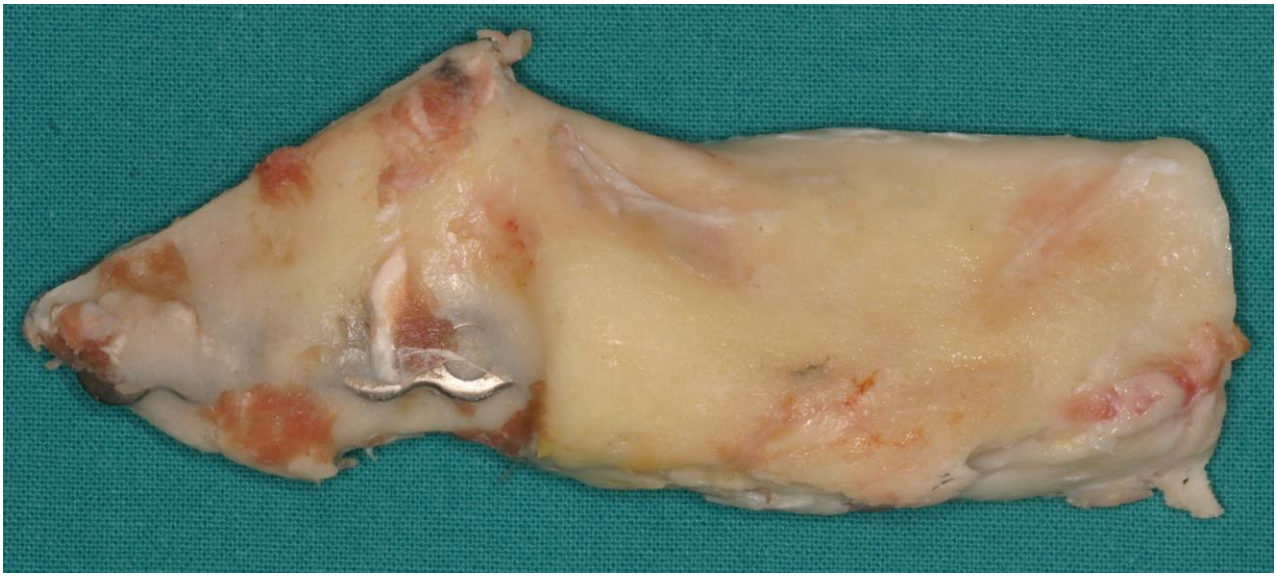

Figure 8a: Specimen 811 – rhBMP-7 + rhTGFβ_3. Lateral view of right hemimandible with reconstruction plate at the lower border covered by bone regenerate and no evidence of erstwhile defect.

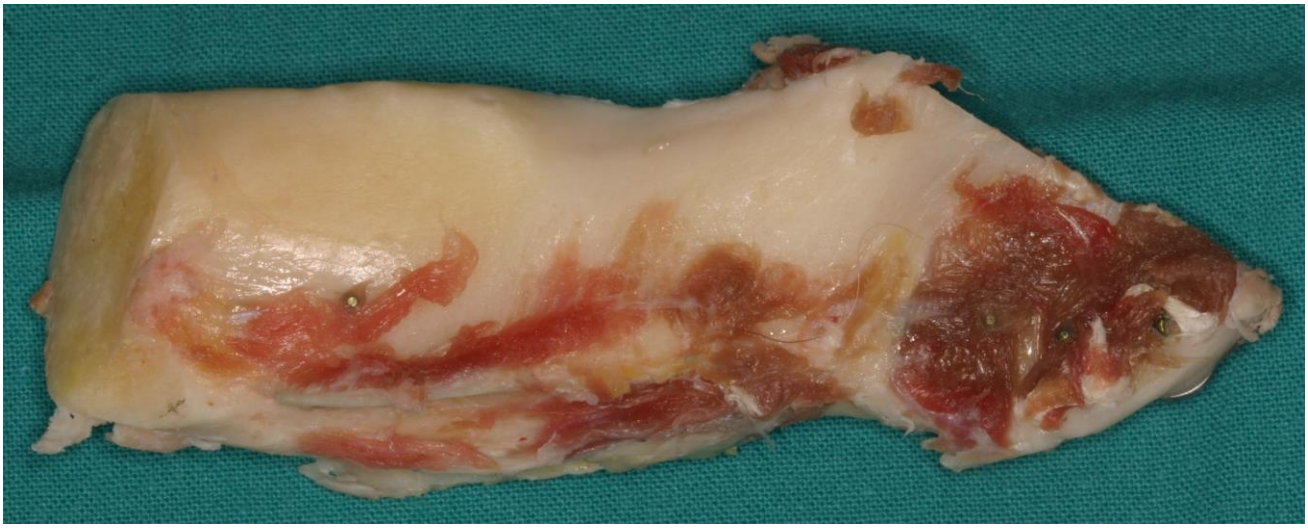

Figure 8b: Specimen 811 – rhBMP-7 + rhTGFβ_3. Lingual view of resected right hemimandible.

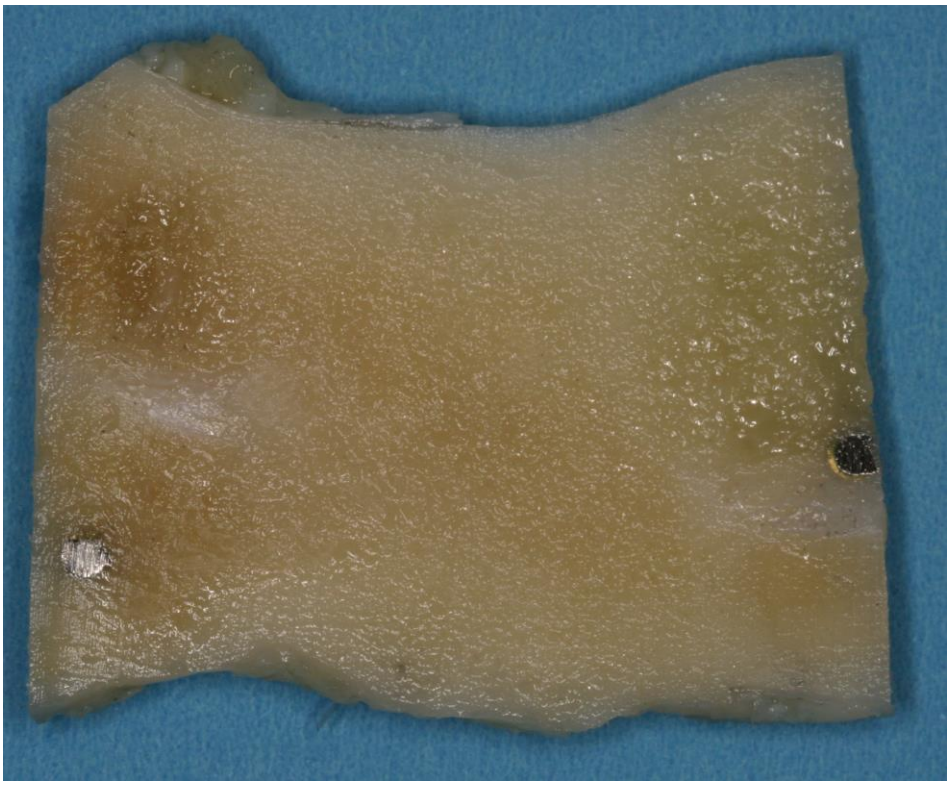

Figure 9: Specimen 005 – rhBMP-7 *solo*. Sagittal section through right hemimandible showing high density of bone regenerate.

4.2 **Qualitative Radiographic analysis**

Assessment of radiographic images was qualitative and considered bone volume, visibility of interface, and trabecular architecture. In all four specimens the defect margins were imperceptible, confirming complete remodelling and integration of the regenerate with the recipient bone. The newly formed bone was isodense (in some cases hyperdense) to the normal mandible (Figures 10-13). The radiographic appearance of all specimens was uniform throughout the defect area, with no areas of radiolucency noted to indicate poor bone formation or density. Cortical continuity was evident both on the buccal and lingual borders of the mandible, with no steps, breaks or radiolucency noted on either aspect of all four specimens. The trabecular architecture was indistinguishable from normal mandible confirming *restitutio ad integrum* of regenerated bone. Results thus confirm the achievement of *"clinically significant osteoinduction"* as the quality of the regenerated bone is identified radiographically as normal bone both in radiodensity and trabecular architecture.[54]

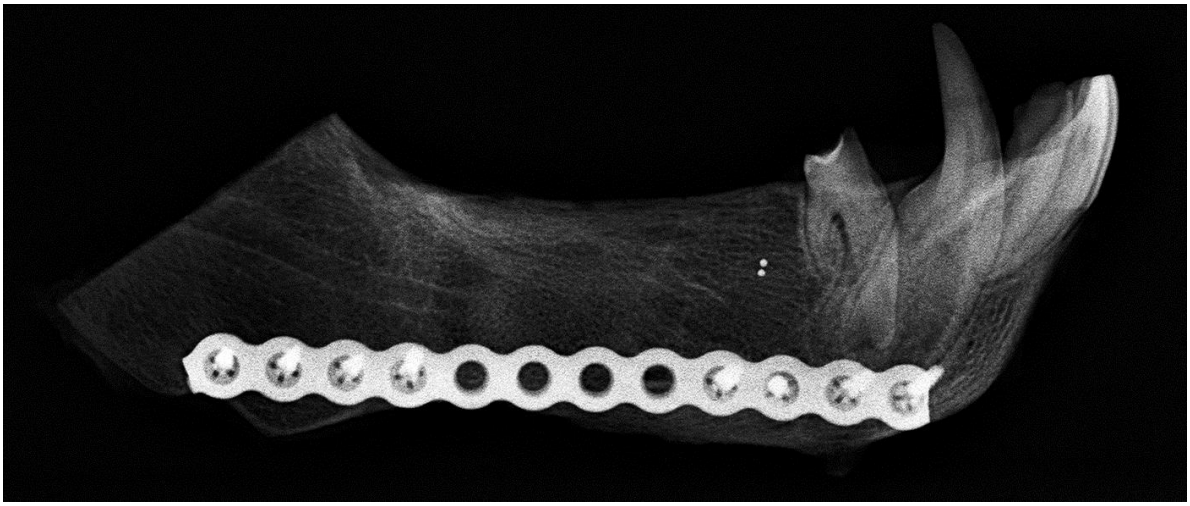

Figure 10a: Specimen 005 - BMP-7 *solo*. Lateral view of right hemimandible revealing loss of margins, isodense regenerate area, trabecular morphology similar to adjacent mandible, and restitution of volume within defect.

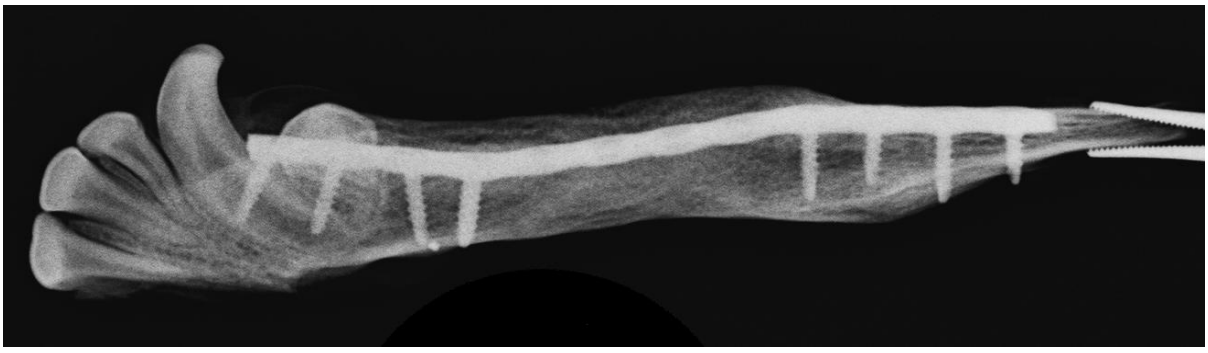

Figure 10b: Specimen 005 - BMP-7 *solo*. Occlusal view showing restored bucco-lingual dimensions with isodense bone regenerate within defect.

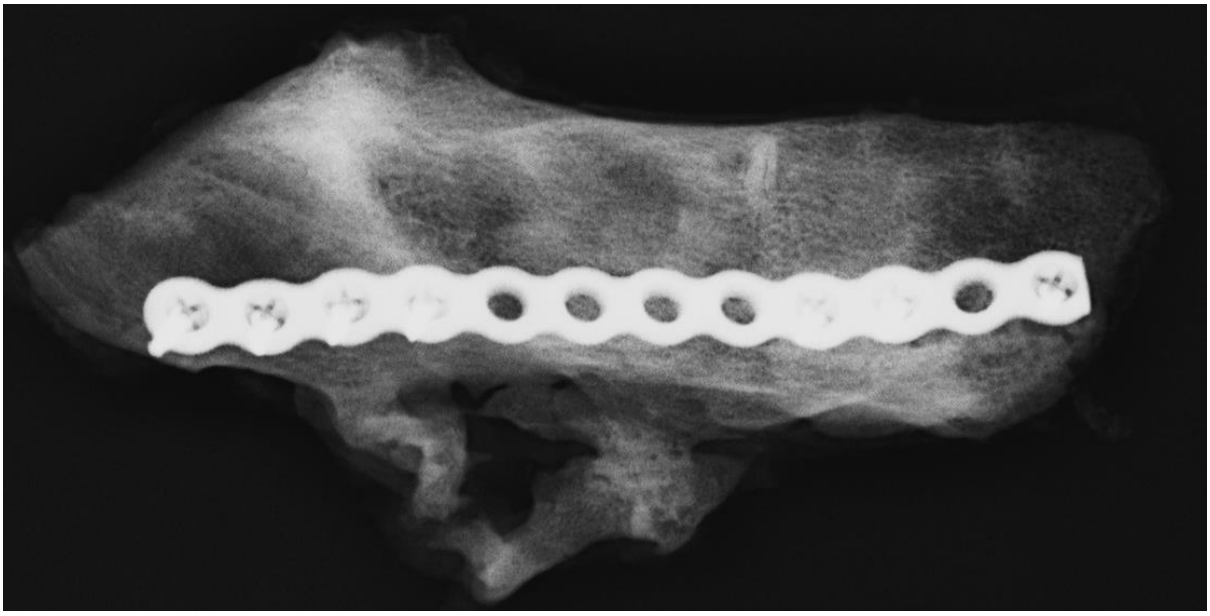

Figure 11a: Specimen 805 - BMP-7 *solo*. Lateral view showing exuberant growth of regenerate at the lower border. The regenerate area appears more radiodense than the native mandible.

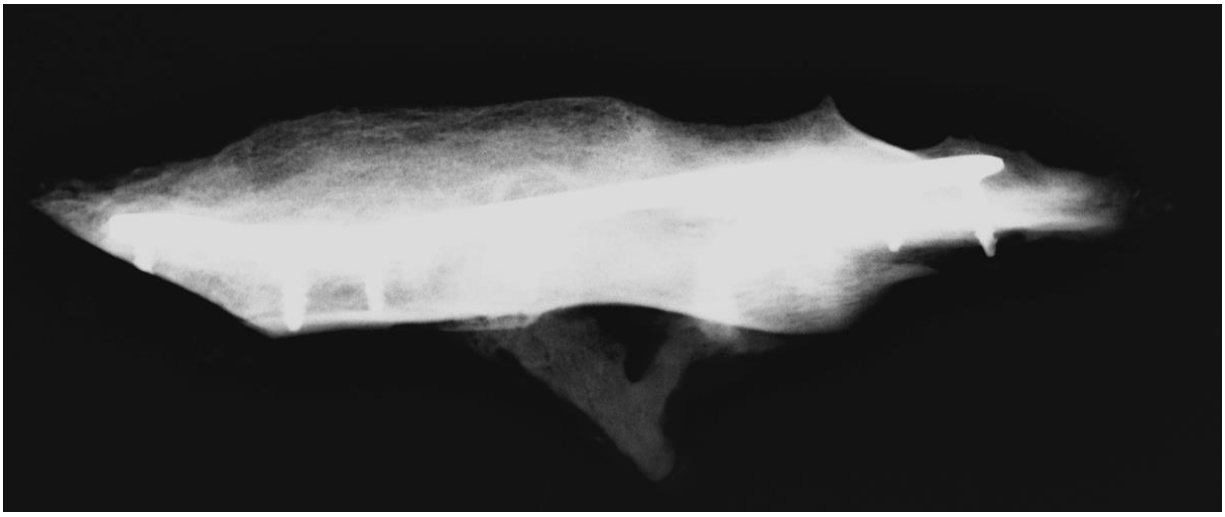

Figure 11b: Specimen 805 - BMP-7 *solo*. Superior view with increased bucco-lingual dimension.

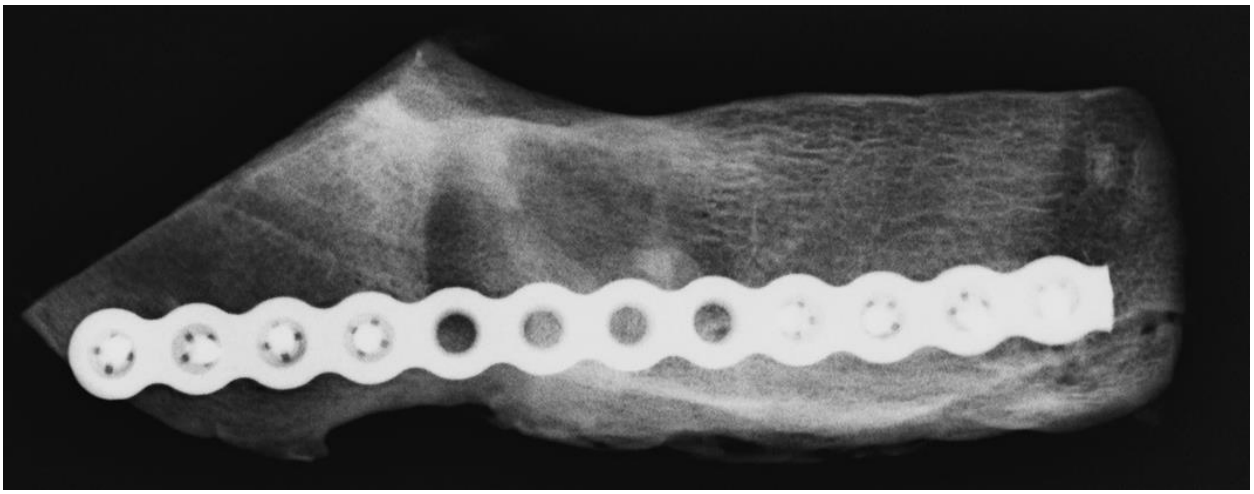

Figure 12a: Specimen 811 - BMP-7 + TGF-β_3. Lateral view right hemimandible with imperceptible defect margins and contiguous trabeculae.

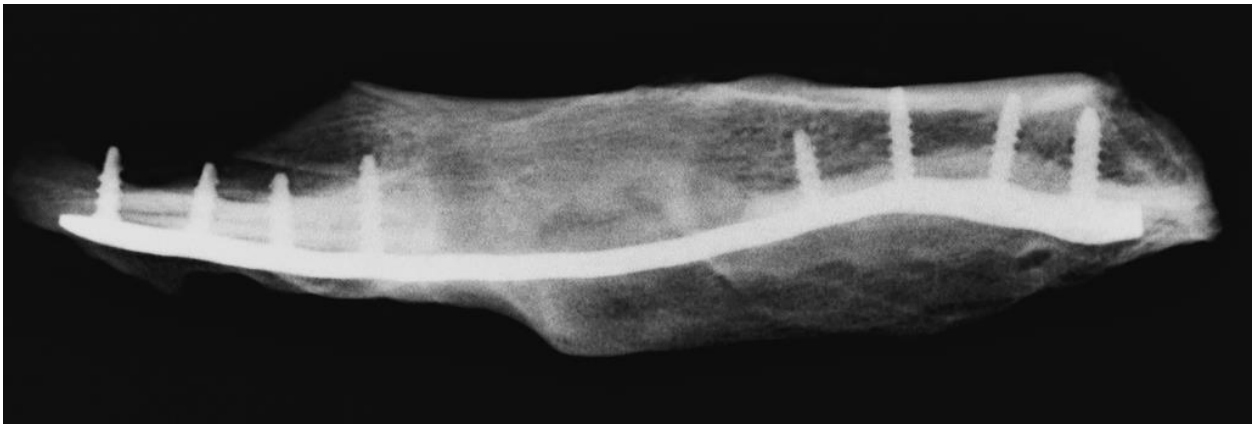

Figure 12b: Specimen 811 - BMP-7 + TGF-β_3. Occlusal view showing increased bucco-lingual dimensions with hyperdense bone regenerate.

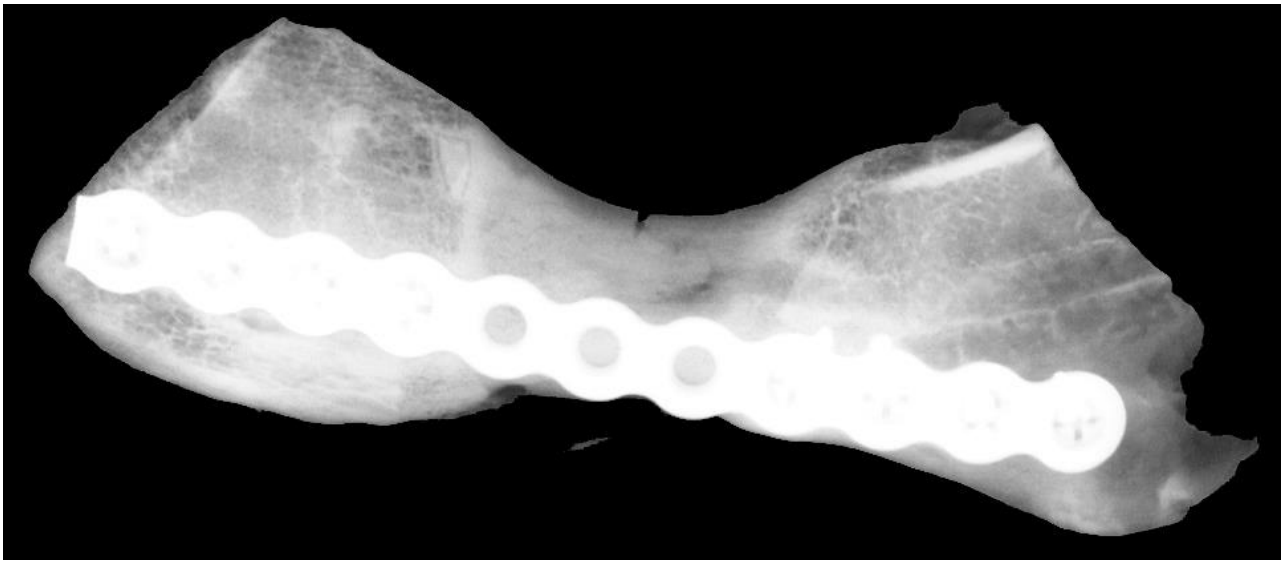

Figure 13: Specimen 816 - BMP-7 + TGF-β_3. Lateral view showing decreased alveolar height but bone regenerate isodense.

4.3 **Histomorphometry**

Representative photomicrographs of sagittal histological sections at 5x magnification are shown in figures 14-17a through each specimen. Low power histology confirms regeneration with highly mineralized bone trabeculae. Trabecular density is greater than the adjacent normal mandible. The superior and inferior cortical plates are restored. Bone formation can be seen along the recipient margins with more delicate mineralizing trabeculae of woven bone towards the centre of the previous defect area. High power magnification (10x) shows newly formed trabecular bone facing vascularised marrow with adipose tissue. Osteoblasts can be seen along the margin of woven bone (Figures 18a & b).

In specimen 005 implanted with rhBMP-7 *solo*, the mean amount of new bone formation within the defect was 229.1 ± 12.7 mm^2 (range 220-238mm^2), which is 43 ± 4.9% (range 40-47%) of the regenerate area, and 40.5 ± 6.4% (range 36-45%) of the total defect area. Thus the mineralised tissue occupied 43% of the total area of regenerate and 40.5% of the original defect area. In specimen 805 implanted with rhBMP-7 *solo*, the mean amount of new bone formation within the regenerate is 444.7 ± 68.7 mm^2 (range 396-493mm^2) which is 50.7 ± 7.8% (range 45-56%) of regenerate, and 48.5 ± 3.5% (range 41-56%) of the original defect area. In specimen 811 implanted with rhBMP-7 and rhTGF-β_3, the mean amount of new bone formation is 376.6 ± 68.6mm^2 (range 328-425mm^2) which is 53.3 ± 4.9% (range 50-57%) of regenerate area, and 48.9 ± 4.9% (range 45-52%) of the total defect area. In specimen 816 also implanted with rhBMP-7 and rhTGF-β_3, the average amount of new bone formation is 249.0 ± 22.6mm^2 (range 233-

265mm^2) which is 73.3 ± 7.1% (range 68-78%) in regenerate area, and 57.3 ± 7.1% (range 52-62%) of the total defect area (Table 1).

IMPLANT DEVICE	Bone area in regenerate (mm^2) Mean ± SD (Range)	ROI 1 * (%) Mean +/- SD (Range)	ROI 2 **(%) Mean +/-SD (Range)
BMP-7 SOLO – 005	229.0 ± 12.7 (220-238)	43.0 ± 4.9 (40-47)	40.5 ± 6.4 (36-45)
BMP-7 SOLO – 805	444.7 ± 68.7 (396-493)	50.7 ± 7.8 (45-56)	48.5 ± 3.5 (41-56)
BMP 7 + TGF-β3 – 811	376.6 ± 68.6 (328-425)	53.3 ± 4.9 (50-57)	48.9 ± 4.9 (45-52)
BMP 7 + TGF-β3 – 816	249.0 ± 22.6 (233-265)	75.3 ± 7.1 (68-78)	57.3 ± 7.1 (52-62)

Table 1: Mineralised bone areas

**Percentage of regenerate area consisting of mineralised bone*

***Percentage of original defect area consisting of mineralized bone*

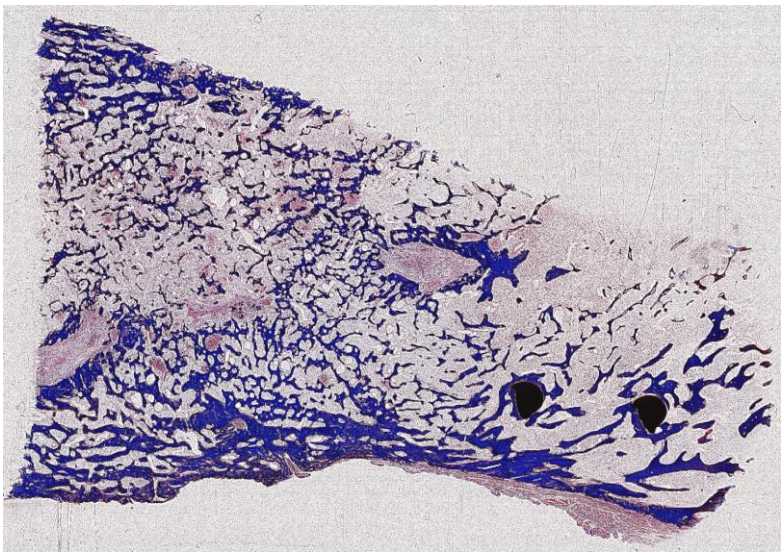

Figure 14: Specimen 005 – rhBMP-7 *solo*. Photomicrograph of undecalcified sagittal section stained with Goldner's trichrome (5x).

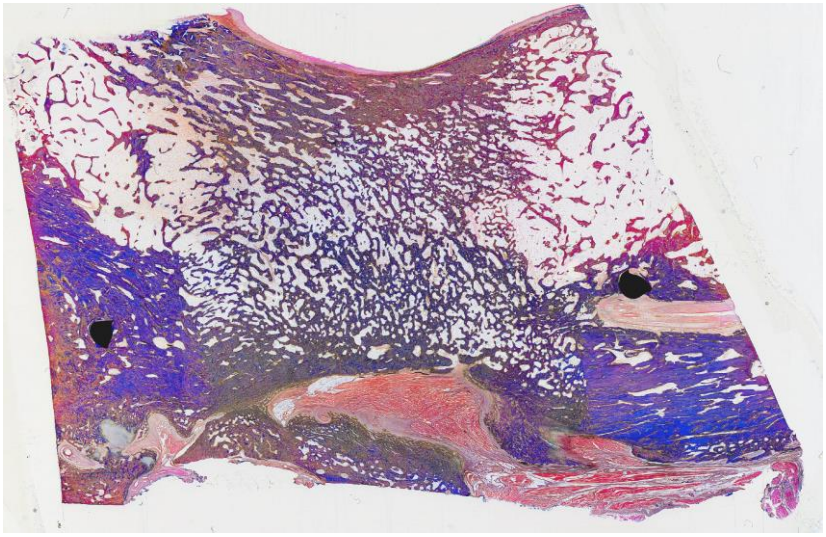

Figure 15: Specimen 805 BMP-7 *solo*. Photomicrograph through undecalcified sagittal section stained with a Goldner's trichrome (5x). Regenerated tissue shows higher trabecular bone density than the native mandible.

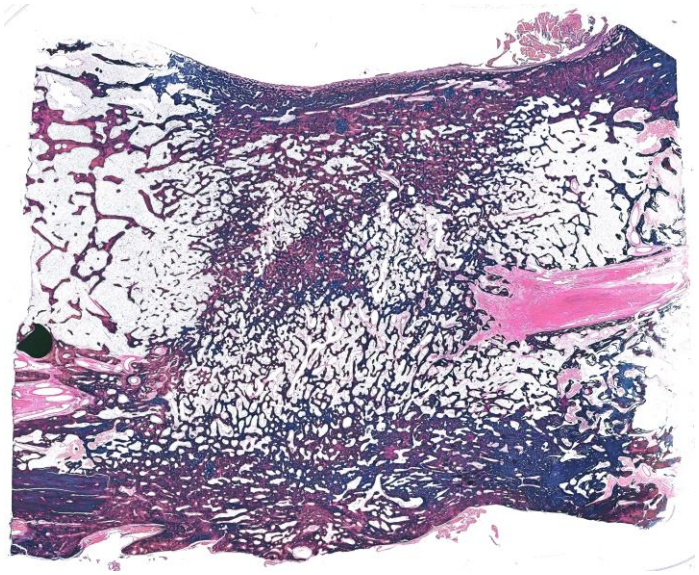

Figure 16: Specimen 811 BMP-7 + TGF-β_3. Photomicrograph through undecalcified sagittal section stained with a Goldner's trichrome (5x). Cortical regeneration and increased trabecular density within the defect.

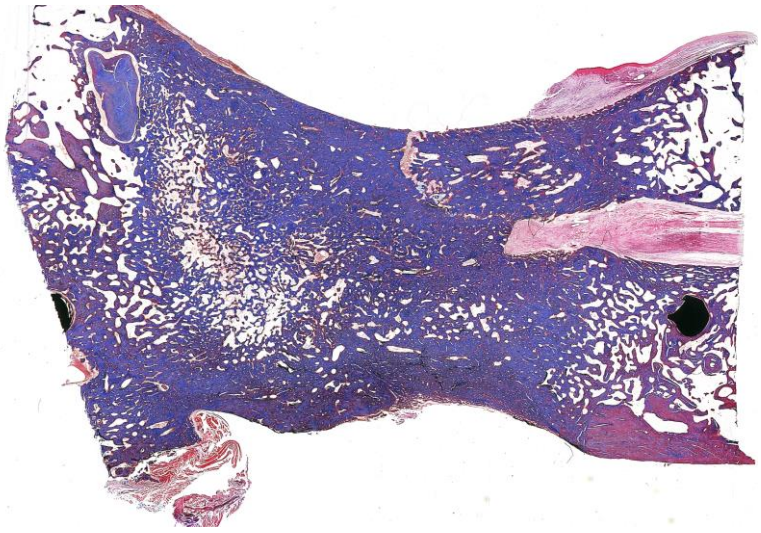

Figure 17: Specimen 816 BMP-7 + TGF-β_3. Photomicrograph through undecalcified sagittal section stained with a Goldner's trichrome (5x). Exceptional bone density within defect area.

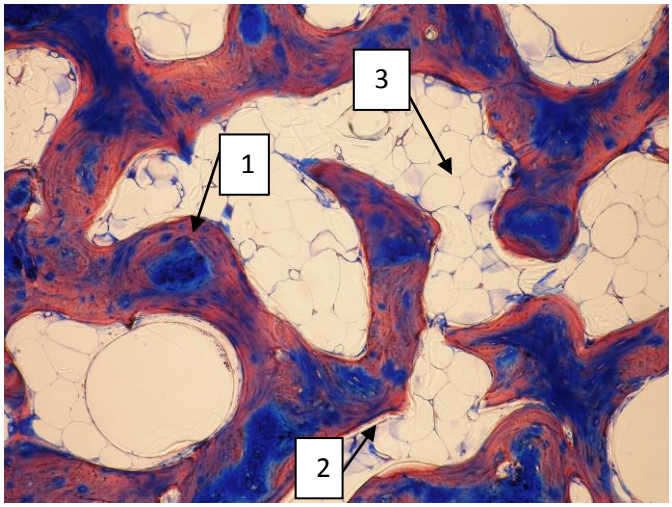

Figure 18a: Specimen 805 - BMP-7 *solo*. High power photomicrograph (10x) through undecalcified sagittal section stained with a Goldner's trichrome. Newly formed trabecular bone faces vascularized marrow with adipose tissue. Arrow 1: Mineralized tissue, arrow 2: osteoblasts visible along margins of bone, arrow 3: adipose tissue.

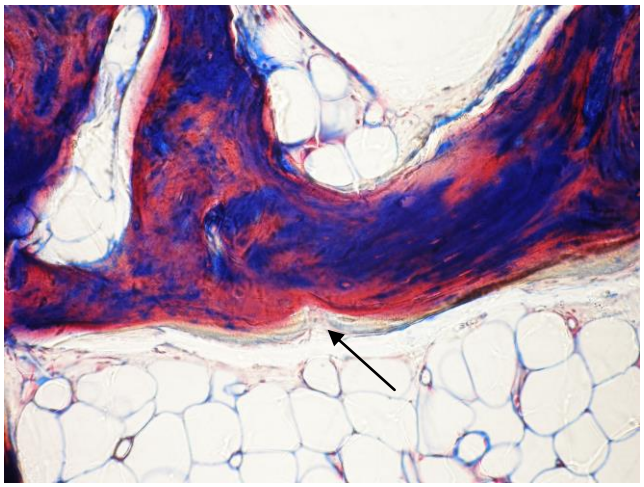

Figure 18b: Specimen 805 - BMP-7 *solo*. High power photomicrograph (10x) through undecalcified sagittal section stained with a Goldner's trichrome. Osteoblasts visible along margin of mineralised bone (arrow).

5 Limitations

The small number of animals available for the study was a significant limitation. With only two animals in each study group, it was not possible to identify differences between the treatment groups. The lack of a control group is another limitation, however given the scarcity of animals available for scientific research, and that previous work in calvaria confirmed that defects of 2.5cm in diameter do not heal spontaneously, it was decided that a control group was unnecessary, since a 2.5cm segmental defect in the mandible is a non-healing defect. Within the limit of the study however, binary application of BMP-7 and TGF-β_3 (ratio of 20:1) showed significant restitution of integrity as evaluated histologically and radiographically (Figures 10-18), but above all, superb morphological healing with substantial bone regeneration and cortication of the newly formed segment of the mandibular defect with superior bone density across the treated defect area (Figures 5-9).

6 DISCUSSION

This study found that all the implanted devices regenerated bone and restituted the osseous micro and macro anatomy of the treated mandibular defect. Comparing the area of bone regenerate in all four specimens, specimen 805 implanted with rhBMP-7 *solo* produced the largest area of bone regenerate within the defect. Following this was specimen 811 which was implanted with rhBMP-7 and rhTGF-$\beta_{3.}$ Specimen 816 (rhBMP-7 and rhTGF-β_3) had bone areas slightly lower than 811, and the least amount of bone was produced in specimen 005 which was implanted with rhBMP-7 *solo*. Specimen 816 was volumetrically deficient but the density of the regenerate was significantly higher in this specimen than all others. This small study failed to identify a difference in bone regeneration between the two treatment groups.

Many studies have used histology as the arbitrate of success or failure. The contention of this study is that radiography is a far more important tool to assess whether a regenerate has achieved preclinical success. High power histology demonstrating new bone formation is often used to erroneously conclude success. Although histology is a useful adjunct, it often hides the bone architecture, density and mineralization where high power images are zoomed in on only small areas of bone formation. Radiographic examination of the entire defect provides unequivocal evidence of mineralized tissue regeneration within a defect, the volume of tissue regenerated, the amount of remodelling that has occurred (thus resulting in the loss of an obvious defect interface) and finally the restitution of the micro anatomical structure of osseous trabeculation.

The assessment of all specimens was subjective and qualitative to replicate the clinical scenario of bone graft assessment. This confirmed that new bone regeneration had occurred within the mandible defects. The bone was isodense to the adjacent normal bone indicating good regenerated bone quality. Remodelling was so extensive that the defect margins had become imperceptible. The main difference between the various specimens was that of the bone height within the defect, where specimen 805 showed greatest bone height, followed by 811 and 005, with 816 having the lowest levels of bone height. Although the bone height of 816 was lowest, this specimen defect appeared to be most radiopaque. These results were supported by the histomorphometry analysis. Finally trabecular morphology is a final confirmation of remodelling and once again the regenerate was indistinguishable from the adjacent mandible.

The results of the study have reconfirmed that the morphogens of the TGF-β superfamily regenerate tissue within surgically created defects that is histologically identifiable as mineralized bone. More telling is the radiographic assessment which shows outstanding *restitutio ad integrum* of the surgically created defect. This allows us to conclude that clinically significant osseoinduction has been achieved.

Binary application of osteoinductive growth factors has previously shown to significantly increase the volume of bone regenerate in orthotopic and heterotopic sites (calvarium) of non-human primates.[24, 26] In this small study no performance advantage was found between the single morphogens and binary treated defects, beside the radiographic and histomorphometric examinations as outlined under the limitations of the study.

A final fundamental question still remains: Are these results translatable into successful therapy for human patients? It is becoming clear that this may not be the case as previous attempts at therapeutic osseoinduction in humans have shown. Although described as clinically successful by its author, critical examination of published radiographic evidence (Figure 1) cannot support this contention. Moreover, increasing concern regarding efficacy and safety of Infuse (rhBMP-2) raised in several articles by *Spine Journal*[50], led to a 16 month investigation into Medtronic (Medtronic Inc., Minneapolis, U.S.A.) the makers of Infuse. This investigation found questionable ties between the company and the physician consultants commissioned with testing, reviewing and reporting on Medtronic products. From this investigation by the Senate Finance Committee, it was found that the studies published by Medtronic were inaccurately representing the risks of InFuse in addition to placing added weight on alternative treatment side effects. Medtronic was found in violation of the trust of patients in their medical care and as a result sullied the reputation of bone inductive agents.[55]

Resolving these challenges will require renewed vigour and courage on the part of scientists, physicians, patients, and regulatory authorities.

7 CONCLUSION

The osteogenic proteins of the TGF-β superfamily, *solo* or in combination, regenerate osseous defects of the mandible in *Papio ursinus*. Qualitative assessment of the regenerate reveals that clinically significant osteoinduction has been achieved.

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