

THE REPAIR OF SEGMENTAL MANDIBULAR DEFECTS BY A
BONE MORPHOGENETIC PROTEIN BONE DEVICE IN MAN.

Carlo Ferretti

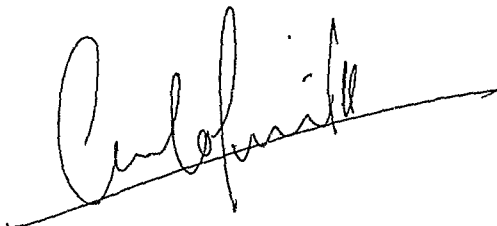
A dissertation submitted to the Faculty of Health Sciences,
University of the Witwatersrand, Johannesburg, in partial
fulfilment of the requirements for the degree of
Master of Dentistry in the branch of Maxillofacial and Oral Surgery.

Johannesburg, 1999

THE REPAIR OF SEGMENTAL MANDIBULAR DEFECTS BY A
BONE MORPHOGENETIC PROTEIN BONE DEVICE IN MAN.

Carlo Ferretti

I, Carlo Ferretti, hereby declare that this dissertation is my own work and has not been submitted before for any qualification or examination.

A handwritten signature in black ink, appearing to read 'Carlo Ferretti', written over a horizontal line.

Carlo Ferretti

PRESENTATIONS AND AWARDS RESULTING FROM THIS WORK

The Repair of Mandibular Segmental Defects by a BMP Bone Device in Man: Preliminary Results. Congress of the South African Society of Maxillofacial and Oral Surgeons at Sun City, South Africa; August 1997.

Awarded the Leibinger Prize for the best post-graduate paper at the Congress of the South African Society of Maxillofacial and Oral Surgeons at Sun City, South Africa; August 1997.

A portion of this research was televised internationally on the programme Beyond 2000.

To my patients,
who entrusted me with their treatment
and allowed me the privilege to perform this research.

ACKNOWLEDGEMENTS

To my supervisor, Professor Ugo Ripamonti, for introducing me to scientific research and showing me that the pursuit of relevant Science requires hard work, creative thought, but above all passion.

To the staff of the Bone Research Laboratory for always providing unfaltering assistance, particularly during the extraction of BMPs and the preparation of histological sections.

To Professor John Lownie, the consultants and my colleagues in the Division of Maxillofacial and Oral Surgery, who allowed me to include many of their patients in my research.

TABLE OF CONTENTS

DECLARATION

PRESENTATIONS AND AWARDS RESULTING FROM THIS
WORK

DEDICATION

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

ABSTRACT

1.	INTRODUCTION	1
2.	MATERIALS AND METHODS	13
	SECTION I: Preparation of the Osteogenic Device	13
	PART 1: Extraction of Native BMPs from Bovine Bone	13
	1.1 Preparation of Bovine Demineralized Bone Matrix	14
	1.1.1 Preparation of Bovine Bone Powder	14
	1.1.2 Demineralization of Bovine Bone Powder	15
	1.2 Extraction of BMPs from Bovine Demineralized Bone Matrix	15
	1.2.1 Heparin affinity chromatography	17
	1.2.2 Hydroxyapatite chromatography	17
	1.2.3 Sephacryl S-200 gel filtration chromatography	18
	1.3 Testing of Osteogenic Activity	19
	1.3.1 Preparation of Rat Heterotopic Implants	19

1.3.2 Bioassay of Bovine BMPs in Long Evans Rats	19
1.3.3 Histology on Harvested Implants	20
1.3.4 Alkaline Phosphatase Assay	21
1.3.5 Identification of Native Osteogenic Fractions	22
PART 2: Preparation of Delivery System	23
2.1 Preparation of Human Demineralized Bone Matrix	23
PART 3: Formulation of Osteogenic Device	23
3.1 Combining Bovine BMPs with Human Demineralized Bone Matrix	23
SECTION II: Clinical Procedures	25
Part 1: Patient Selection	25
1.1 Pre-operative Assessment	25
1.2 Patient Profile	25
1.2.1 Patient 1	26
1.2.2 Patient 2	26
1.2.3 Patient 3	27
1.2.4 Patient 4	27
1.2.5 Patient 5	28
1.2.6 Patient 6	28
1.2.7 Patient 7	29
1.2.8 Patient 8	30

1.2.9 Patient 9	30
1.2.10 Patient 10	31
1.2.11 Patient 11	31
1.2.12 Patient 12	32
1.2.13 Patient 13	32
PART 2: Surgical Procedures	33
2.1 Recipient Site Preparation	33
2.2 Autologous Bone Procurement and Preparation	33
2.3 Osteogenic Device Preparation and Placement	34
PART 3: Post-operative Procedures	34
3.1 Immediate Post-operative Care	34
3.2 Post-operative Assessment and Follow-up	35
PART 4: Histological Evaluation of Reconstructed Segments	36
4.1 Biopsy of Reconstructed Segments	36
4.2 Tissue Processing for Histology	36
4.3 Morphometric Analysis	37
3. RESULTS	38
SECTION I: Testing and Identification of Inductive Factors	38
PART 1: Bioassay of Native Bovine BMP	38
PART 2: Identification of Osteogenic Fractions	38
SECTION II: Clinical Results	39

Part 1: Follow-up	39
Part 2: Aesthetics	39
Part 3: Function	39
Part 4: Radiographic Assessment	40
Part 5: Recipient Site Complications	41
Part 6: Donor Site Complications	41
SECTION III: Biopsy Analysis Results	42
Part 1: Histological examination	42
1.1 BMP Device Patients with Successful Osteogenesis	42
1.2 BMP Device Patients with Failed Osteogenesis	43
1.3 Autologous Bone Graft Patients with Successful Osteogenesis	43
1.4 Autologous Bone Graft Patients with Failed Osteogenesis	43
Part 2: Histomorphometry	44
ANNEXURE A	45
FIGURES	47
4. DISCUSSION	72
LIST OF REFERENCES	88

ABSTRACT

Bone induction with naturally sourced BMPs has been demonstrated repeatedly in heterotopic and orthotopic sites of non-human primates. This has spawned the investigation of bone regeneration in mandibular defects of human patients. This was compared to osteogenesis in patients treated with autologous bone grafts, considered the gold standard for the reconstruction of skeletal defects.

The osteogenic device was formulated as a combination of human demineralized bone matrix as a carrier and naturally sourced BMPs. BMPs were extracted from bovine bone with chaotropic agents and subsequently purified by a sequential chromatographic process. BMPs (quantified as alkaline phosphatase units of activity) were loaded onto human demineralized bone matrix. The device was combined with sterile saline and applied to the defects as a paste. Autologous bone was obtained from the iliac crest and prepared as a particulate cancellous bone and marrow graft in a bone mill. These were loaded into perforated titanium mesh trays secured to the remaining mandible. Patients were followed-up clinically and radiographically at 1, 6 week, 3, 6, 12 month intervals. A trephine biopsy of the implants was performed at 3 months post-implantation and the specimens examined on undecalcified bone sections.

The osteogenic device induced bone formation in 2 of 6 patients treated. Histological examination of successful implanted BMP devices exhibited mineralised bony trabeculae with copious osteoid seams. These were lined by contiguous osteoblastic layers. Bone deposition directly onto non-vital matrix provided unequivocal evidence of osteoinduction. Bone induction occurred without a chondrogenic phase. Of the 7 patients grafted with autologous bone 5 had histological evidence of osteogenesis. Morphometric analysis of the histological sections showed that, when successful, BMP treated defects had highly active osteogenesis compared to autologous bone. However, the high proportion of inactive BMP implants indicates the need for further development to produce an osseoinductive system which performs reliably in this clinical context. Surprisingly, 2 autologous bone grafts also failed. This may be as a result of the more advanced age of the patients and the inclusion of periosteum in the surgical resection. Moreover, all the autologous implants demonstrated volumetric decline over the follow-up period. Histomorphometric analysis showed that in all patients successful grafts had a far higher fibrovascular tissue content than the failed grafts. This suggests that the titanium mesh system may be biologically flawed and act as a barrier to angiogenesis. These results provide the first histological evidence of the ability of naturally sourced BMPs to induce bone formation in mandibular defects in human subjects.

1. INTRODUCTION

The reconstruction of skeletal defects of the axial and appendicular skeleton poses a considerable challenge to modern surgery. The gold standard for skeletal reconstruction at the end of the twentieth century remains autologous bone. The realisation that autologous bone provided a reliable source of replacement bone was first made several centuries ago, however the clinical application of this knowledge only began in the early 19th century (De Boer, 1988). World War I provided a major impetus to the birth of a systematic approach to reconstruction of maxillofacial defects based on biological principles. Kazanjian (1918) is credited with the development of maxillofacial reconstructive techniques based on non-vascularised free bone grafts which are still in use today. The reconstruction of ever more complex defects in compromised situations exposed the limitations of non-vascularised bone grafts and it became evident that there was a need to develop techniques which allowed for the transfer of vascularised bone with or without soft tissue. The use of vascularised fibula for reconstruction of contralateral fibular defects was pioneered by Taylor in 1975 and subsequently Hidalgo adapted the technique for the reconstruction of mandibular defects as recently as 1989. Today there are several options available for vascularised reconstruction of the mandible including rib (Sarafin et al., 1977), radial forearm flap (Yang et al.,

1981, Soutar et al., 1983), iliac crest (Taylor, 1982), and scapula (Sullivan et al., 1989). It is considered the zenith in the science of maxillofacial reconstructive surgery.

The reconstruction of segmental defects of the mandible remains a challenging endeavour despite major technological developments and advances in the understanding of the biology of bone grafting. Success requires due consideration of several important points. Reconstructive efforts must bear in mind that the host tissue-bed is often compromised, either as a result of severe post-traumatic scarring or radiation induced hypovascularity. The environment is further rendered hostile to grafting as a result of the potential for salivary contamination accompanied by microbial invasion. In addition, unlike long bones which must withstand mainly compressive forces, a mandibular graft must be able to withstand flexional and shearing forces. Thus the successful reconstruction must fulfil several criteria (Carlson et al., 1996):

1. Mandibular continuity must be re-established.
2. The correct alveolar height must be established.
3. Arch form and width must be reconstituted.
4. Osseous bulk must be restored and maintained.
5. Facial form must be restored.

6. The possibility for future prosthetic rehabilitation of dental function must be provided.

In an attempt to meet these requirements several graft systems have been developed which include the following options:

- Autogenous bone en bloc
- Particulate cancellous bone and marrow (PCBM) grafts supported in a titanium mesh or an allogeneic freeze dried mandible.
- Pedicled osteomyocutaneous grafts
- Composite grafts supported by microvascular anastomosis

The use of particulate cancellous bone and marrow grafts was initiated by Mowlem in 1944 and subsequently popularised by Boyne (1969) who applied the technique for the reconstruction of several maxillofacial defects and acquired extensive experience during the Vietnam war. As a result of the work of several researchers the mechanism of bone formation in particulate cancellous bone and marrow grafts has been elucidated (Axhausen, 1956; Burwell, 1962; Craig Gray and Elves, 1983; Marx and Kline, 1983). Osteogenesis occurs in two phases, phase I bone being deposited by the transplanted osteoblasts and endosteal cells, while phase II bone is produced by osteoblastic cells induced from the host soft tissue bed which remodel the graft and replace the phase I bone with mature osteons. In contrast, block

grafts undergo complete necrosis of the cellular elements and new bone formation occurs over several years by creeping substitution (osteconduction) from the edges of the defect by cellular elements derived from host bone (Burchardt, 1982; 1987). In addition vascularisation of particulate cancellous bone and marrow grafts occurs in a matter of days as opposed to months or years for block grafts (Burchardt, 1982, 1987; Thorogood and Gray, 1975). A recent human study provided further evidence of the significantly faster healing in particulate cancellous bone and marrow grafts than in block grafts (Shirota et al., 1996). Moreover, particulate cancellous bone and marrow graft in supporting trays has several technical advantages over other graft systems. It allows for more exact reproduction of facial form and function and is technically far simpler than microvascular techniques. Consequently it has been used extensively for mandibular reconstruction in several centres (Boyne, 1969; Dumbach, et al. 1994; Carlson and Marx, 1996; Tideman, et al. 1998). However, particulate cancellous bone and marrow grafts are not a panacea for all situations and the use of vascularised grafts is still required in many patients, particularly those who have received radiation therapy for the management of malignancies and have compromised soft tissue bed cellularity and vascularity (Manson, 1994). Despite the relative success of the available reconstructive options, they all are associated with considerable drawbacks. They require the use of autologous bone and soft tissue which may result in

considerable donor site morbidity (often more severe than the recipient site) (Marx and Morales, 1988; Hoffmann, et al. 1998; Beirne, et al. 1996) and occasionally patient mortality (Brazaitis, et al. 1994) as a result of tissue harvest. Moreover the reconstruction of particularly large defects can be problematic due to the limited volume of autologous tissue available.

These problems have provided the impetus for the development of alternatives to autologous bone for the reconstruction of osseous defects. The first steps towards an understanding of the complex biology of osseous regeneration came from a surprising source. Charles Huggins observed that the implantation of living urinary tract or urinary bladder epithelium in the rectus abdominis muscle of dogs resulted in the formation of bone. This phenomenon was termed epithelial osteogenesis (Huggins, 1931). The existence of growth and morphogenetic factors in the extracellular matrix of bone was initially postulated by Lacroix (1945). He proposed that endochondral ossification was under the influence of a group of substances which he termed *osteogenin*. Further evidence for their existence was the discovery that new bone was formed by non-vital, demineralised bone matrix implanted in muscle pouches of rodents (Urist, 1965; Reddi and Huggins, 1972). Subcutaneous implantation of demineralized bone matrix results in a sequential cascade of biochemical and morphogenetic events that culminates

in the formation of endochondral bone (Reddi, 1981). It is reminiscent of the sequence of events occurring during embryonic endochondral bone differentiation. The developmental cascade is characterised by fibronectin deposition on the matrix to enhance cellular adhesion, chemotaxis and attachment of mesenchymal stem cells, cellular proliferation in response to mitogenic signals and differentiation of chondroblasts, vascular invasion and chondrolysis followed by differentiation of osteoblasts and formation of bone (Reddi, 1981). This phenomenon was termed bone formation by induction and it provided the first evidence of the existence of molecular initiators within the extracellular matrix of bone responsible for the initiation of the osteogenic cascade. These elusive morphogens were called bone morphogenetic proteins (BMPs). A concerted research effort was initiated to unravel the complexities of the molecular control of bone formation and regeneration during embryonic development and during post-fetal life. The identification and isolation of these factors proved to be a difficult task since these factors were present in small quantities and were intimately bound to the extracellular matrix of bone (ECM). Several important technical developments contributed to the elucidation of the role of BMP's in bone formation.

The first was the finding that the osteogenic activity of demineralized bone matrix could be eliminated by dissociative extraction with chaotropic agents

such as 4M guanidinium hydrochloride or 8 M urea and the development of a functional bioassay in the subcutaneous space of a rat to monitor the specific biology of osteogenic activity (Sampath and Reddi, 1981). Neither the soluble extract, nor the residual insoluble collagenous bone matrix (ICBM) induced an endochondral cascade in ectopic bioassays. Biological activity could only be restored by reconstituting the ICBM with the soluble signal, demonstrating the presence of putative inductive molecules in the protein extracts.

Secondly, the application of heparin affinity chromatography to the protein extracts from bovine demineralized bone matrix (Sampath, et al. 1987) allowed the isolation and purification to homogeneity by electroendosmotic elution in sufficient quantities to allow for the amino acid sequencing of the first osteogenic protein designated osteogenin (Luyten, et al. 1989).

Finally the use of DNA cloning techniques resulted in the expression of recombinant human BMP (Wozney, et al., 1988; Celeste, et al. 1990; Ozkaynak, et al., 1990). Since the cloning and expression of human osteogenin or BMP-3 several other BMPs have been identified, cloned and expressed. These include BMP-2, BMP-4, BMP-5, BMP-6, BMP-7 or OP-1 (osteogenic protein 1), BMP-8 or OP-2 (Ripamonti and Reddi, 1994 for review).

The BMP/OP family show sequence homologies with members of the transforming growth factor- β (TGF- β) superfamily (Wozney, et al., 1988; Celeste, et al. 1990; Ozkaynak, et al., 1990). The TGF- β superfamily encompasses a group of structurally related proteins affecting a wide range of differentiating processes during embryonic development (Heine et al., 1989; Rosa et al., 1988; Sporn and Roberts, 1990) . Indeed, several recent reports have provided important clues to the multiple roles BMPs play in the regulation of epithelial/mesenchymal interactions during embryonic development in addition to their traditional role of bone induction (Lyons et al. 1990, Vukicevic et al. 1994).

All the BMP members are capable of singly inducing endochondral bone formation in the ectopic bioassay and in orthotopic sites (Reddi, 1992). Moreover, recent evidence has also demonstrated the ability of TGF- β to initiate endochondral bone formation in heterotopic sites in baboons. (Ripamonti et al., 1996a; Ripamonti et al., 1997a; Duneas et al., 1997).

The induction and regeneration of cartilage and bone by BMPs recapitulates events that occur during the normal course of embryonic development. The therapeutic application of BMPs exploits a functionally conserved process originally deployed during fetal osteogenesis (Reddi, 1981). The potential to

harness the osteoinductive ability of BMPs and to deploy it for reconstructive osteogenesis may provide novel molecular approaches to the reconstruction of human skeletal defects and has captured the imagination of reconstructive surgeons.

Abundant evidence of the osteoinductive efficacy of BMPs has emerged from trials in several animal models. The application of recombinant or natively sourced BMPs has found to successfully heal critical sized defects, in amongst others, the ulna of dogs (Nilsson et al., 1986) and rabbits (Bostrom et al., 1996), calvarium in pigs (Lindholm et al., 1994) and baboons (Ripamonti et al., 1993a; Ripamonti et al., 1996b), femurs in sheep (Gerhart et al., 1993), and mandibles in rats (Hedner et al. 1995), dogs (Toriumi et al. 1991) and monkeys (Boyne, 1996). The latter trial studied the ability of recombinant human BMP-2 to regenerate bone in critical sized mandibular defects in rhesus monkey. Complete osseous regeneration was reported in all animals. Similar successful results were obtained in the canine model. The promising results obtained in animal trials have laid the foundation for the application of this new biotechnology in the field of human skeletal reconstruction in both the craniofacial and the orthopaedic setting.

Initial trials deployed human demineralized bone matrix in block and powder form. An early trial applied demineralized bone matrix to 3 children treated

for spinal scoliosis by vertebral fusion and reported clinical success (Sharrard and Collins, 1961). This was followed by the further use of demineralized bone matrix in degenerative joint and disk disease (Urist And Dawson, 1981), periodontal defects (Sonis et al., 1983) and nasal reconstruction (Toriumi et al., 1990). Xenogeneic (calf) demineralized bone matrix was applied to a phalangeal defect in a 14 year old girl. Clinical success was reported accompanied by histologic confirmation of osteoinduction (Upton et al., 1984).

Application of the principle of induced osteogenesis in the craniofacial region was pioneered by a group from Harvard Medical School (Glowacki et al., 1981). Allogeneic demineralized bone matrix was used in 34 patients for contour augmentation, intraosseous implants and for bone regeneration in a patient with cloverleaf skull deformity. Although claims of osteoinduction were made based on radiographic findings of bone formation from the centre of the grafted area, there was sparse histologic confirmation of this. The same group reported a further 78 patients that had received demineralized bone matrix for the treatment of congenital craniofacial skeletal defects and for the reconstruction of jaw defects (Mulliken et al., 1981; Kaban et al., 1982).

BMPs were first applied for clinical trials of osseous regeneration subsequent to the development of biotechnology to extract, isolate and purify native BMPs and consequently, the cloning and expression of recombinant human BMPs. This allowed the tailoring of therapeutic strategies by increasing the dose of delivered morphogen per gram of device, thereby improving the clinical performance over demineralized bone matrix. Applications were initially in the orthopaedic setting. Twelve patients with refractory non-unions of the femur were successfully treated by a combination of internal fixation and implants of human, native BMPs (Johnson et al., 1988). A subsequent report by the same group showed that human, native BMPs delivered with autolyzed, antigen-extracted, allogeneic bone successfully treated segmental defects of long bones (Johnson et al., 1992).

Use in the craniofacial region is less common but is gaining in popularity. Native bovine BMPs were used in a large series of diverse maxillofacial applications including implant placement, osteotomies, fractures and reconstruction (Sailer and Kolb, 1994a). The authors reported clinical success in compromised situations, but unequivocal evidence of induction was absent. Furthermore, the purification of BMPs was not to homogeneity and several other protein were implanted with the BMPs (Sailer and Kolb, 1994a). The same group reported on their experience with the reconstruction of human

calvarial defects using native bovine BMPs with human demineralized bone matrix carrier in conjunction with lyophilised cartilage (Sailer and Kolb, 1994b). Radiographic evidence of calcification was reported without histologic confirmation of induction. It is important to note that none of the publications reported any adverse local or systemic effects from the use of BMPs in the animal and human trials.

The aim of this study was to test the viability of a reconstructive device consisting of naturally sourced bovine BMPs with a human demineralized bone matrix as delivery system for the reconstruction of selected mandibular segmental defects in human patients.

2. MATERIALS AND METHODS

SECTION I: Preparation of the Osteogenic Device

Part 1: Extraction of Native BMPs from Bovine Bone

The methods used to extract and purify BMPs and to prepare demineralised bone matrix were developed and refined over the last two decades by several researchers in the field. (Sampath and Reddi, 1981; Sampath *et al.*, 1987; Luyten *et al.*, 1989; Ripamonti *et al.*, 1992). The majority of BMP in its native state is tightly bound to the collagen of the extracellular matrix of bone (Sampath *et al.*, 1987). A small fraction is associated with the mineral component of bone. The extraction strategy exposes the collagenous matrix to chaotropic agents such as urea or guanidinium to effect the solubilisation of the tightly bound BMPs. However, this system of protein solubilization is not BMP specific and many other proteins are extracted simultaneously. The conditions are then altered to promote the binding of BMPs to an immobilised heparin column, a first step in the purification of BMPs. The eluted BMPs are then applied to a hydroxyapatite column, to achieve further purification. Finally, gel filtration is employed to fractionate proteins on the basis of their respective molecular weights. At this stage, BMPs are still impure, but further

purification is avoided, as it results in loss of biological activity and low yields of proteins purified to homogeneity. Moreover, previous work in primates has established the optimal biological activity of purified BMPs after gel filtration chromatography (Ripamonti et al., 1992; Ripamonti et al., 1993a). Gel filtered fractions are collected, reconstituted with rat insoluble collagenous bone matrix and implanted in the subcutaneous space of the rat to identify osteogenic fractions. Osteogenic fractions, as determined by histology and tissue alkaline phosphatase activity, are pooled and exchanged into a medium of choice before clinical use.

1.1 Preparation of Bovine Demineralized Bone Matrix

1.1.1 Preparation Bovine Bone Powder

Approximately 150 kg of fresh bovine femurs and tibiae were obtained from a local abattoir and stored at -20 °C until further use. The epiphyses were resected using a circular saw and discarded. The diaphyses were sectioned into 1 cm discs and the exposed marrow was removed. The diaphyseal discs were cooled by immersion into liquid nitrogen and fractured into smaller fragments with a hammer. The bone fragments were dehydrated in absolute ethanol at 4° C, and defatted in ether at 4° C. Bone fragments were air-dried in a fume

hood. Dehydrated, defatted bone fragments were again cooled to -70°C using liquid nitrogen and crushed in a ring mill apparatus. The resultant bone powder was sieved to a discrete particle size of 75 to 420 μm .

1.1.2 Demineralisation of Bovine Bone Powder

5 kilograms of bovine bone powder were placed in a polypropylene extraction tank fitted with a porous polyethylene sieve (70 micron pore-size) to its bottom. The bone powder was demineralised in five volumes of 0.5 N hydrochloric acid at room temperature. Continuous stirring and pH monitoring was maintained throughout demineralisation. Successive five-volume additions of 0.5 N hydrochloric acid were made, until the pH of the slurry stabilised at a value below 0.1, indicating that demineralisation was complete. Spent hydrochloric acid was drained between successive additions. Demineralised bone matrix was washed three times with five volumes of distilled water and the pH of the slurry adjusted with five volumes of 50 mM Tris to pH 7.2 - 7.4.

1.2 Extraction of BMPs from Bovine Demineralised Bone Matrix

Extraction of BMPs from demineralised bone matrix was performed using the previously prepared bovine bone matrix. The extraction buffer consisted of 8 M urea, in 50 mM Tris pH 7.4, containing 100 mmol NaCl and the following enzyme inhibitors:

- 100 mM ϵ -aminocaproic acid
- 6.8 mM N-ethyl maleimide
- 5 mM benzamidine hydrochloride
- 0.5 mM phenylmethylsulphonyl fluoride.

Three volumes of extraction buffer were added to the demineralised bone matrix. Extraction took place with continuous stirring at room temperature for 17 hours. The extract was filtered off, and the insoluble collagenous bone matrix was re-extracted with a second addition of three volumes of extraction buffer as before. The first and second extracts were processed separately. Extracts were exchanged into 6 M urea, 50 mM Tris pH 7.4 containing 150 mM NaCl, by the process of ultrafiltration. This was accomplished on a 5 000 Da cutoff polyvinylidene fluoride membrane-based ultrafiltration cartridge of 6 square foot surface area (Millipore, USA). The extract was circulated at a flow volume of 6 L/minute, and at a pressure of 15 to 30 bar. 6 M urea, 50 mM Tris buffer pH 7.4, was added to the concentrate in successive steps, to achieve a

salt concentration of 150 mM in the extract, conditions which still allow the binding of BMPs to the heparin-Sepharose affinity chromatographic step which follows.

1.2.2 Heparin Affinity Chromatography

A heparin-Sepharose CL-6B column of 5 cm diameter by 20 cm height was employed (Pharmacia Fine Chemicals, Uppsala Sweden). The extract was passed through the column at 6 cm/hr and the column was washed with 6 M urea, 50 mM Tris-chloride buffer pH 7.4 containing 150 mM NaCl, until no proteins were detected in the eluate. This was determined by continuous photometric monitoring at 280 nm. Heparin bound proteins were eluted in a stepwise fashion with 6 M urea, 50 mM Tris pH 7.4 containing 500 mM NaCl. The eluted proteins were detectable at 280 nm, and were collected and exchanged into 6M urea, 50 mM Tris pH 7.4, 10 mM phosphate, using a 300 ml capacity diafiltration apparatus loaded with a 10 kDa cutoff membrane (YM-10, Amicon Corp., MA, USA).

1.2.2 Hydroxyapatite Chromatography

The heparin binding fraction was loaded onto a hydroxyapatite column (16 mm x 20 cm, Ultrogel, IBF, USA) equilibrated in 10 mM phosphate, 6 M urea, 50 mM Tris pH 7.4. The column was washed with 10 mM phosphate buffer, and eluted with 100 mM phosphate, 6 M urea, 50 mM Tris pH 7.4. This fraction was exchanged with 4 M guanidinium chloride, 50 mM Tris pH 7.4.

1.2.3 Sephacryl S-200 Gel Filtration Chromatography

The fraction from the previous step, in 4 ml of 4 M guanidinium chloride, was loaded onto two tandem columns (2.5 x 100 cm) of Sephacryl S-200 (200 kDa exclusion limit), and run at 6 cm per hour. This chromatographic procedure results in the separation of proteins based on their molecular weights. 20 ml fractions were collected, and 250 μ l set aside to be assayed in duplicate for osteogenic activity in the rat. The resultant osteogenic fractions (27 -31 kDa) were pooled, concentrated and exchanged into 10 mM HCl using a 50 ml capacity diafiltration apparatus loaded with a 5 000 dalton cutoff membrane (Amicon Corp. MA, USA). Concentration and exchange was repeated three times to achieve removal of all guanidinium in the pooled fraction. The total amount of protein in the pooled, exchanged medium was determined by the Lowry assay (Lowry *et al.*, 1951).

1.3 Testing of Osteogenic Activity

1.3.1. Preparation of Rat Heterotopic Implants

25 mg of allogeneic insoluble collagenous bone matrix prepared from the diaphysis of long bones of Long Evans rats, was placed into sterile 5 ml polypropylene tubes. Protein fractions in 10mM HCl were added to the matrix and vortexed thoroughly to ensure homogeneous permeation of the matrix. Chondroitin-C sulphate (100 μ l) of a concentration of 10 mg/ml and 50 μ l rat tail type I collagen in (5 mg/ml, 0.5 M acetic acid) were added (Muthukumaran *et al.*, 1988). The addition of soluble type I collagen and chondroitin sulphate reduces the friability of lyophilised pellets, resulting in solid plano-convex pellets ideally suited for implantation. Two to three volumes of chilled absolute ethanol was added and the tube vortexed. Precipitation was allowed to proceed at -20°C for 30 minutes. Tubes were centrifuged at 500 xg for 20 minutes, to pelletise contents, and supernatant ethanol discarded. The pellets were washed 3 times with 85% ethanol to remove the chaotrope and lyophilised *in vacuo* at -70° C.

1.3.2 Bioassay of Bovine BMPs in Long Evans Rats

The rat subcutaneous site was chosen to assess osteogenic activity (Reddi and Huggins, 1972). This model has been used for several years due to its simplicity of preparation and ease of tissue harvest at the conclusion of the experiment.

Rats were anaesthetised with an intravenous mixture of 5:1 ketamine/rompun. The anterior aspect of the thorax was shaved and prepared for surgery. *Via* a midline skin incision two pouches were created in a subcutaneous plane immediately above each pectoralis muscle. Implants were inserted into pouches, one implant per pouch. The incision was closed with 4/0 resorbable sutures (Vicryl, Ethicon Inc., New Jersey). The rats were housed in suspended cages in the rodent section of the Central Animal Services of the university, and kept under daily clinical observation. The rats were euthanased in a CO₂ chamber and the implants harvested on day 12 post-implantation. The harvested implants were sectioned in half, one fragment for histology the other for biochemical analysis.

1.3.3 Histology on Harvested Implants

Specimens were fixed in Bouin's fluid, dehydrated through graded changes of ethanol and infiltrated under vacuum (for 5 days) with historesin and embedded in historesin (LKB, Bromma, Sweden). 4 μ m sections were cut with a motorized microtome (Polycut-S, Reichert-Jung, Germany) and stained with toluidine blue.

1.3.4 Alkaline Phosphatase Assay

The implant fragment was homogenized in 2 ml of ice-cold 3mM sodium bicarbonate containing 0.15 M NaCl, pH 7.8. This was centrifuged and the supernatant retained for alkaline phosphatase activity determination. The alkaline phosphatase activity of the supernatant is considered an index of bone formation by induction (Reddi and Huggins, 1972; Reddi and Sullivan, 1980). Tissue alkaline phosphatase activity was determined by the colourimetric detection of the yellow coloured nitrophenyl phosphate resulting from enzymatic action by alkaline phosphatase on the substrate, para-nitrophenol phosphate (PNPP), at pH 9.3.

Substrate solution :5 mM PNPP in distilled water

Buffer :0.1 M sodium barbitol, pH 9.3

1 ml of substrate solution and 1 ml of buffer solution were placed in tubes, and temperature equilibration was achieved in a water-bath at 37° C. Blanks were prepared by the addition of 0.1 N NaOH to tubes containing substrate and buffer. The assay sample was then added, and the reaction allowed to proceed for 30 minutes before stopping it with the addition of 2 ml 0.1 N NaOH. The absorbance of samples was determined spectrophotometrically at 400 nm. Alkaline phosphatase activity was expressed as units/mg solubilised explant protein. One unit of alkaline phosphatase activity is defined as that which generates 1 μ mol nitrophenyl phosphate/30 min at 37° C. This was calculated based on the extinction coefficient value of nitrophenyl phosphate, which is 218.58.

1.3.5 Identification of Native Osteogenic Fractions

The protein fractions which resulted following gel filtration were subject to *in vivo* assay as described in 1.3.2. Confirmation of osteogenic activity was obtained following histological examination as described in 1.3.3. Quantification of the osteogenic potency of the active BMP fractions was obtained by alkaline phosphatase assay as described in 1.3.4. The bovine BMP fractions with the highest osteogenic activity in rats were selected for human implantation. One unit of osteogenic activity was defined as that amount

which elicits 1 unit of alkaline phosphatase activity per mg solubilised bone explant protein in 12 day old ossicles.

Part 2: Preparation of Delivery System

1.1 Preparation of Human demineralized Bone Matrix

The delivery system for the device was prepared from frozen human cortical bone chips which was a kind gift of Bone S.A (Johannesburg, South Africa). Cortical chips were dehydrated in absolute ethanol at 4° C, and defatted in ether at 4° C. The bone chips were air-dried in a fume hood. Dehydrated defatted bone chips were cooled to - 70° C using liquid nitrogen and crushed in a ring mill apparatus. The crushed powder was sieved to particle size 75 to 420 µm and demineralized as described for bovine bone powder. The demineralized human bone matrix was stored at - 20° C until device formulation.

Part 3: Formulation of Osteogenic Device.

3.1 Combining Bovine BMPs with Human Demineralized Bone Matrix

The strategy used to formulate the human osteogenic device was based on loading a known osteogenic activity onto a specific mass of carrier material, which was human demineralised bone matrix. Osteogenic activity was determined by the level of alkaline phosphatase activity elicited during bone formation in the rat subcutaneous assay. The dose response curve for bovine BMPs in the rat subcutaneous assay is known. Also, the ED_{50} , the dose at which the BMPs exhibit half maximal activity under rat bioassay conditions, was calculated to be 2.0 alkaline phosphatase units per 25 mg carrier matrix. Since the rat bioassay is performed with collagenous implants comprising 25 mg of insoluble collagenous bone matrix, then the activity required to formulate 1 g of osteogenic device bearing an ED_{50} dose is $(1000/25) \times 2 = 80$ units per gram of collagen. Since it is difficult to extrapolate responses to BMPs in human patients, a safety factor of 0.5 was used to formulate the device. Hence, the rat ED_{50} value was halved arbitrarily and an osteogenic device, consisting of 40 alkaline phosphatase units per gram was formulated. The BMPs, in 10 mM HCl (each ml of HCl containing 5.69 alkaline phosphatase units of activity) were added to human demineralised bone matrix under sterile conditions in a laminar flow hood to obtain a final loading of 40 U / gram of matrix. The mixture was lyophilised *in vacuo* to dryness, packed into sealed 50ml Nunc tubes and irradiated with gamma radiation (2.5 mRAD).

SECTION II: Clinical Procedures

Part 1: Patient Selection

1.1 Preoperative Assessment

All patients presented at the maxillofacial clinics of the academic hospitals of the University of the Witwatersrand, Johannesburg. Patients were selected who had mandibular segmental defects following avulsive trauma or the surgical ablation of benign tumours. Inclusion in the trial required that the patient be medically fit to undergo the envisaged surgery. All patients had preoperative full blood count and urea and electrolyte measurements and any haematological or biochemical abnormalities were corrected. Panoramic radiography of the mandible was obtained for all patients. Furthermore, the patient had to give consent to being included in the trial after having read and understood the patient information and consent form (Annexure A). Once selected the patients were allocated alternately to the control (autologous bone graft) or the trial (BMP device) group. A brief overview of the patients selected and the defect size follows.

1.2 Patient profiles

1.2.1 Patient 1

25 year male

The patient suffered a gunshot injury of the mandible resulting in a comminuted fracture and a continuity defect of the right body of the mandible. Initial treatment was debridement and closed reduction. Following a 3 month period for recuperation the patient returned for mandibular reconstruction to restore masticatory function.

Radiography showed a continuity defect of the right body of the mandible extending from the 47 area to the 45 area and superior displacement of the proximal segment (Fig. 1).

The defect was reconstructed with 2.5 grams of BMP device.

1.2.2 Patient 2

39 year male.

The patient sustained a gunshot injury to the face in August 1990. Bullet entered beneath the left mastoid and exited through the right cheek. Primary treatment consisted of the placement of a reconstruction plate to span the defect via an extraoral approach. In 1991 the patient presented with a septic

plate which was removed in May 1991. Once the sepsis had been controlled the patient presented for definitive reconstruction of the mandibular defect. The defect was approximately 3cm in length extending from the right angle to the 46 area (Fig. 2).

The defect was reconstructed with a titanium mesh and a particulate cancellous bone and marrow graft obtained from the right ilium.

1.2.3 Patient 3

21 year female

This patient presented with an large ameloblastoma extending from the right sigmoid notch to the 44 region. The mandible was resected from the 43 to the right condyle (Fig. 3) and a primary reconstruction was performed. The condyle was reconstructed with a costochondral graft harvested from the right sixth rib. The mandibular defect was reconstructed with a particulate cancellous bone and marrow graft procured from the left ilium supported in a titanium mesh.

1.2.4 Patient 4

56 year male

The patient had a partial mandibular resection for a malignant ameloblastoma in November 1994. He presented for definitive reconstruction of a mandibular segmental defect extending from the right angle of the mandible, across the midline, to the region of the 32 (Fig. 4). The mandibular defect was reconstructed with a titanium mesh and an autologous bone particulate cancellous bone and marrow graft which was procured from the left and right iliac crests.

1.2.5 Patient 5

20 year female.

The patient had an extensive continuity defect of the mandible following resection of a very large ameloblastoma. The defect extended from the 37 to the right angle (Fig. 5) and was temporarily reconstructed with a titanium reconstruction plate. The patient returned 2 months later for definitive reconstruction of the mandible.

The defect was reconstructed with titanium mesh and 10 grams of BMP device.

1.2.6 Patient 6

46 year female

The patient had a hemimandibulectomy in November 1994 for ameloblastoma and presented for definitive reconstruction of the mandible. The defect extended from the left condyle to the 42 (Fig. 6) and was temporarily reconstructed with a reconstruction plate. Definitive reconstruction was accomplished with a costochondral graft and titanium mesh supporting 7.5 grams of BMP device.

1.2.7 Patient 7

26 year male.

Patient suffered multiple gunshot wounds on the 6/12/95. A low velocity gunshot wound of the left aspect of the face and multiple gunshots to the dorsal aspect of the torso were sustained. The entry wound was over the left temporal area and the exit in the submental area. Surgery immediately post-injury entailed the debridement of the comminuted fracture of the left angle and extraction of the 36, 37, and 38. The mandible was immobilized by means of interdental eyelets and maxillo-mandibular fixation. Following a 3 month period of recuperation the patient returned for definitive reconstruction of a continuity defect of the left body of the mandible extending from the 36 area to the 38 area (Fig. 7).

The defect was reconstructed with titanium mesh and 2.5 grams of BMP device.

1.2.8 Patient 8

49 year female

The patient presented with an extensive cemento-ossifying fibroma of the mandible which required resection. The resultant continuity defect extended from the right angle to the 35 (Fig. 8). The defect was reconstructed primarily with a titanium mesh and a particulate cancellous bone and marrow. Autogenous bone was harvested from the left iliac crest.

1.2.9 Patient 9

14 year female

The patient had a hemimandibulectomy on the 11/9/1994 for a desmoplastic fibroma. The resultant defect extended from the right condyle to the 42 (Fig. 9). The defect was spanned by a reconstruction plate. The reconstruction was effected with a costochondral graft and titanium mesh with a particulate bone cancellous marrow graft. A corticocancellous graft was procured from the

external surface of the left posterior ilium. Additional cancellous bone was removed from the medullary space.

1.2.10 Patient 10

41 year male

Patient presented for reconstruction of his mandible following partial mandibular resection for an ameloblastoma. The mandibular segmental defect extended from the 43 area to the neck of the left condyle (Fig. 10) and was spanned by a reconstruction plate. Definitive reconstruction was performed with a titanium mesh and autologous bone particulate cancellous bone and marrow graft which was procured from the left and right iliac crests.

1.2.11 Patient 11

32 year male

The patient suffered a high velocity gunshot injury to the mandible in Moçambique several weeks prior to presentation. Rudimentary primary treatment was administered in Moçambique. He was referred for definitive reconstruction. He presented with severe antero-posterior mandibular

deficiency, and a deranged occlusion. The mandibular continuity defect extended from the left angle to the 47 (Fig. 11). The defect was reconstructed with titanium mesh and autologous bone procured from the right iliac crest.

1.2.12 Patient 12

27 year male

The patient suffered a gunshot injury of the left angle which was treated primarily by debridement and closed reduction. The continuity defect extended from the left angle to the 36 (Fig. 12). The defect was reconstructed with 2.5 grams of BMP device.

1.2.13 Patient 13

42 year male

The patient suffered a gunshot injury of the right mandible which was initially treated with debridement and closed reduction. The defect extended from the right angle to the 46 (Fig. 13). Reconstruction was accomplished with 2.5 grams of BMP device.

Part 2: Surgical Procedures

2.1 Recipient Site Preparation

All surgery was performed under general anaesthesia. Following induction patients received 1 gram of intravenous cefazolin sodium (Eli Lilly, Isando, South Africa). Interdental eyelets were placed, the occlusion established and the patient placed in maxillo-mandibular fixation. Exposure of the defect was *via* an extraoral, submandibular approach (Fig. 14). Care was taken to ensure that the oral mucosa was not breached or where immediate reconstruction was performed that watertight primary closure of the oral mucosa was obtained. The osseous margins were revised to expose the endosteal core and the titanium mesh (Leibinger, Germany) was tailored to fit the defect (Figs. 15 and 16). The mesh was secured with at least 4 bicortical titanium screws (Leibinger, Germany) in each segment. The mesh was then filled with autologous bone (control group) or bone morphogenetic device (trial group).

2.2 Autologous Bone Procurement and Preparation

Autologous bone was harvested primarily from the anterior iliac crest. If larger volumes were required bilateral anterior iliac crests were harvested or

alternatively posterior ilium was used in one patient. The bone was procured using standard approaches and a block of corticocancellous bone was harvested. Haemostasis at the donor site was obtained with bone wax. A suction drain was placed and primary, layered closure obtained. The bone was placed in a Tessier bone mill (Leibinger, Germany) and milled to form a particulate cancellous bone and marrow graft (Fig. 17). The bone was compressed in a 20 ml syringe and then introduced into the mesh tray.

2.3 Device Preparation and Placement

The device was placed in a sterile receiver. By carefully adding sterile saline a thick paste was formed (Fig. 18). The paste was then placed in the mesh taking care to ensure that the device remained within the confines of the mesh. Primary, layered soft tissue closure was obtained to ensure a good soft tissue bed enveloped the mesh and its contents.

Part 3: Post-operative Procedures

3.1 Immediate Post-operative Care

All patients were maintained on intravenous cefazolin sodium (Kefzol, 1 gram 8 hourly) and papaveratum (Omnopon, Roche, Johannesburg, South Africa, 15 - 20 mg 6 hourly). The patients were maintained on intravenous fluids for 48 hours and oral fluids were instituted after 24 hours. Routine oral toilet and wound care was maintained. Control patients were mobilized as soon as possible (in general after 48 hours) and all suction drains and urinary catheters were removed after 24 hours. A liquid diet was commenced after 48 hours.

3.2 Post-operative Assessment and Follow up

Patient were seen for review at 1 week, 6 weeks, 3 months, 6 months and 1 year post surgery. Maxillo-mandibular fixation was removed after 6 weeks and a soft diet was commenced. Clinical assessment included review of extraoral and intraoral surgical sites. Palpation of the reconstructed segments was performed to evaluate the resilience of the regenerated alveolus. Occlusal stability was monitored at all follow-up appointments. Panoramic radiography was performed at 1 week, 3 months, 6 months and 1 year review. The radiographs were assessed for signs of ossification and stability of the reconstruction mesh and screws.

Part 4: Histological Evaluation of Reconstructed Segments

4.1 Biopsy of Reconstructed Segment

An intraoral biopsy under local anaesthesia was performed on all patients at 3 months post-reconstruction. The reconstructed alveolus was exposed via a 1 cm crestal incision. A 5 mm³ biopsy of the tissue within the mesh was harvested with a scalpel. Primary mucosal closure was obtained with 3/0 chromic. Analgesics and antibacterial mouthwash were provided to the patient for 5 days.

4.2 Tissue Processing for Histology

The specimens were fixed in 70% absolute ethanol for 1 week and then dehydrated in ascending grades of ethanol. These were then embedded, undecalcified, in a polymethylmethacrylate resin (K-Plast, Medim, Germany). Undecalcified serial sections were cut at 6 µm using tungsten-carbide knives and a motor-driven microtome (Polycut-S, Reichert-Jung, Germany). Sections were stained using the free floating method with Goldner's trichrome stain for undecalcified bone and mounted onto clear slides.

4.3 Morphometric Analysis

Goldner's trichrome stained sections were examined with a Provis AX70 research microscope (Olympus Optical Co., Tokyo, Japan) equipped with a calibrated Zeiss Integration Platte II with a 100 lattice points (Ripamonti, 1991; Ripamonti et al., 1997a). The volumes of matrix or bone graft, mineralized new bone, osteoid and fibrovascular tissue (as a percent) in the specimen were determined with the point counting technique (Parfitt, 1983). Thickness (in μm) of newly formed osteoid seams was measured using a computerised image analysis system (Flexible Image Processing System, Council for Scientific and Industrial Research, Pretoria, South Africa) connected to a capturing video camera (WV-CP410/G Panasonic, Osaka, Japan).

3. RESULTS

SECTION I: Identification and Testing of Osteoinductive Fractions

Part 1: Bioassay of Extracted Bovine BMPs

The implanted pellets (25mg rat insoluble collagenous bone matrix reconstituted with Sephacryl S-200 fractions) yielded firm, vascularized ossicles when harvested on day 12. Histological evaluation revealed copious endochondral bone formation in the pellets reconstituted with fractions 27-31 kDa.

Part 2: Identification of Osteogenic Fractions

The alkaline phosphatase activity of the Sephacryl S-200 fractions are shown in Figure 19. Fractions of 27 kDa to 31 kDa were osteogenic and demonstrated the highest alkaline phosphatase activity. These were consequently pooled for clinical application

SECTION II: Clinical Results

Part 1: Follow-up

Patients 1, 3 - 7, 9 - 12, and 13 were followed-up successfully for a time period ranging from 13 months to 3.5 years. Patients 2 and 8 were lost to follow-up at 6 and 5 months respectively.

Part 2: Aesthetics

Patients 1- 5, 7, 10, 12 and 13 had good restoration of facial aesthetics (Fig. 20). Of these, patients 3, 4, 5 and 10 had hemimandibles reconstructed while the remainder had small defects (2-5 cm) which were easy to reconstruct. Patients 6, 9 and 11 had fair facial aesthetics (Fig. 21) while patient 8 had poor aesthetics (Fig. 22) due to incorrect anteroposterior placement of the mesh.

Part 3: Function

All patients except for 2, 8, 11, and 12 have been rehabilitated dentally to date. Patients 1, 3, 4, 7, 10, and 13 received chrome cobalt prostheses and now maintain a normal solid diet (Fig. 23). Patients 6 and 9 have similarly been

rehabilitated but as a result of poor stability of the prostheses are maintaining a semi-solid diet. Patient 5 had a very large defect and only two lower molars remaining with which to secure a prostheses. Although the patient received a prostheses, she found it to be so unstable that she is incapable to function with it. Consequently she maintains a soft diet. Patient 11 has received 5 osseointegrated fixtures and is in the process of prosthetic rehabilitation (Fig. 24).

Part 4: Radiographic assessment

Assessment of ossification within the defects was difficult due to the overlying radio-opaque mesh. Therefore, changes as a result of ossification or resorption were only detectable if overt. Patients who received autologous bone grafts showed good initial radioopacity, particularly in the smaller defects (Fig. 25). The larger defects however showed extensive resorption over the follow-up period (Fig. 26 A and B).

Trial patients demonstrated no radio-opacity immediately following grafting. Patient 1 showed almost complete ossification of the defect 3.5 years post-surgery (Fig. 27), and patient 13 shows partial filling of the defect by bone 1

year post-surgery. The other trial patients (5, 6, 7, 12) showed little or no signs of ossification within the defect (Fig 28 A and B).

Part 5: Recipient Site Complications

All intraoral wounds healed well other than patients 3 and 8 who had primary reconstructions. Patient 3 had a minor intraoral dehiscence which was closed without further complications. Patient 8 had a major dehiscence as a result of the over-contoured reconstruction mesh. Several attempts at closure failed, following which the patient was lost to follow-up. Patient 5 required a reduction of the titanium mesh as it was very prominent in the mouth and causing discomfort.

Extraoral wounds all healed well other than patients 2, 3 and 12. Patients 2 and 3 developed minor suture abscesses which resolved with debridement and wound care. Patient 12 developed a small abscess due to a loose screw which resolved following screw removal.

Part 6: Donor Site Complications

All donor sites healed uneventfully. However patients 4 and 10 had persistent minor gait disturbances and required canes to assist with walking initially, but

were able to walk unassisted following physiotherapy. Patient 6 was reconstructed with the osteogenic device, however she required a costochondral graft to replace the missing condyle. She developed a mild but persistent neuralgia from the rib donor site.

SECTION III: Biopsy Analysis Results

Part 1: Histological examination

1.1 BMP Device Patients with Successful Osteogenesis

Patient 1 demonstrated copious amounts of mineralised new bone lined with copious osteoid seams (Fig. 29). Osteoblastic rimming of the seams was also evident. Unequivocal evidence of induction was provided by the presence of vital, cellular osteoid deposited directly on fragments of demineralized bone matrix (Fig 30). The intervening stroma consisted of a rich vascular fibrous connective tissue. Patient 13 demonstrated limited areas of induction. These areas similarly showed vital new bone lined by osteoid seams. The bulk of the stroma however consisted of thick, avascular collagenous tissue containing persistent fragments of demineralized bone matrix which had failed to induce osteogenesis.

1.2 BMP Device Patients with Failed Osteogenesis

Patients 5, 6, 7, and 12 showed no histological evidence of osteogenic activity in the examined histological specimens. There was persistence of multiple fragments of acellular, demineralized bone matrix. The spaces between the fragments are smaller and filled with dense, avascular collagenous tissue (Fig. 31).

1.3: Autogenous Bone Graft Patients with Successful Osteogenesis

Patients 2, 3, 8, 9 and 11 showed areas of vital bone within the transplanted autogenous bone. These areas have osteocyte filled lacunae and osteoid seams overlying the vital bone. Occasional areas of osteoid and mineralised vital bone are seen in direct apposition to fragments of non-vital bone. The stroma is highly vascular and consists of loose fibrous connective tissue. The degree of osteogenesis is highly variable between patients (Fig. 32 and 33).

1.4: Autogenous Bone Graft Patients with Failed Osteogenesis

Patients 4, and 10 had no evidence of osteogenesis. There were persistent fragments of non-vital, transplanted bone, demonstrating empty lacunae and

no osteoid production. The intervening stroma was dense and poorly vascularised (Fig. 34).

Part 2: Histomorphometry

The volume fraction, as a percent, of mineralised bone and osteoid in biopsies of all instances (BMP and autologous bone) at 3 months are shown in Fig. 35. The mean induced new bone (mineralised bone and osteoid) volume in the two successful trial patients was 46.94 % (SD 14.23). Control patients with successful osteogenesis had a volume of regenerated new bone of 24.61 % (SD 11.94). Osteoid seam thickness in μm of the same biopsies is shown in Fig. 36. The mean osteoid thickness in trial patients was 29.37 μm (SD 0.59) and in control patients 21.45 μm (SD 17.01). The matrix and bone graft remnant as a percent of total volume is seen in Fig. 37. The volume of matrix in failed patients from both trial and control groups was 53.67 % (SD 15.34) as compared to 20.55 % (SD 9.32) for successful patients. Conversely the fibrovascular tissue volume (Fig. 38) is higher in successful grafts at 55.91 % (SD 13.94) of both groups of patients, while it is 46.33 % (SD 15.34) in the unsuccessful transplant procedures.

ANNEXURE A

Patient information and consent form

As you know a piece of your jaw is missing. In order to restore the shape of your face and to allow you to chew properly again you will require a second operation to rebuild your jaw. The standard treatment for this at present is to take a piece of bone from your hip, crush it up and place it in a metal holder shaped like your jaw which is then screwed to the ends of your jaw.

We are asking you to take part in a study which will test a new material for the rebuilding of bone defects. This material is human bone powder (which has been specially treated so that the chance of transmitting any disease is non-existent) mixed with a special protein (naturally occurring in all bones) which promotes bone formation.

Our previous studies using this new material in animals have shown excellent healing of bone defects, far better than any material presently in use. Furthermore, human studies at other centres have confirmed that this material may be used quite safely in humans.

You will be allocated to 2 groups randomly: one group will have the jaw rebuilt with a piece of their own hip, the other group will receive the new material. The treatment both groups will receive will be identical, the only difference will be the material used to rebuild the jaw.

Before the operation normal X - rays and special X - rays will be taken to check the size of the defect in your jaw. Following your discharge from the hospital you will be required to return for regular checkups at 1 week, 6 weeks, 3 months, 6 months and 1 year after the operation. At these appointments we will monitor your progress and the healing of your wound. It will be necessary to take further X-rays at these appointments to check bone healing. Three months after the operation we will need to take a small piece of the newly formed bone (this is necessary so that we may get a more accurate idea of how the bone is healing). This is a very short procedure and will be done under local anaesthetic. You will experience very little discomfort and will be in hospital for one day. It is very important that these appointments are kept because it is only then that we can get the important information we need. The potential benefits for those who receive the new material include:

- Defects that heal quicker allowing a return to normal function sooner.
- Markedly diminished pain and discomfort as it will not be necessary to take a piece of hip.
- Less time spent in hospital.

However, regardless of the way your jaw is restored all patients that take part in this study will increase our knowledge, resulting in better treatment of jaw defects in the future.

Participation in this study is voluntary and you are free to refuse to participate or to withdraw your consent and to discontinue participation at any time. Such refusal or discontinuance will not affect your regular treatments or medical care in any way. A signed copy of this consent form will be made available to you

I have fully explained the procedures, identifying those which are investigational, and have explained their purpose. I have asked if any questions have arisen regarding the procedures and have answered these questions to the best of my ability.

Date: _____ Doctor: _____

I have been fully informed of the procedures to be followed, including those which are investigational. I have been given a description of the attendant discomforts, risks and benefits to be expected and the appropriate alternative procedures. In signing this consent form, I agree to this method of treatment and I understand that I am free to refuse to participate or to withdraw my consent and discontinue my participation in this study at any time. I understand also that if I have any questions at any time, they will be answered.

Date: _____ Patient: _____

or Guardian/ Next of Kin: _____

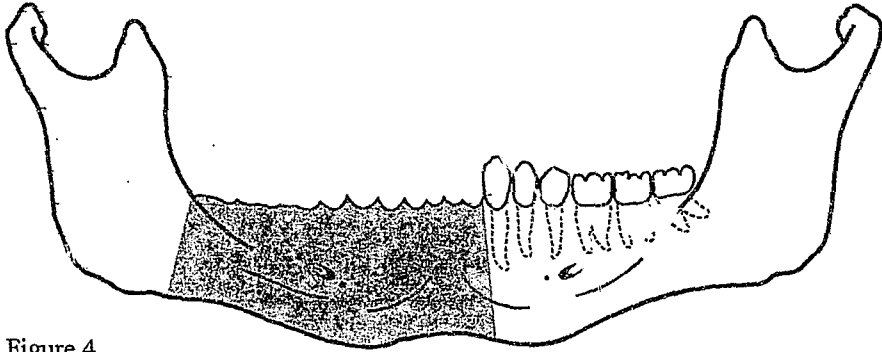


Figure 4.

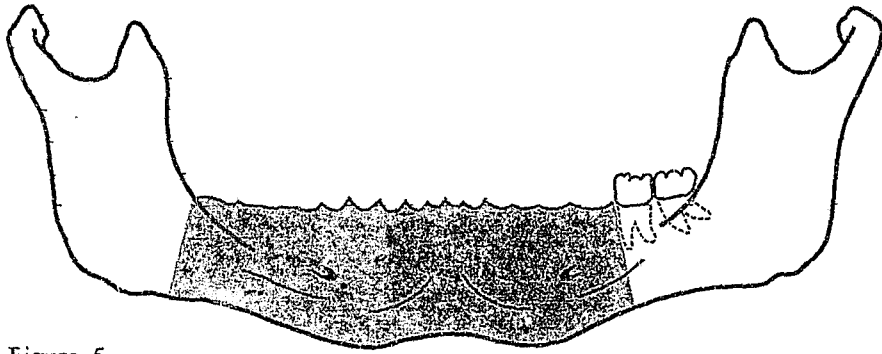


Figure 5.

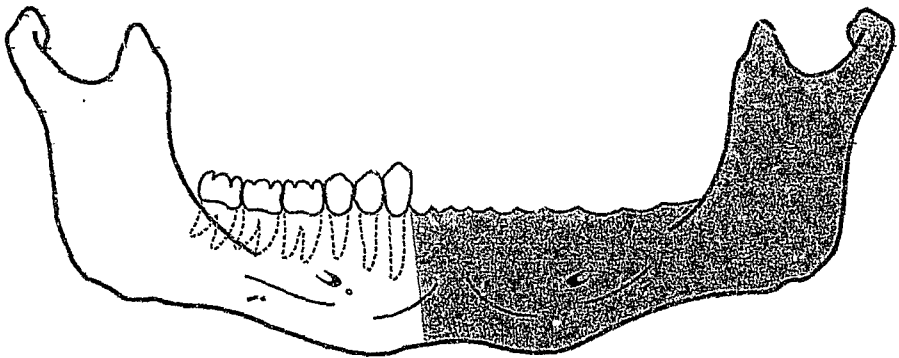


Figure 6.

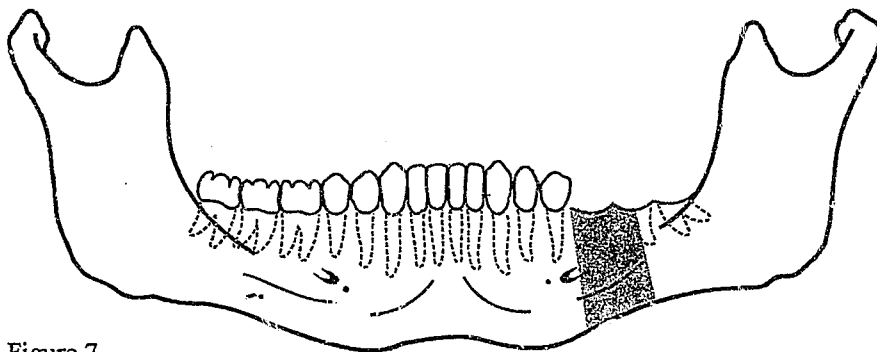


Figure 7.

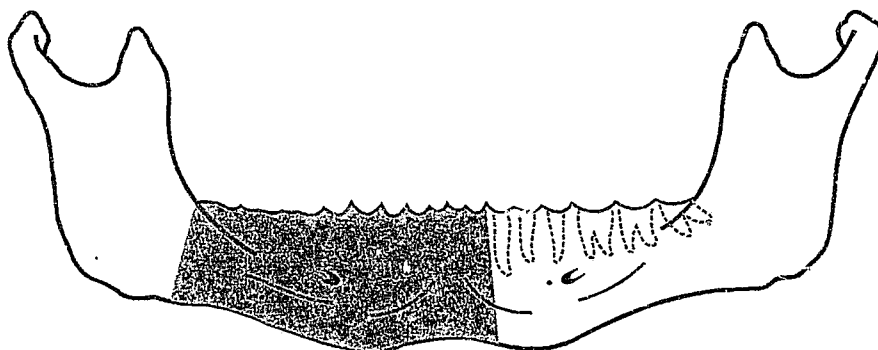


Figure 8.

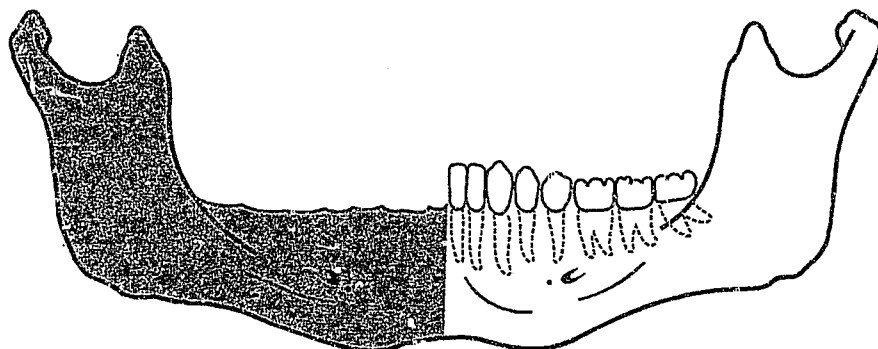


Figure 9.

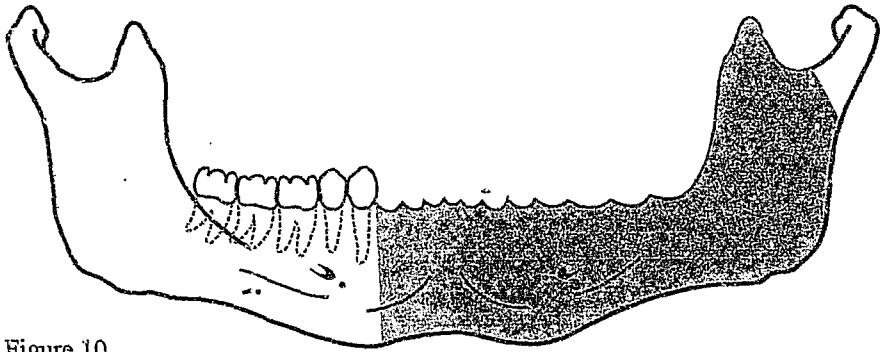


Figure 10.

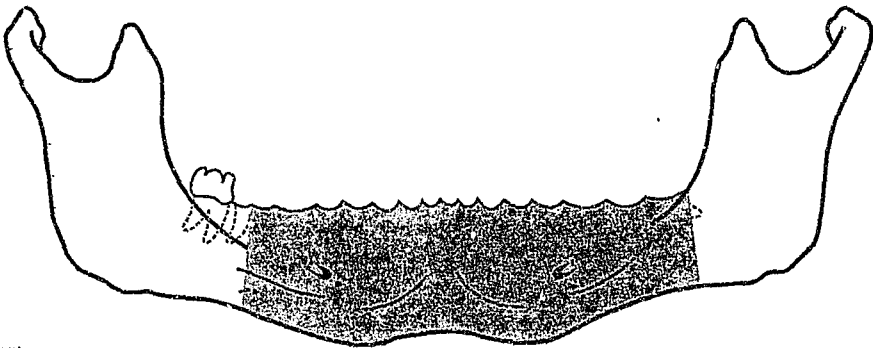


Figure 11.

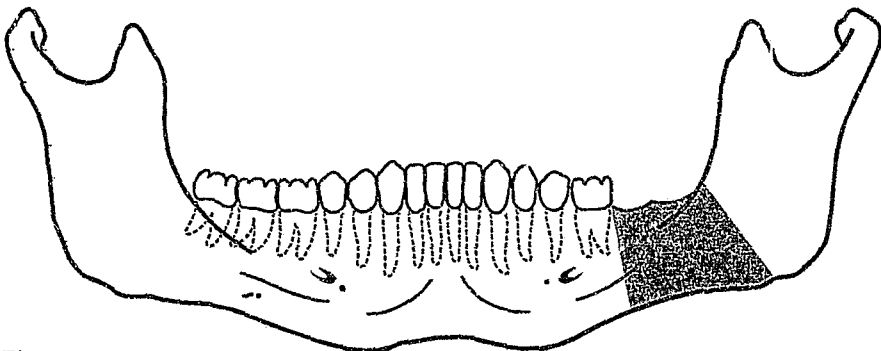


Figure 12.

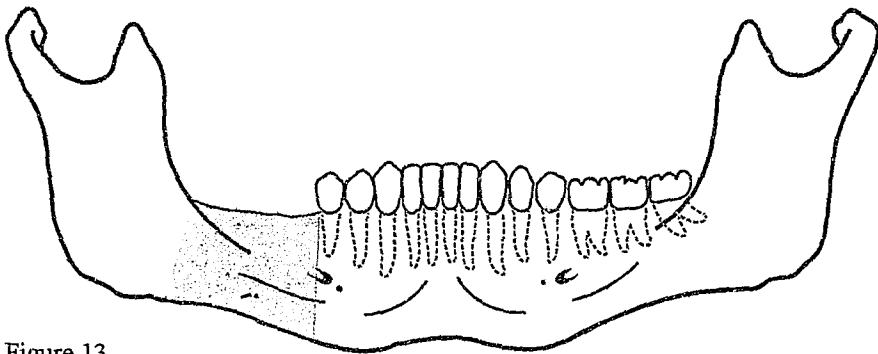


Figure 13.

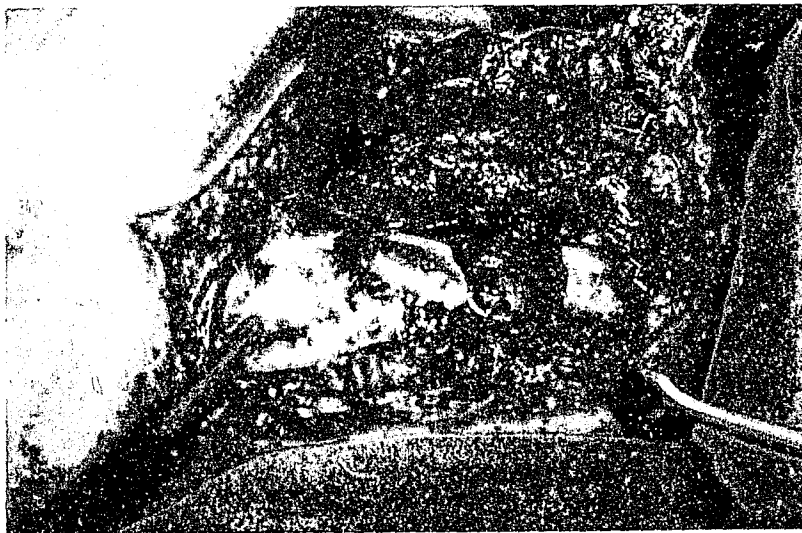


Figure 14. The defect is exposed via a submandibular incision. The bone ends have been refined to expose the central endosteum.

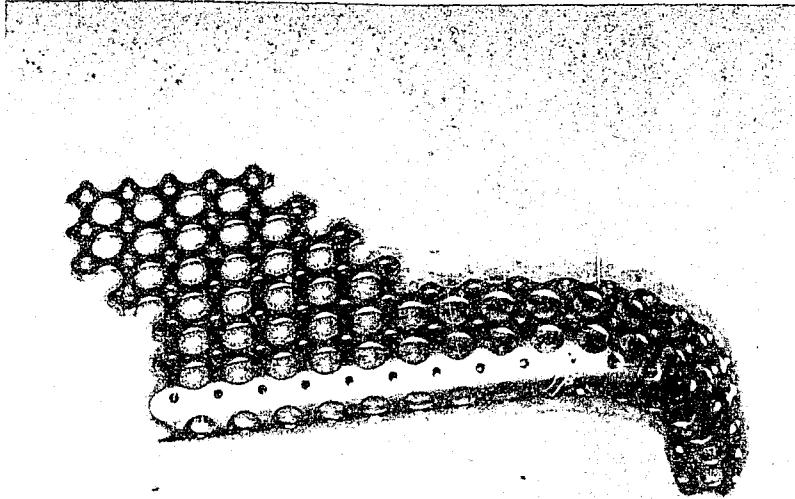


Figure 15. The titanium mesh prior to modification to the required morphology.



Figure 16. Once adequately contoured, the mesh is secured to the host bone with titanium screws.

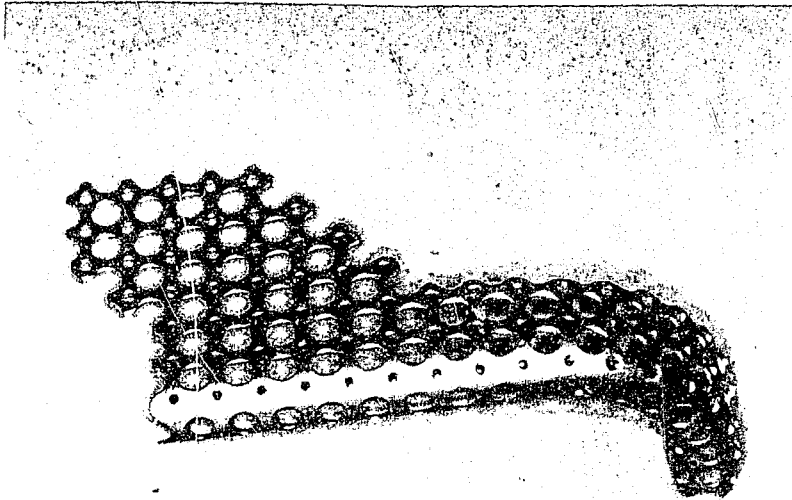


Figure 15. The titanium mesh prior to modification to the required morphology.



Figure 16. Once adequately contoured, the mesh is secured to the host bone with titanium screws.

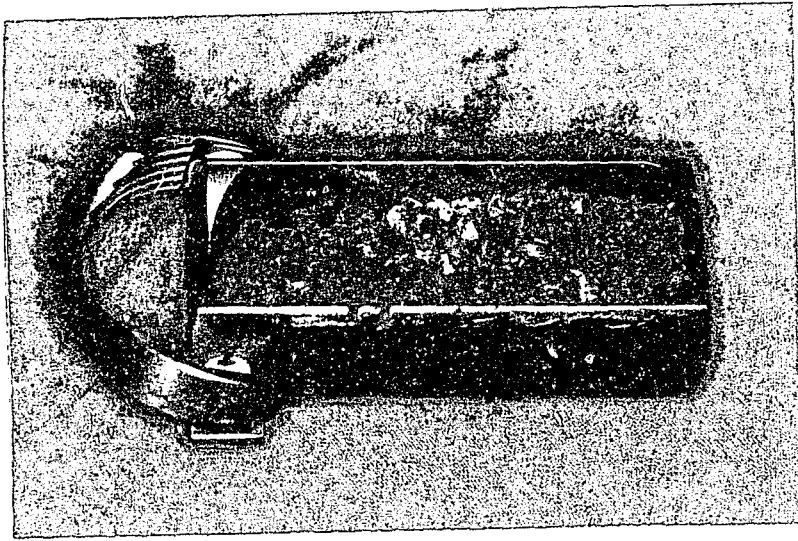


Figure 17. The particulate cancellous bone and marrow graft shown here is prepared by passing autologous iliac crest bone through a bone mill.

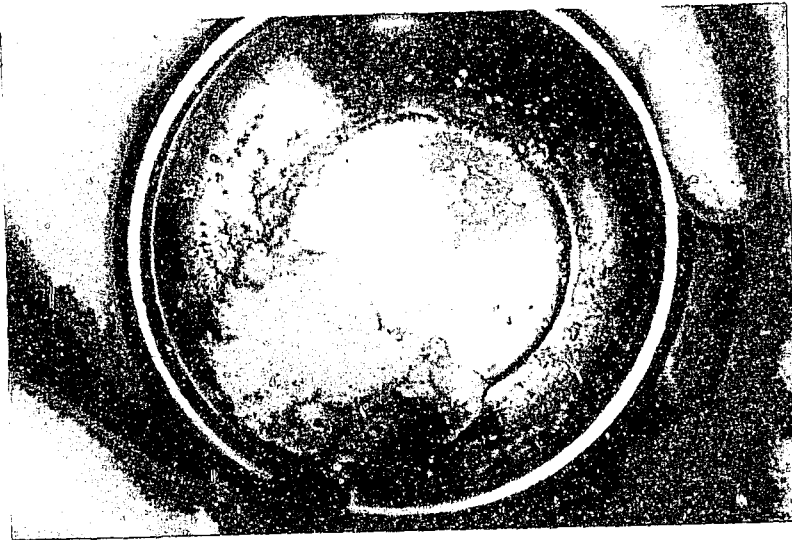


Figure 18. The BMP device is prepared in theatre by adding sterile saline incrementally to form a thick paste.

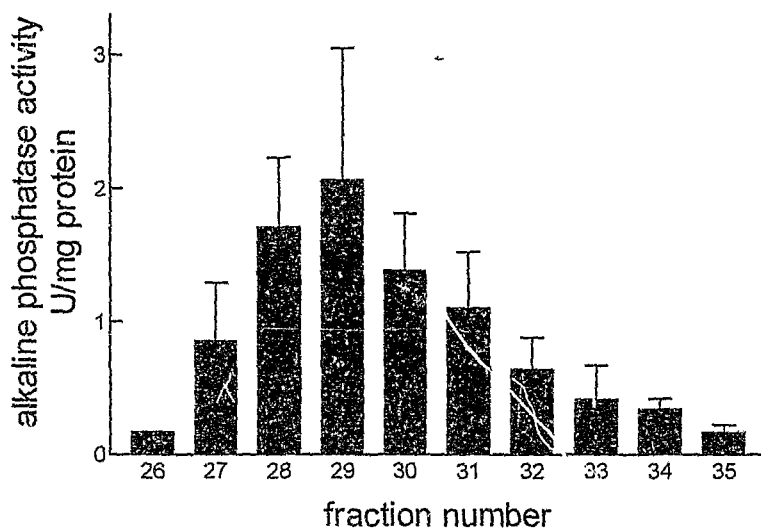


Figure 19. Alkaline phosphatase activity of 12 day implants of rat insoluble collagenous bone matrix (ICBM) reconstituted with Sephacryl S-200 fractions and bioassayed in the subcutaneous space of Long Evans rats. Osteogenic activity is confined to fractions 27-31. Fractions 27 to 31 were pooled, concentrated and exchanged into 10 ml of 10 mM HCl. The activity was expressed relative to volume of solvent (10 mM HCl). This value was 5.69 U/ml. The total activity purified was 569 units.



Figure 20. Patient 3, preoperative (a) and postoperative (b) frontal and profile views, demonstrating good restoration of facial form.



Figure 21. Patient 11, preoperative (a) and postoperative (b), frontal and profile views demonstrating a fair rehabilitation of facial form. This patient had a very large mandibular defect following an avulsive, high velocity gunshot injury. As a result of concomitant soft tissue loss, the mandibular antero-posterior position could not be re-established resulting in a slightly deficient mandibular profile.

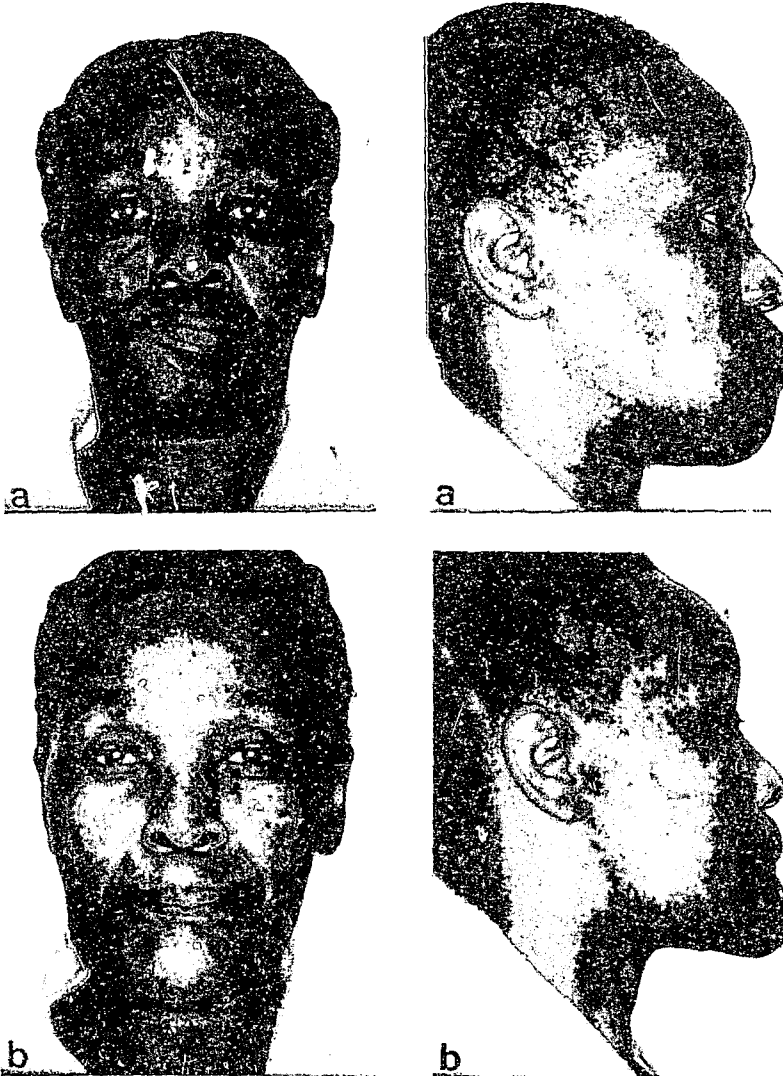


Figure 22. Patient 8, preoperative (a) and postoperative (b) frontal and profile views showing a poor aesthetic result. The titanium tray was positioned too far anteriorly, creating a prognathic profile. Moreover, as a result of the incorrect tray position, this patient developed an intraoral dehiscence.

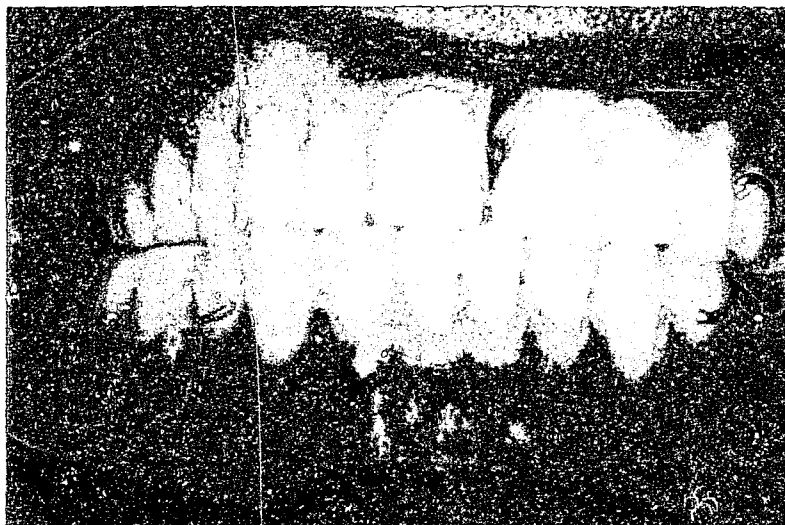


Figure 23. Patient 1 with chrome cobalt prostheses in situ. 8 patients received similar prostheses which provided good to excellent functional results.

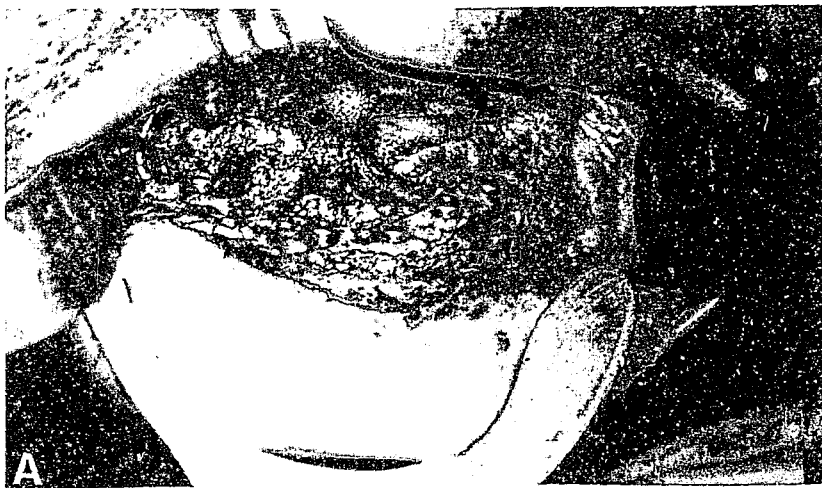


Figure 24. Patient 11 received 6 osseointegrated fixtures (A), 6 months following reconstruction. Radiographic view of the fixtures in situ (B).



Figure 25. Patient 2. Radiograph 5 months following reconstruction with autologous bone shows excellent radioopacity within the defect and good reconstitution of alveolar height. This patient was subsequently lost to follow-up.

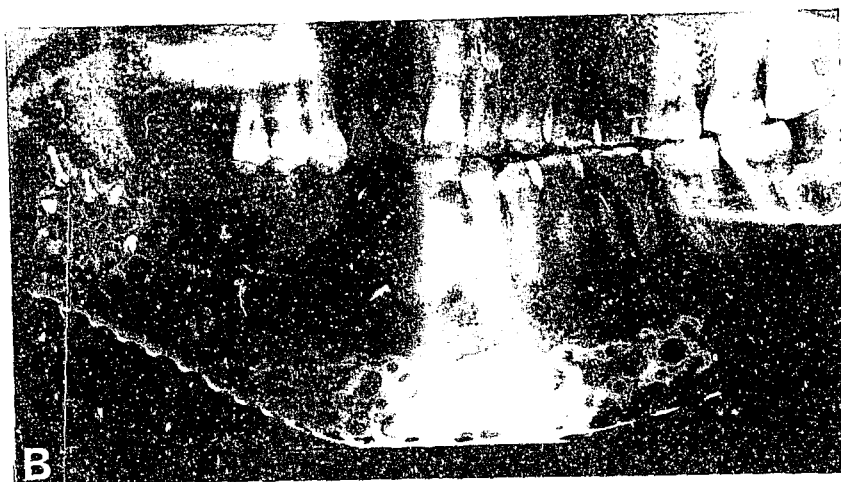
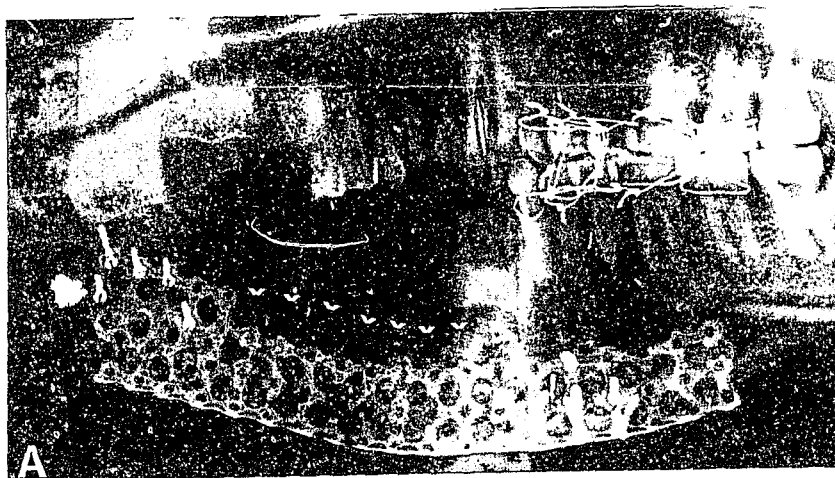


Figure 26. Patient 4. The immediate post-operative radiograph (A) of a defect reconstructed with autologous bone shows excellent radio-opacity in the defect which extends above the mesh (arrows). The radiograph taken 1 year post-operatively (B) shows complete loss of radioopacity. No radiographic evidence of the presence of bone can be seen.

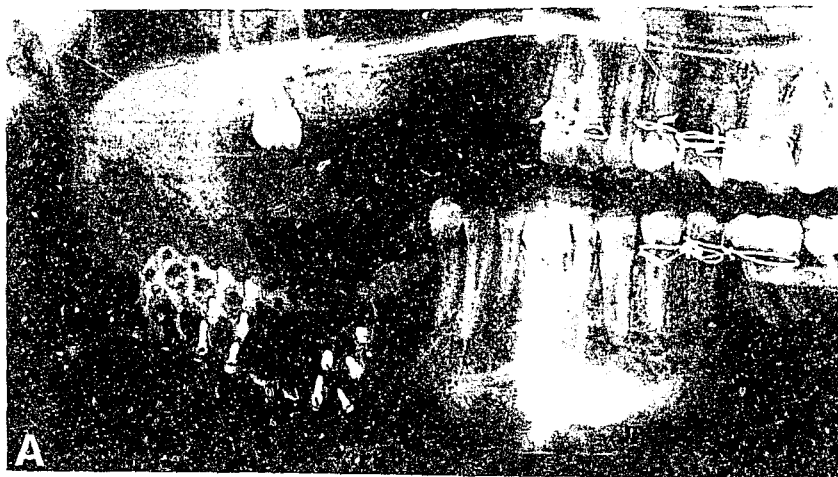


Figure 27. Patient 1. The immediate postoperative radiograph (A) shows the mesh spanning the radiolucent defect. The radiograph 3.5 years post reconstruction (B) with 2.5 grams of BMP device shows the defect almost completely filled with radioopaque bone.

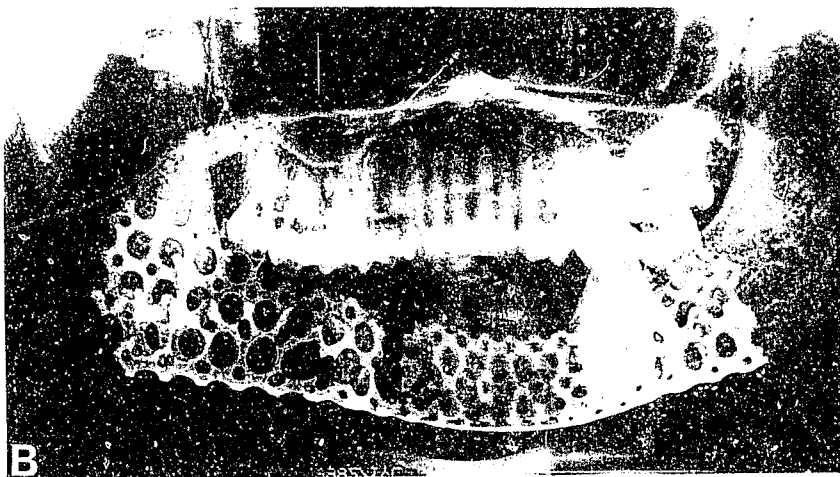
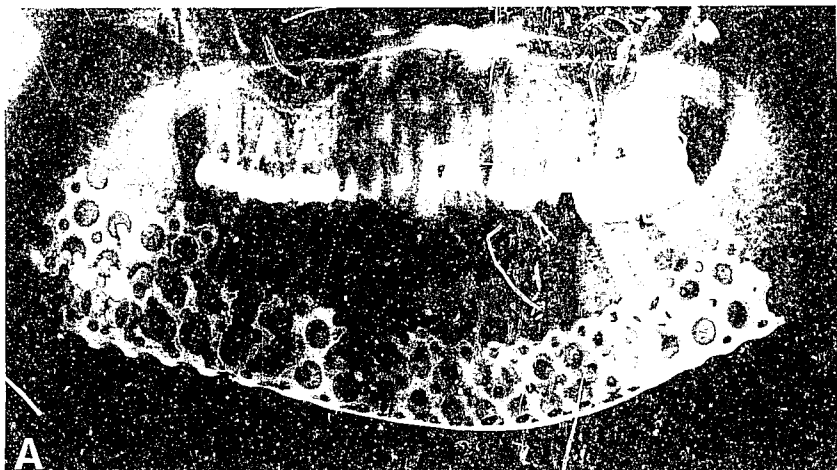


Figure 28. Patient 5. Immediate post-operative radiograph (A) shows an extensive defect bridged by a titanium mesh grafted with 10 grams of BMP device. One year post-operatively there is no evidence of ossification within the defect (B).



Figure 29. Photomicrograph of biopsy from Patient 1, 3 month post reconstruction with 2.5 grams of BMP device. The trabeculae of mineralised bone are highly cellular and are lined by thick osteoid seams. Osteoblastic rimming on the osteoid seams is evident (black arrows). Large vessels are evident in the stroma (line arrows). Goldner's trichrome, original magnification X 40.

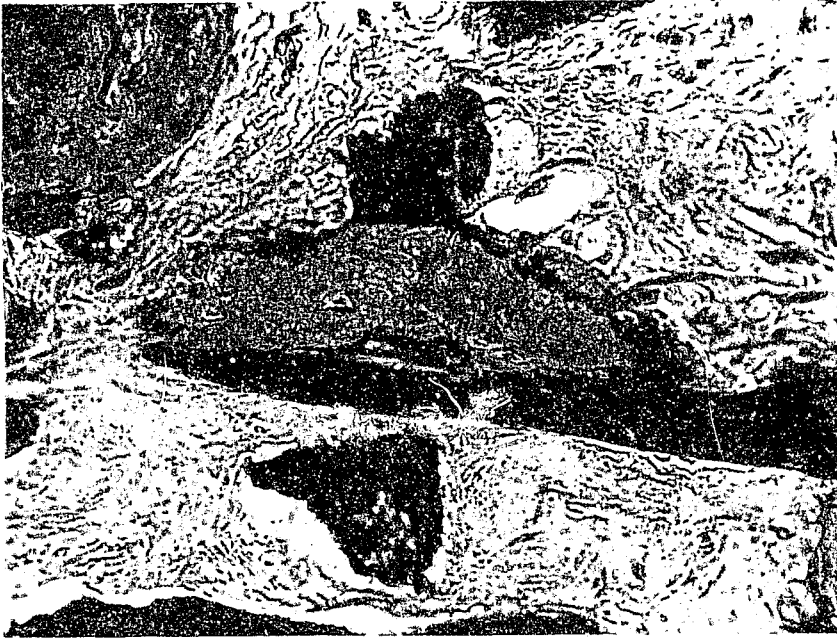


Figure 30. Photomicrograph of the biopsy of Patient 1 treated with BMP device showing vital cellular bone lined with an osteoid seam in apposition to a non-vital fragment of demineralized bone matrix. This provides unequivocal evidence of induction. Goldner's trichrome, original magnification X 100.



Figure 31. Photomicrograph of the biopsy of Patient 5, 3 months post reconstruction with 10 grams of BMP device. There is persistence of the demineralized bone matrix with no evidence of induction. The matrix volume is high and there sparse intervening stroma which is fibrous and hypovascular. Goldner's trichrome, original magnification X 40.



Figure 32. Photomicrograph of the biopsy of Patient 3 following reconstruction with autologous bone. Cellular, vital bone is encircled by non-vital graft bone. Ample osteoid seams indicate good osteogenic activity. The stroma is highly vascular. Goldner's trichrome, original magnification X 40.



Figure 33. Photomicrograph of a biopsy of an autologous bone graft. A non-vital fragment of autologous bone is covered with a thick, cellular osteoid seam. It is likely that this osteogenesis is the result of osteoinductive effects exerted by the graft. Note the close proximity of the developing vasculature (arrow). Goldner's trichrome, original magnification X 100.

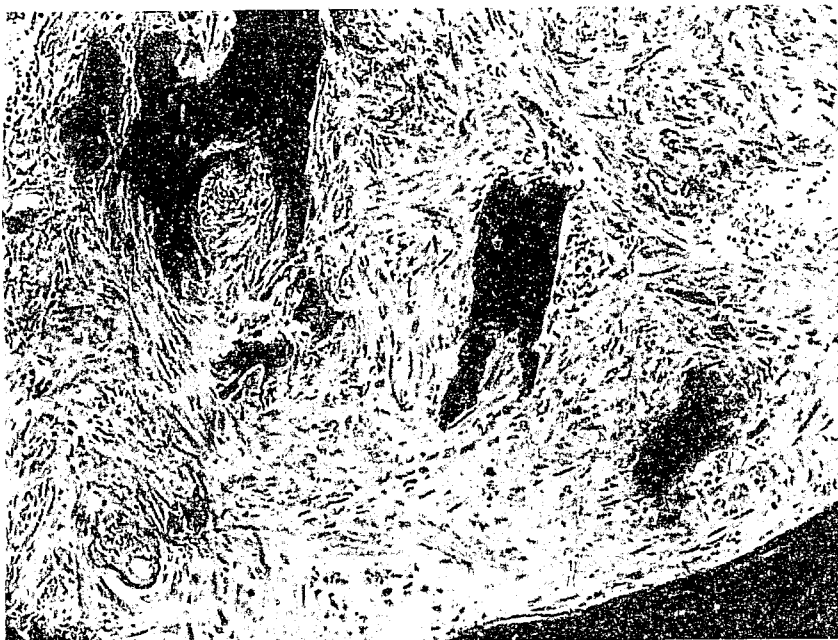


Figure 34. Photomicrograph of the biopsy from Patient 4 who was reconstructed with autologous bone. There are several fragments of non-vital, acellular transplanted bone lying in a dense, fibrous, avascular stroma. Goldner's trichrome, original magnification X 40.

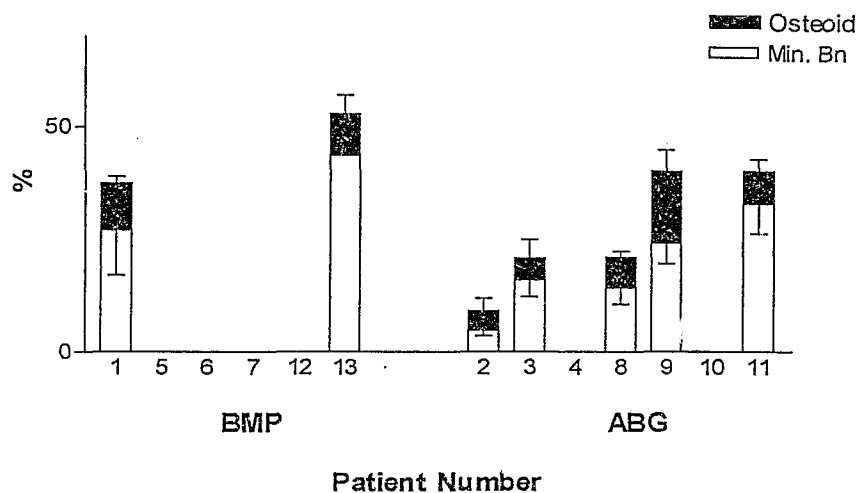


Figure 35. Regenerated tissue volumes (as a percentage) in three month biopsies of autologous bone grafts (ABG) and BMP devices (BMP). Min. Bn - Mineralised Bone

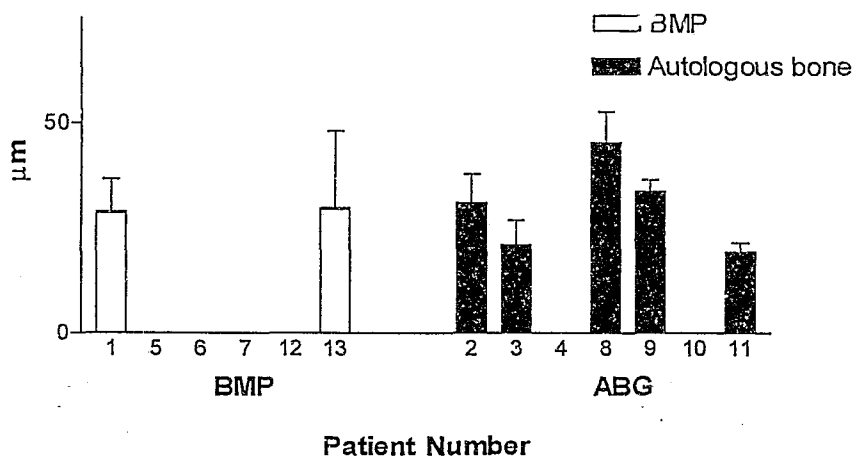


Figure 36. Osteoid seam thickness in μm .

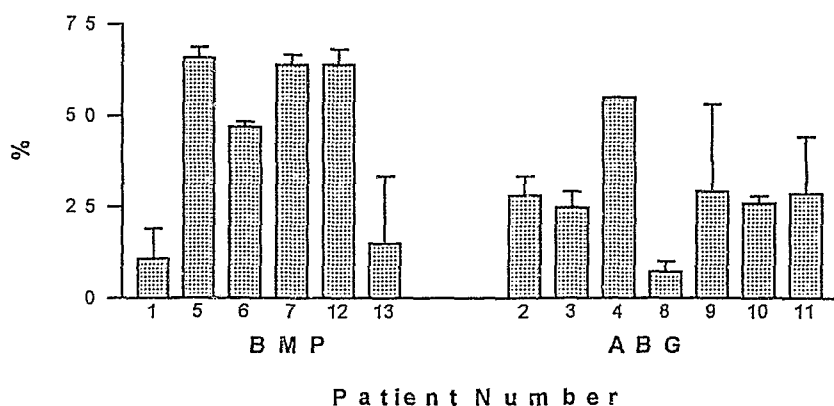


Figure 37. Remnant non-vital bone graft and demineralised bone matrix volumes (as a percentage) in biopsies 3 months post reconstruction with autologous bone graft (ABG) and BMP device respectively. The matrix volume is higher in all patients with failed grafts (4, 5, 6, 7, 10, 12).

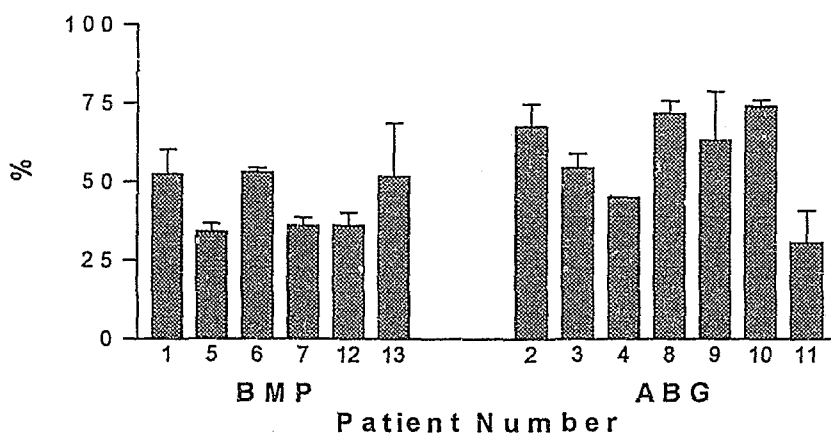


Figure 38. Fibrovascular tissue volumes (as a percentage) in 3 month biopsies of ABG and BMP device patients. The fibrovascular tissue volumes in successful grafts (1, 2, 3, 8, 9, 11, 13) is higher than in failed grafts.

4. DISCUSSION

Mandibular reconstruction is a challenging endeavour because not only must the problems of bone regeneration be addressed but this must be accomplished in an environment often hostile to osteogenic cells as a result of excessive scarring, contamination by oral flora, and radiotherapy. Reconstruction of the morphology of the mandible places an additional burden on the reconstructive task. This has however been accomplished relatively successfully with a variety of systems, the most successful of which requires the use of autologous bone in one form or the other. The procurement of autologous bone and its use at an orthotopic site for the reconstruction of skeletal defects has become routine. It is the gold standard against which all other reconstructive materials should be judged. The problems of donor site morbidity, weakened donor site and volume limitations have resulted in the continuous search for alternatives to autologous bone for osseous regeneration.

The most promising solution has evolved from the landmark discovery in 1965 by Marshall Urist that demineralized bone matrix induces the formation of endochondral bone in heterotopic sites in rodents (Urist, 1965). It took, however, more than 20 years of continuous research to fully identify the molecular signals capable of the induction of endochondral bone (Ripamonti

and Reddi, 1995 for review). Dissociative extraction of demineralized bone matrix with chaotropic agents eliminates the endochondral cascade (Sampath and Reddi, 1991). This fact has provided evidence for the presence of putative inductive molecules in the soluble extract. Consequently the molecules responsible for bone induction, the bone morphogenetic proteins (BMPs), have been isolated, purified to homogeneity and cloned (Wozney et al., 1988; Luyten et al., 1989; Özkaynak et al., 1990; Celeste et al., 1990). Native and recombinant human (rh) BMPs combined with a suitable delivery system induce bone formation in heterotopic and orthotopic sites in several experimental models (Sampath and Reddi, 1981; Luyten et al., 1989; Ripamonti et al., 1991; Toriumi et al., 1991; Ripamonti et al., 1992; Boyne, 1996). The therapeutic potential of naturally derived BMPs has been tested in several clinical trials with relative success (Johnson et al., 1988; Johnson et al., 1992; Sailer and Kolb, 1994a; Sailer and Kolb, 1994b). Against this background the principle of osteoinduction has been applied to the reconstruction of mandibular defects and compared to defects reconstructed with autologous bone.

The results show that there was a higher failure rate amongst trial patients (4 out of 6) when compared to control patients (2 out of 7). However, the successful BMP implants were highly active and regenerated larger volumes

of bone compared to the autologous bone grafts. Statistical significance could not be shown however, due to inadequate number of patients. For this reason, purely qualitative inferences and conclusions will be made.

It is necessary to discuss the apparently poor performance of the trial device and to propose possible ways for improvement. Poor trial device performance may be attributed, but not limited, to the following:

- Suboptimal dose of BMP
- The physical impediment of the titanium mesh on the host response, amongst which angiogenesis is possibly the most critical.

This is the first trial of a BMP based inductive device to reconstruct large defects in the mandible. Of the six patients treated with the device, two showed clear histological evidence of induction. There was copious osteoid and mineralised bone deposition directly in contact with non-vital demineralized bone matrix. This provides unequivocal evidence of osteoinduction. Osteoid seams were lined with plump contiguous osteoblasts. The intervening stroma was collagenous and highly vascular. However four trial patients failed to initiate an inductive cascade (at least in the area of the biopsies). In these patients there was persistent demineralized bone matrix with no vital bone, and a dense avascular fibrous stroma.

The reasons for osteoinductive failure in four patients must be sought by reviewing the prerequisites for osteoinduction. Bone regeneration in clinical contexts requires three key components: a morphogenetic signal, a suitable carrier matrix with which this signal is to be delivered and that acts as a scaffold for new bone to form, and responding host cells capable of differentiating into bone cells following the binding of BMPs to specific cell surface receptors (Ripamonti and Duneas, 1998). Osteoinduction is the result of a highly complex, integrated process involving cell-to-cell and cell-extracellular matrix interactions. The importance of the extracellular matrix in bone induction is emphasised by the realisation that reconstitution of the soluble signal (BMPs) with the insoluble substratum restores the biological activity of BMPs (Sampath and Reddi, 1981). The extra cellular matrix binds growth and differentiating factors, protects them from proteolytic degradation and modulates a controlled slow release. The developmental cascade involved in bone formation is characterised by chemotaxis of mesenchymal cells, proliferation in response to mitogenic signals and differentiation of cartilage, angiogenesis and bone differentiation (Reddi, 1981). The importance of angiogenesis during osteogenesis has already been stressed (Trueta, 1963). Similarly a critical step in the osteoinductive cascade is vascular invasion and the attachment of responding mesenchymal cells to the matrix, and their

differentiation to osteoblasts under the stimulus of matrix released bone morphogenetic proteins (Foidart and Reddi, 1980; Ripamonti et al., 1993).

In the demineralized bone matrix-dependant bone induction model, angiogenesis heralds chondrolysis and subsequently osteogenesis (Ripamonti, 1997). The origin of the cells which respond to BMP signals and ultimately differentiate into osteoblasts has not been satisfactorily explained. However, there is compelling evidence to suggest that osteoblast are derived from local mesenchymal cell populations (Reddi, 1981). It is also possible that the invading "osteogenetic vessels" may provide a temporally regulated flow of cell populations capable of expression of the osteogenic phenotype (Ripamonti et al., 1993). Pericytes have been proposed as target cells for osteoblastic differentiation (Urist et al., 1983). In vitro evidence demonstrating the ability of BMPs to modulate the phenotype of endothelial cells implies that they may be capable of responding to bone matrix molecules inductive signals (Heliotis and Ripamonti, 1994). Moreover, BMP-3 has an affinity for several basement membrane components, most notably collagen type IV (Paralkar et al., 1990) which may act as a delivery system by sequestering bone morphogenetic proteins and presenting them to responding mesenchymal cells and osteoprogenitors to initiate osteogenesis (Paralkar et al., 1990). Clearly responding cells lured by the chemotactic gradient must have access to the site

where they are to differentiate to the osteogenic phenotype. We cannot exclude the possibility of a blockade phenomenon exerted by the titanium mesh on migrating cells resulting in their inability to reach and bind to the BMP-coated matrix. A further function of the blockade phenomenon may be to interrupt the developing chemical gradient which is critical to initiate cellular migration. This is borne out strongly by the histological evidence: all failed implants were hypovascular. Moreover the histomorphometric data suggests that fibrovascular tissue content of all the successful grafts (trial and control) was higher than in failed grafts. As a consequence of delayed or absent vascular invasion bound BMPs are bathed in blood clot components and subsequently undergo proteolysis.

The initiation of bone formation by BMPs is dependant on a critical threshold concentration of BMPs (Ripamonti et al., 1993b). Once this concentration is exceeded, regenerated bone volumes increase commensurate with increase in the dose of morphogen until a plateau is reached. S-200 baboon osteogenin fractions induce more tissue regeneration than larger doses of heparin-Sepharose fractions (Ripamonti et al., 1993a). The performance of rhOP-1 at various doses has clearly demonstrated that increasing doses result in larger volumes of regenerated tissue (Ripamonti et al., 1996). Further evidence of this relationship is the dose response curve for BMPs in the rat subcutaneous

assay. Dosage strategies for native BMPs in animal trials have ranged from 100mg native BMP / 100mg gelatin for a 2.5cm defect in the ulna of dogs (Nilsson et al., 1986); 60-100mg native BMP for 14mm cranial defects in pigs (Lindholm et al., 1994); 280µg (Sephacryl S-200 fraction) and 1.8mg (heparin sepharose and hydroxyapatite fractions) of osteogenin / 500mg baboon insoluble collagenous bone matrix (Ripamonti et al., 1993a) in 25mm calvarial defects in baboons; and 50 µg of osteogenin / aliquot of hydroxyapatite granules for heterotopic implantation (Ripamonti et al., 1992) in Long Evans rats. Native BMPs have been applied for human osteogenesis in the following manner: 50-100mg per femoral defect delivered with a polylactic acid copolymer strip or gelatine capsule (Johnson et al., 1988), 100mg BMP per strip of autolyzed, antigen-extracted, allogeneic bone for treatment of long bone defects (Johnson et al., 1992), and 1mg of a crude naturally sourced BMP extract / cm³ of granular bone matrix (Sailer and Kolb, 1994a and b). In order to explain the erratic performance of the BMP device in the present work, it would be useful to make some sort of comparison to strategies adopted by other authors. Unfortunately, the measurement units chosen by the above authors to measure and quantify the amount of implanted BMP are not ideal for the following reasons:

- Because natively sourced BMP isolates contain impurities, the mass measure may be inappropriate.

- A description in mass units does not reflect the potency of the isolated BMP fraction. Hence, in works published to date using this measure, their results are rendered incomparable to ours in relation to BMP content.
- The mass measure does not describe specific BMP activity.

For these reasons, it was decided to use the more appropriate measure of *specific activity* (Sampath and Reddi, 1983). This form of quantification describes the osteogenic activity of a unit of isolate, regardless of its purity. The high degree of similarity with respect to bone physiology and remodelling between man and baboon makes the baboon ideally suited for the study of comparative bone physiology and repair with relevance to man (Schnitzler et al., 1993). To regenerate bone in calvarial defects in baboons, 131 units of alkaline phosphatase activity were loaded per gram of delivery system (Ripamonti et al., 1992). Although many clinical trials with BMPs failed to find any adverse side-effects it is unclear at present if orthotopically applied BMPs in human patients could result in uncontrolled osteogenesis or if wide dissemination of BMPs in the body may cause adverse effects (particularly in light of the recent discovery of the widespread presence of BMP mRNA in varied tissues of the human body)(Lyons et al. 1990, Vukicevic et al. 1994). Moreover, the minimum dose required to regenerate bone in human patients is unknown. To develop our dosage strategy a careful balance was sought between the requirements of bone induction and the avoidance of unwanted

side-effects. Consequently a cautious dosage strategy was adopted and 40 units of alkaline phosphatase activity were loaded per gram of delivery system. This possibly resulted in the delivery of a feeble osteogenic impulse to the defect and limited osteogenesis.

The capacity of bone to regenerate following injury without scarring is perhaps unique amongst the tissues of adult organisms (Reddi, 1994). This phenomenon is accomplished by activation of a sequential cascade of events which culminates in the local differentiation of mature bone. This cascade recapitulates events that occur during embryonic bone development. However, the regenerative potential of this system is finite and most skeletal defects will not heal without surgical intervention. The recognition of this fact has stimulated the development of bone graft surgery. The cellular events that follow the transplantation of an autologous bone graft were first described by Axhausen in 1907 and Barth in 1908 (Chase and Herndon, 1955; Manson, 1994). The transplant bed is initially filled with coagulated blood, followed by the development of an inflammatory response characterised by vascular buds infiltrating the transplant bed. By the second week fibrous granulation tissue becomes increasingly dominant in the transplant bed (Burchardt, 1987). The cellular elements in cortical bone grafts necrose and vascular ingrowth occurs through the pre-existing vascular channels or Volkmann's canals preceded by

osteoclastic resorption along the haversian channels. Complete vascularization of cortical grafts occurs after two months, at least twice the period required for cancellous grafts (Deleu and Trueta, 1965). The bone graft is simultaneously resorbed by osteoclasts and new bone is deposited in the transplant by osteoblastic migration from the recipient bone ends towards the midpoint of the grafted segment, a process termed osteoconduction. In contrast cancellous bone grafts due to their open architecture undergo rapid vascular invasion. Degeneration of the constituents of the marrow spaces provides large channels for neoangiogenesis (Burwell, 1964) or alternatively, end-to-end anastomosis of graft to host vessels may occur (Deleu and Trueta, 1965). Complete vascularisation is achieved in 2 to 3 days.

Angiogenesis is intimately associated with osteogenesis and is a prerequisite for successful bone graft incorporation (Trueta, 1963; Stevenson et al., 1996). During embryonic osteogenesis the vascular invasion of the cartilage anlage heralds the differentiation of a new cellular phenotype, the osteogenic cell which will finally differentiate into osteoblastic cells (Reddi, 1981). The importance of this vascular invasion was stressed by Trueta, referring to it as “osteogenetic vessels” and further proposed that osteoblasts were of endothelial derivation (Trueta, 1963). Another cell of vascular origin which may be an osteoblast progenitor cell is the pericyte (Diaz-Flores et al., 1992;

Brighton et al., 1992). Pericytes are contractile cells found in the microvasculature of connective tissue. Moreover, pericytes and osteoblasts have a close spatial and temporal relationship during embryonic osteogenesis. Finally, the morphological similarities between pericytes and osteoblasts have added credence to this hypothesis. It is therefore abundantly clear that successful bone grafting requires the presence of highly vascular host bed. Cancellous grafts enjoy a distinct advantage over cortical grafts due to their ability to be rapidly vascularised.

New bone formation following bone transplantation is derived from two principal sources: from the graft and from the host. Graft derived osteogenesis relies on the transplantation of viable bone forming cells which include osteocytes, endosteal osteoblasts, and periosteal cells. This new bone is termed phase I bone (Axhausen, 1956) and is composed predominantly of osteoid and woven bone. Osteocytic contribution to osteogenesis is minimal (Craig Gray and Elves, 1982) and the majority of osteocytes, particularly in cortical grafts, necrose as a result of their inability to access the developing vasculature for their metabolic requirements. Periosteal cells (particularly of the inner cambial layer) account for approximately one third of graft osteogenesis. This is especially so in younger organisms where the cambial layer is highly cellular (Craig Gray and Elves, 1982; Burchardt, 1987). The

major contribution to graft osteogenesis is however from transplanted endosteal cells which produce two thirds of graft derived bone (Burwell, 1964, Craig Gray and Elves, 1982). Consequently cancellous bone autografts, due to their rich endosteal cell content, are ideally suited for transplantation.

The contribution of the recipient site to osteogenesis following bone transplantation is incompletely understood (Burchardt, 1987). It is postulated that woven phase 1 bone is replaced by lamellar bone or phase 2 bone by osteoblasts derived from osteoprogenitor cells in the recipient bed (Axhausen, 1956; Marx and Kline, 1983). This is possibly due to osteoinductive events initiated by the graft (Marx and Saunders, 1986). The coupling of phase 1 to phase 2 bone formation is mediated by BMPs (Marx and Saunders, 1986). Histological evidence of induction was indeed noted in some of the control patients. If the recipient tissue bed is hypocellular or hypovascular, coupling fails. The graft is doomed to resorb and the final reconstructed segment will consist of a volume deficient ossicle. At best, phase 2 bone replaces vital, phase 1 bone in a ratio of 1:1 (Marx and Saunders, 1986). This emphasises the importance of ensuring a maximal phase 1 response by transplanting highly cellular cancellous bone and compacting it into the recipient site.

Surprisingly, of the seven patients that received autologous bone grafts, two demonstrated complete absence of osteogenesis. The histological pattern of the failed grafts suggests that the primary reason for poor or absent osteogenesis was failure of the primary angiogenic response. The stroma of these grafts was dense fibrous tissue with sparse intervening vessels as compared to the successful grafts whose stroma was highly vascular. Although areas of vascularity were noted in failed grafts, none of the successful grafts were avascular. This suggests that vascularisation may be only one of the prerequisites for successful bone transplantation. The small number of patients makes it difficult to identify trends. However, the two failed patients were the eldest males in the group and both had suprapariosteal resections of large ameloblastomas. Although the eldest patient in the group displayed successful osteogenesis, she had a primary reconstruction following a subperiosteal tumour resection. Graft failure may therefore be the end result of the cumulative effect of several factors. It is well established that there is an age-associated decline in bone density and bone repair capacity (Quarto et al., 1995). The former is probably a result of an increased rate of resorption, coupled with a decreased rate of bone deposition. The latter may be as a result of decreased numbers of osteoprogenitor cells and impaired ability to differentiate towards an osteogenic lineage (Quarto et al., 1995). Failure of

osteogenesis in these two patients may therefore be the consequence of the cumulative effect of the following factors:

- Surgical resection of periosteum.
- The age associated paucity of the cellular content in the transplant
- The age associated decrease in cellular activity of the transplanted cells.
- Similarly to the trial patients, the obstructive effects of the titanium mesh may have lead to failure of the graft to initially induce an angiogenic stimulus and to subsequently initiate osteogenesis.
- Finally, the recipient bed may have been unable to initiate and sustain phase 2 bone as a result of surgical scarring following the suprapariosteal dissections.

The autologous grafts had a considerable decrease in volume during the follow-up period. The concept that mechanical stress is important for the maintenance of bone graft strength and volume was first postulated by Wolff in 1892 (Manson, 1994). The influence of mechanical loading on bone grafts may extend to the initial encouragement of osteogenesis. The use of rigid bone plates in combination with bone grafts in primates results in decreased density and mineral content of bone grafts (Kennady et al., 1989). It is likely that bone plates prevent the transmission of functional stresses to the bone graft resulting in a disuse osteoporosis termed "stress shielding". Particulate

cancellous bone and marrow grafts contained within a titanium mesh may be subject to the same phenomenon resulting in a progressive loss of bone mass and density combined with failure of osteogenesis. Some investigators have advocated the removal of stress shielding hardware once bony healing has occurred (Kennady et al., 1989, Alexander et al., 1993). A similar principal may be required for titanium meshes.

The technique of reconstructing defects with a titanium mesh allowed for successful rehabilitation of facial aesthetics and mandibular function. However, defects crossing the midline or larger than a hemimandible were more challenging to restore aesthetically. Symmetry was particularly difficult to recreate in defects extending across the midline. In addition, with extensive defects, the antero-posterior positioning of the mandible was more difficult to re-establish. One patient had a poor result as a consequence of the incorrect antero-posterior position of the mesh resulting in an excessively protruding mandible and intraoral dehiscence due to soft tissue tension.

In conclusion, this trial has provided the first histological confirmation of osteoinduction in mandibular segmental defects in human patients treated with native bovine BMP. The present formulation was however unable to induce osteogenesis in four of the trial patients two of which had particularly large

defects. Furthermore, histological verification of the ability of autologous particulate cancellous bone and marrow grafts to support active osteogenesis was obtained. It was evident however, that autologous bone in this formulation resulted in volumetrically deficient reconstructions. Moreover the failure of osteogenesis in two control and four trial patients may indicate that the titanium mesh system although technically sound, may be biologically flawed. Taking this into account, the potential exists to create an osteoinductive device which may ultimately preclude the need to harvest autologous bone to repair skeletal defects in human patients. Considerable work needs to be done in the field of human osteoinduction to investigate several key issues. Firstly, minimum dosage strategies required to provide optimal clinical performance need to be identified. Secondly, the application of recombinant morphogens may improve the clinical performance of inductive devices. Thirdly, the exciting recent discovery of the synergic action between TGF- β 1 and OP-1 (Ripamonti et al., 1997; Duneas et al., 1998) may allow for the development of synergistic molecular therapeutics for the rapid regeneration of cartilage and bone in clinical contexts. Fourthly, bio-engineered customised autografts may be developed in the future by exploiting the phenomenon of osseointegration in heterotopic sites. Finally, the development of an inorganic, porous, non-immunogenic and carvable biomaterial with the inherent ability to

withstand mechanical stresses will allow for the optimal delivery of BMPs in clinical contexts.

LIST OF REFERENCES

Alexander, R., and Theodos, L. (1993) Fracture of the bone-grafted mandible secondary to stress shielding. *J. Oral. Maxillofac. Surg.* 51: 695-697

Axhausen, W. (1956) The osteogenic phases of regeneration of bone. A historical and experimental study. *J. Bone. Joint. Surg. [A]* 38: 593-600

Beirne, J.C., Barry, H.J., Brady, F.A., Morris, V.B. (1996) Donor site morbidity of the anterior iliac crest following cancellous bone harvest. *Int. J. Oral. Maxillofac. Surg.* 25: 268-271

Bostrom, M., Lane, J.M., Tomin, E., Browne, M., Berberian, W., Turek, T., Smith, J., Wozney, J. and Schildhauer, T. (1996) Use of bone morphogenetic protein-2 in the rabbit ulnar nonunion model. *Clin. Orth. Rel. Res.* 327: 272-282

Boyne P.J. (1969) Restoration of osseous defects in maxillofacial casualties. *JADA.* 78: 767-776

Boyne, P.J. (1996) Animal studies of application of rhBMP-2 in maxillofacial reconstruction. *Bone* 19: 83S-92S

Brazaitis, M.P., Mirvis, S.E., Greenberg J., Ord, R.A. (1994) Severe retroperitoneal haemorrhage complicating anterior iliac bone graft acquisition. *J. Oral. Maxillofac. Surg.* 52: 314-316

Brighton, C.T., Lorch, D.G., Kupcha, R., Reilly, T.M., Jones, A.R., and Woodbury, R.A. (1992) The pericyte as a possible osteoblast progenitor cell. *Clin. Orthop. Rel. Res.* 275: 287-299

Burchardt, H. (1983) The biology of bone graft repair. *Clin. Orthop. Rel. Res.* 174: 28-42

Burchardt, H (1987) Biology of bone transplantation. *Orthop. Clin. North. Am.* 18(2): 187-96

Burwell, R.G. (1964) Studies in the transplantation of bone VII. The fresh composite of homograft-autograft of cancellous bone: An analysis of factors leading to osteogenesis in marrow transplants and in marrow-containing bone grafts *J. Bone. Joint. Surg. [B]* 46:110-140

Carlson, E.R. and Marx, R.E. (1996) Mandibular reconstruction using cancellous cellular bone grafts. *J. Oral. Maxillofac. Surg.* 54: 889-897

Celeste, A.J., Ianazzi, J.A., Taylor, R.C., Hewock, R.M., Rosen, V., Wang, E.A. and Wozney, J.M. (1990) Identification of transforming growth factor- β family members present in bone inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA* 87: 9843-9847

Chase, S.N. and Herndon, C.H. (1955) The fate of autogenous and homogenous bone grafts. A historical review. *J. Bone. Joint. Surg.* 37A:809-841

Craig Gray, J. and Elves, M.W. (1982) Donor cell contribution to osteogenesis in cancellous bone grafts. *Clin. Orthop. Rel. Res.* 163: 261-271

De Boer, H.H. (1988) The history of bone grafts. *Clin. Orth. Rel. Res.* 226: 292-298

Deleu, J. and Trueta, J. (1965) Vascularization of bone grafts in the anterior chamber of the eye. *J. Bone. Joint. Surg.* 47[B]: 319-329

Diaz-Flores, L., Gutierrez, R., Lopez-Alonso, A., Gonzales, R., and Varela, H. (1992) Pericytes as a supplemental source of osteoblasts in periosteal osteogenesis. *Clin. Orthop. Rel. Res.* 275: 280-286

Dumbach, J., Rodemer, H., Spitzer, W.J., Steinhauser, E.W. (1994) Mandibular reconstruction with cancellous bone hydroxylapatite and titanium mesh. *J. Craniomaxillofac. Surg.* 22: 151-155

Duneas, N., Crooks, J. and Ripamonti, U. (1997) Transforming growth factor- β 1: Induction of bone morphogenetic protein genes expression during endochondral bone formation in the baboon, and synergistic interaction with osteogenic protein-1 (BMP-7). *Growth Factors.* 15: 259-277

Gerhart, T.N., Kirker-Head, K.A., Kriz, M.J., Holtrop, M.E., Hennig, G.E., Hipp, J., Schelling, S.H. and Wang, E. (1993) Healing segmental femoral defects in sheep using recombinant human bone morphogenetic protein. *Clin. Orthop. Rel. Res.* 293: 317-326

Glowacki, G., Kaban, L.B., Murray, J.E., Folkman, J. and Mulliken, J.B. (1981) Application of the biological principle of induced osteogenesis for craniofacial defects. *Lancet.* 1: 959-983

Hedner, E. and Linde, A. (1995) Efficacy of bone morphogenetic protein (BMP) with osteopromotive membranes: an experimental study in rat mandibular defects. *Eur. J. Oral. Sci.* 103: 236-241

Heine, U.I., Muuoz, E.F., Flanders, K.L., Ellingsworth, L.R., Sporn, M.B., Roberts, A.B. (1989) Role of transforming growth factor- β in the development of the mouse embryo. *J. Cell. Biol.* 105: 2861-2867

Heliotis, M., and Ripamonti, U. (1994) Phenotypic modulation of endothelial cells by bone morphogenetic protein fractions in vitro. *In Vitro Cell. Dev. Biol.* 30A: 353-355

Hidalgo, D. (1989) Fibula free flap: a new method for mandibular reconstruction. *Plast. Reconstr. Surg.* 84: 71-79

Hoffmann, J., Ehrenfeld, M., Hwang, S., Schwenzer, N. (1998) Complications after microsurgical tissue transfer in the head and neck region. *J. Cranio-Maxillofac. Surg.* 26: 255-259

Huggins, C.B. (1931) The formation of bone under the influence of epithelium of the urinary tract. *Arch. Surg.* 22: 377-408

Johnson, E.E., Urist, M.R. and Finerman, G.A.M. (1988) Bone morphogenetic protein augmentation grafting of resistant femoral non-unions. Clin. Orthop. Rel. Res. 230: 257-265

Johnson, E.E., Urist, M.R. and Finerman, G.A.M. (1992) Resistant nonunions and partial or complete segmental defects of long bones. Treatment with implants of a composite of human bone morphogenetic protein (BMP) and autolyzed, antigen-extracted, allogeneic (AAA) bone. Clin. Orthop. Rel. Res. 277: 229-237

Kaban, L.B., Mulliken, J.B., Glowacki, J. (1982) Treatment of jaw defects with demineralized bone implants. J. Oral. Maxillofac. Surg. 40: 623-626

Kazanjian, V.H., Burrows, H. (1918) Treatment of gunshot wounds of the face accompanied by extensive destruction of the lower lip and mandible. Br. J. Surg. 6: 74-85

Kennady, M.C., Tucker, M.R., Lester, G.E., and Buckley, M.J. (1989) Stress shielding effect of rigid internal fixation plates on mandibular bone grafts. Int. J. Oral Maxillofac. Surg. 18: 307-310

Lacroix, P. (1945) Recent investigations on the growth of bone. *Nature*. 156: 576

Lindholm, T.C., Lindholm, T.S., Marttinen, A. and Urist, M.R. (1994) Bovine bone morphogenetic protein (bBMP/NCP)- induced repair of skull trephine defects in pigs. *Clin. Orthop. Rel. Res.* 310: 263-270

Lowry, O.H., Rosebrough, W.J., Farr, A.L., and Randall, R.J., (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275

Lyons, K.M., Pelton, R.W., Hogan, B.L.M. (1990) Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). *Development*. 109:833-844.

Luyten, F.P., Cunningham, N.S., Muthukumaran, R.G., Hammonds, R.G., Nevins, W.B., Wood, W.I., Reddi, A.H. (1989) Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. *J. Biol. Chem.* 264: 13377-13380

Manson, P. (1994) Facial bone healing and bone grafts. A review of clinical physiology, in Bone Repair and Regeneration. Clinics in Plastic Surgery 21(3):331-346.

Marx, R.E., and Kline, S.N. Principles and methods of osseous reconstruction. In: International Advances in Surgical Oncology, ed. by Murphy, B.L., Liss, London, (1983) pp. 167-228

Marx, R.E., and Saunders, T.R. Reconstruction and rehabilitation of the cancer patient. In: Reconstructive preprosthetic oral and maxillofacial surgery, ed. by Fonseca, R.J., and Davis, W.H., Saunders, Philadelphia (1986) pp. 1068-1072

Marx, R.E., and Morales, M.J. (1988) Morbidity from bone harvest in major jaw reconstructions. J. Oral. Maxillofac. Surg. 48: 196-203

Mulliken, J.B., Glowacki, J., Kaban, L.B., Folkman, J. and Murray, J.E. (1981) Use of demineralized allogeneic bone implants for the correction of maxillocraniofacial deformities. Ann. Surg. 194: 366-371

Nilsson, O.S., Urist, M.R., Dawson, E.G., Schmalzried, T.P. and Finerman, G.A.M. (1986) Bone repair induced by bone morphogenetic protein in ulnar defects in dogs. *J. Bone. Joint. Surg.* 68 (B): 635-642

Özkaynak, E., Rueger, D.C., Drier, E.A., Corbett, C., Ridge, R.J., Sampath, T.K. and Opperman, H. (1990) OP-1 cDNA encodes an osteogenic protein in the TGF- β family. *EMBO J.* 9: 2085-2093

Paralkar, V.M., Nandedkar, A.K.M., Pointer, R.H., Kleiman, H.K., and Reddi A.H. (1990) Interaction of osteogenin, a heparin binding bone morphogenetic protein, with type IV collagen. *J. Biol. Chem.* 265: 17281-17284

Parfitt, A.M. (1983) Stereologic basis of bone histomorphometry: Theory of quantitative microscopy and reconstruction of the third dimension. In: *Bone histomorphometry: Techniques and interpretation*, ed. by Recker R.R., CRC Press, Boca Raton, FL, U.S.A., pp. 53-87.

Quarto, R., Thomas, D., and Liang, C.T. (1995) Bone progenitor cell deficits and the age-associated decline in bone repair capacity. *Calcif. Tissue. Int.* 56: 123-129

Reddi, A.H., Huggins, C.B. (1972) Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc. Natl. Acad. Sci. USA. 69: 1601-1605

Reddi, A.H., Sullivan, N. (1980) Matrix induced endochondral bone differentiation: Influence of hypophysectomy, growth hormone and thyroid stimulating hormone. Endocrinology 107: 1291-1299

Reddi, A.H. (1981) Cell biology and biochemistry of endochondral bone development. Collagen. Rel. Res. 1: 209-226

Reddi, A.H. (1992) Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr. Opin. Cell Biol. 4: 850-855

Reddi, A.H. (1994) Bone and cartilage differentiation. Curr. Opin. Gen. Dev. 4: 737-744

Ripamonti, U. (1991) Bone induction in non-human primates. An experimental study on the baboon (Papio ursinus). Clin. Orthop. 269: 284-294

Ripamonti, U., Ma, S., Cunningham, N.S., Yeates, L., and Reddi A.H. (1992) Initiation of bone regeneration in adult baboons by osteogenin, a bone morphogenetic protein. *Matrix* 12: 369-380

Ripamonti, U., Ma, S., Cunningham, M.S., Yeates, L., and Reddi, A.H. (1993a) Reconstruction of the bone-bone marrow organ by osteogenin, a bone morphogenetic protein, and demineralized bone matrix in calvarial defects of adult primates. *Plast. Reconstr. Surg.* 91: 27-36

Ripamonti, U., Van Den Heever, B., and Van Wyk, J. (1993b) Expression of the osteogenic phenotype in porous hydroxyapatite implanted extraskelally in baboons. *Matrix* 13: 491-502

Ripamonti, U. and Reddi, A.H. (1994) Periodontal regeneration: potential role of bone morphogenetic proteins. *J. Periodont. Res.* 29: 225-235

Ripamonti, U and Reddi, A.H. (1995) Bone Morphogenetic Proteins: Applications in Plastic and Reconstructive Surgery. *Adv. Plas. Reconstr. Surg.* 11: 47-74

Ripamonti, U., Bosch, C., Van Den Heever, B., Duneas, N., Melsen, B. and Ebner, R. (1996a) Limited chondro-osteogenesis by recombinant transforming growth factor- β_1 in calvarial defects of adult baboons (*Papio ursinus*). J. Bone. Miner. Res. 11: 938-945

Ripamonti, U., Van Den Heever, B., Sampath, T.K., Tucker, M.M., Rueger, D.C., and Reddi A.H. (1996b) Complete regeneration of bone in the baboon by recombinant human osteogenic protein-1 (hOP-1, Bone Morphogenetic Protein-7). Growth Factors 123: 273-289

Ripamonti, U., Duneas, N., Van Den Heever, B., Bosch, C. and Crooks, J. (1997a) Recombinant transforming growth factor- β_1 induces endochondral bone in the baboon and synergizes with recombinant osteogenic protein-1 (bone morphogenetic protein-7) to initiate rapid bone formation. J. Bone. Miner. Res. 12: 1584-1595

Ripamonti, U. (1997b) Molecular therapeutics for bone repair and regeneration. S. A. Bone. Joint. Surg. 7: 21-32

Ripamonti, U., and Duneas, N. (1998) Tissue morphogenesis and regeneration by bone morphogenetic proteins. Plast. Recon. Surg. 101: 227-239

Rosa, J., Roberts, A.B., Danielpour, D., Dart, F.F., Sporn, M.B. and Dawid, I.B (1988) Mesoderm induction in amphibians: the role of TGF- β 2-like factors. *Science* 239: 783-786

Sailer, H.F. and Kolb, E. (1994a) Application of purified bone morphogenetic protein (BMP) in cranio-maxillo-facial surgery. BMP in compromised surgical reconstructions using titanium implants. *J. Craniomaxillofac. Surg.* 22: 2-11

Sailer, H.F. and Kolb, E. (1994b) Application of purified bone morphogenetic protein (BMP) preparations in cranio-maxillo-facial surgery. Reconstruction in craniofacial malformations and post-traumatic or operative defects of the skull with lyophilized cartilage and BMP. *J. Craniomaxillofac. Surg.* 22: 191-199

Sampath, T.K., Reddi, A.H. (1981) Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. *Proc. Natl. Acad. Sci. USA.* 78: 7599-7602

Sampath, T.K., and Reddi, A.H. (1983) Homology of bone-inductive proteins from human, monkey, bovine, and rat extracellular matrix. *Proc. Natl. Acad. Sci. USA.* 80: 6591-6595

Sampath, T.K., Muthukumaran, N., Reddi, A.H. (1987) Isolation of osteogenin, an extracellular matrix-associated bone inductive protein, by heparin affinity chromatography. *Proc. Natl. Acad. Sci. USA.* 84: 7109-7113

Sarafin, D., Villareal-Rios A., Georgia, D.E. (1977) A rib containing free flap to reconstruct mandibular defects. *Br. J. Plast. Surg.* 30: 263-266

Schnitzler, C.M., Ripamonti, U., Mesquita, J.M. (1993) Histomorphometry of iliac crest trabecular bone of adult male baboons held in captivity. *Calcif. Tissue. Int.* 52: 447-454

Shirota, T., Ohno, K., Motohashi, M., and Michi, K. (1996) Histologic and microradiologic comparison of block and particulate cancellous bone and marrow grafts in reconstructed mandibles being considered for dental implant placement. *J. Oral. Maxillofac. Surg.* 54: 15-20

Soutar, D.S., Scheker L.R., Tanner N.S.B., McGregor I.A. (1983) The radial forearm flap: a versatile method for intraoral reconstruction. *Br. J. Plast. Surg.* 36:1-8

Sporn, M.B. and Roberts, A.B. (1990) The transforming growth factor- β s. In: Peptide growth factors and their receptors, ed. by Sporn, M.B. and Roberts, A.B., Springer-Verlag, Berlin. Pp. 419-472

Stevenson, S., Emery, S.E., and Goldberg, V.M. (1996) Factors affecting bone graft incorporation. Clin. Orthop. Rel. Res. 324: 66-74

Sullivan, M.J., Baker, S.R., Crompton, R., et al. (1989) Free scapular osteo-cutaneous flap for mandibular reconstruction. Arch. Otolaryngol. Head. Neck. Surg. 115: 1334-1340

Taylor, G.I., Miller, D.M., Ham, F.J. (1975) The free vascularised bone graft: a clinical extension of microvascular techniques. Plast. Reconstr. Surg. 55: 533-544

Taylor, G.I. (1982) Reconstruction of the mandible with free composite iliac bone grafts. Ann. Plast. Surg. 9: 361-376

Thorogood, P.V. and Gray, J.G. (1975) The cellular changes during osteogenesis in bone and bone marrow composite autografts. J. Anat. 120: 27-47

Tideman, H., Samman, N., Cheung, L.K. (1998) Functional reconstruction of the mandible: a modified titanium mesh system. *Int. J. Oral. Maxillofac. Surg.* 27: 339-345

Toriumi, D.M., Kotler, H.S., Luxenberg, D.P., Holtrop, M.E. and Wang, E.A. (1991) Mandibular reconstruction with a recombinant bone inducing factor. *Arch. Otolaryngol. Head. Neck. Surg.* 117: 1101-1112

Trueta, J. (1963) The role of vessels in osteogenesis. *J. Bone. Joint. Surg.* 45B: 402-417

Upton, J., Boyajian, M., Mulliken, J.B. and Glowacki, J. (1984) The use of xenogeneic bone implants to correct phalangeal defects: a case report. *J. Hand. Surg.* 9A: 388-391

Urist, M.R. (1965) Bone: formation by autoinduction. *Science* 150: 893-899

Urist, M.R., Delange, R.J., and Finerman, G.A.M. (1983) Bone cell differentiation and growth factors. *Science* 220: 680-686

Vukicevic, S., Helder, M.N., Luyten, F.P. (1994) Developing human lung and kidney are major sites for the synthesis of bone morphogenetic protein-3 (osteogenin). *J. Histochem. Cytochem.* 42: 869-875

Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M. and Wang, E.A. (1988) Novel regulators of bone formation: Molecular clones and activities. *Science* 242: 1528-1534

Yang, G., Chen, B., Gao, Y., et al. (1981) Forearm free skin flap transplantation. *Natl. Med. J. China.* 61:139

Author Ferretti C

Name of thesis The Repair Of Segmental Mandibular Defects By A Bone Morphogenetic Protein Bone Device In Man

PUBLISHER:

University of the Witwatersrand, Johannesburg

©2013

LEGAL NOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed, or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page) for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.