THE INITIATION OF BONE FORMATION INDUCED INTRINSICALLY BY OSTEOINDUCTIVE HYDROXYAPATITES.

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A dissertation submitted to the faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2005

DECLARATION

I, Lerato Khoali declare that this dissertation is my own work and has not been submitted before for any degree or examination at this or any other University.

Lerato Khoali

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_____Day of_____, 2005.

DEDICATION

To my daughter,

Loyiso Palesa Khoali.

ABSTRACT

The initiation of new bone formation within the porous spaces of hydroxyapatite (HA) implants involves the expression of osteogenic markers belonging to the TGF-β superfamily. To study the genetic expression of these osteogenic markers in relation to the type of HA implant used and implantation period, five different types of porous HA biomaterials were implanted in the *rectus abdominis* muscles of adult baboons *Papio ursinus,* and were harvested at two, three and 12 months. The total RNA of all harvested samples was extracted and analysed using the Northern blot technique. The results showed that *Collagen type IV*, *GDF-10* and *BMP-7* were expressed at the early time points at relatively high levels, and their expression levels were significantly reduced at 12 months. The expression of these makers was not affected by the type of porous HA implant used. The histological sections of these specimens at two and three months showed vascularised connective tissue within the porous spaces of the implants with no bone formation. However, at 12 months there were substantial amounts of bone formed in all the studied implants. The down-regulation of the expressed osteogenic markers at 12 months correlates to the amount of bone formed, suggesting some negative feedback mechanism which may be acting *via* inhibitory Smads proteins in relation to the amount of bone formed. Neither *TGF-β1* nor *BMP*-3 messages were detected in any of the studied samples, It is

possible that these bone markers are not expressed locally within the vicinity of the porous HA implants but are adsorbed to the HA implants from the circulatory system.

ACKNOWLEDGEMENTS

I would like to thank:

My supervisor, Professor Ugo Ripamonti, for his valuable guidance and inspiration in the creation of this project.

Dr Lentsha Ramoshebi, my co-supervisor, for his help and critical input in experimental procedures and write-up.

Dr Laura Roden, my co-supervisor, for her valuable scientific guidance at a difficult time in the project.

Mrs. Thato Chidarikire, for her endless coaching throughout this project and for being a friend.

Miss. Portia Khoboko and Miss. Nina Lewin, for being good supportive friends at a difficult time in the project.

Mr. Malefetsane Sechaba Khoali, my husband, for his love, patience, emotional and financial support throughout this project.

The Department of Orthopaedic Surgery, University of the Witwatersrand, Johannesburg, where I was registered as a student during the execution of this work.

The Bone Research Unit, for supporting me financially during the execution of this work.

This work was supported by funds obtained from the South African Medical Research Council and the University of the Witwatersrand, Johannesburg.

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1 INTRODUCTION

A living bone has an intrinsic capacity to regenerate and repair itself thus making most treatment easy. Nonetheless, there are numerous bone defects that require the use of autografts, and in circumstances where there is not enough bone available for autograft reconstitution, allografts are used. The use of bone grafts brings complications to the patient such as donor site morbidity, graft strength, risks of viral transmission as well as antigenic incompatibility (Yoshikawa *et al.,* 1996; White and Shors, 1986). Owing to these conditions, alternatives have been developed such as the use of osteoconductive biomaterials as a substitute to bone grafts. Studies (Jarcho, 1986; Shors *et al.,* 1989*;* Ripamonti, 1991; 1996; Ripamonti & Reddi, 1994; Whang *et al*., 1998; Saito *et al.,* 2001) have been performed on several materials investigating their compatibility and their ability to act as bone graft replacement devices. These materials range from natural or man-made, comprising of whole or part of living structures or biomedical devices.

1.1 Bone Formation by Induction

The quest to find a biomaterial that can be used as a substitute for bone graft was pioneered over a century ago by Senn (1889), who demonstrated that decalcified bone matrix could be used in treatment of osteomyelitis. Lacroix (1945) postulated that the bone matrix possessed an osteogenic inducer ‘osteogenin’ which was responsible for generating new bone in recipients when implanted intramuscularly or subcutaneously. A major breakthrough was made by Urist (1965), who demonstrated that demineralised bone matrix (DBM) was capable of forming new bone when implanted heterotopically in rodents. Urist described this phenomenon as bone formation by induction (Urist, 1965).

Reddi and Huggins (1972) elicited more information about bone formation by induction when they implanted the DBM in subcutaneous sites of rats and characterised the sequence of events that developed upon implantation. They noted that bone formation resulted from the following events; chemo-attraction of the mesenchymal cells to the implanted DBM, mesenchymal cells differentiation, chondrogenesis, vascular invasion,

chondrolysis, osteoblast differentiation, bone matrix synthesis and mineralisation.

The observation that demineralised bone matrix (DBM) was capable of inducing new bone formation in extraskeletal sites using the rodent bioassay (Urist, 1965; Reddi & Huggins 1972), suggested that there were bone forming proteins within the DBM. This led to attempts of solubilising the proteins of the DBM. However, the organic and inorganic components of the DBM were tightly bound to each other, leading to difficulties in dissociating the two components. This biochemical problem was unlocked by the use of dissociative extractants such as 4M guanidium hydrochloride which successfully extracted and solubilised the bone–inductive proteins from the bone matrix. Once separated, neither the solubilised proteins in the extract nor the remaining insoluble collagenous bone matrix (ICBM) were osteoinductive in the heterotopic bioassay in rodents (Sampath & Reddi, 1981). However, reconstitution of the solubilised protein extract with the ICBM restored the biological activity (Sampath & Reddi, 1981).

More work conducted on the ICBM and the solubilised protein extracts (Ripamonti, 1991), showed that the osteogenic activity in rodents' heterotopic bioassays could only be achieved by reconstituting mammalian solubilised protein extracts with rodent ICBM. Also, osteogenic activity in

baboons could only be achieved by reconstitution of mammalian solubilised protein extractants with baboon ICBM (Ripamonti, 1991). This showed that while the osteogenic proteins are highly conserved between species, the ICBM retains species-specific insoluble immunological components which can inhibit the heterologous bone-induction cascade (Sampath & Reddi, 1983). However, the solubilised protein extracts can be used in any mammalian species, suggesting homology between bone-inductive proteins from different mammalian bone extracellular matrix.

As it was shown that ICBM without the solubilised proteins could not initiate bone formation (Sampath & Reddi, 1981), the study of the solubilised proteins became imperative. These solubilised proteins were extracted from the DBM, characterised, isolated, purified and identified as bone morphogenetic proteins (BMPs) (Wang *et al.,* 1988; Luyten *et al.,* 1989). It was also established that these BMPs belong to the transforming growth factor beta (TGF-β) superfamily of proteins.

1.2 The Transforming Growth Factor beta (TGF-β) Superfamily

The TGF-β superfamily consists of a large group of morphogens which play pivotal roles in tissue morphogenesis and differentiation. Members of this superfamily participate in setting up the basic body plan during embryogenesis in different groups of animals from insects and amphibians to mammals. They control formation of the neural tube, limb, cartilage, bone and sexual organs. They play a major role in the regulation of cartilage and bone during embryonic development and in post-natal life (Reddi, 1994; 1997; 1998; Urist, 1997).

Members of the TGF-β superfamily includes five isoforms of TGF-β (TGF-β1 to TGF-β5) (Sporn & Roberts,1990); bone morphogenetic proteins (BMPs); (Wozney *et al.,* 1988) activins; inhibins (Schwall *et al.,* 1988), the 60A (Wharton *et al.,* 1991); decapentaplegic gene product of *Drosophila melanogaster* (Ferguson & Anderson, 1992), the V*g-1* gene product of *Xenopus laevis* (Weeks & Melton, 1987); *Vg-1*-related murine analogue of murine *Vgr-1* (Lyons *et al.,* 1989), the growth and differentiation factors (GDFs) (Lee, 1991) and the cartilage derived morphogenetic proteins (CDMPs) (Chang *et al.,* 1994).

The critical mediators of the TGF-β signal transduction pathways are defined as Smad proteins. These proteins carry the signal from the membrane bound type I and type II receptors and transduce it to the nucleus (Massagué, 1996). Once in the nucleus Smad proteins interact with other DNA-binding proteins to regulate transcription of specific target genes (Massagué, 1996). Subsequent studies divided Smad proteins into three groups: receptor-activated Smads (R-Smads); inhibitory Smads (I-Smads), and the co-activator Smad 4, which binds to activated R-Smads (Reddi, 1998).

At present eight different Smads have been identified, Smad 1, 5, and 8 are substrates of BMP receptors. Smads 2 and 3 are substrates of TGF-β and activin receptors. Phosphorylation of Smads 1, 5, or 8 activates their interaction with common functional partner Smad 4, and forms a heteromeric complex with it (Massagué, 1996). Smad 4 assists translocation of the receptor–activated Smads into the nucleus where this complex activates BMP-responsive genes. There are two inhibitory Smads, *viz*. 6 and 7. These inhibitory Smads reside in the nucleus and act as a relay to turn-off the type I receptor kinase mediated phosphorylation of Smads 1, 5, and 8 (Massagué, 1996; Reddi, 1998; 2001).

1.2.1 Bone Morphogenetic Proteins (BMPs)

BMPs were the first members of the TGF-β superfamily to be identified as osteoinductive. They were able to induce bone formation when combined with ICBM and implanted intramuscularly or subcutaneously (Reddi, 1992). Approximately 30 BMP isoforms have been identified, sequenced and cloned to date. Of these isolated gene products, BMP-1 is the only one that is not a member of TGF-β superfamily and does not possess any osteogenic activity. Instead BMP-1 is a cleaving procollagen protease, a collagen precursor (Kessler *et al.,* 1996).

One of the first BMPs to be isolated and purified was called osteogenin (Wozney *et al.*, 1988). As more studies were done on BMPs, it was observed that the amino acid sequence of the tryptic peptides of osteogenin had a primary structure which was identical to the amino acid sequence deduced from the cDNA clones of human BMP-3 (Wozney *et al.,* 1988; Luyten *et al.*, 1989) implying that osteogenin and BMP-3 are one and the same protein. Later native BMP-3 (osteogenin) was isolated and purified to homogeneity from baboon bone matrix (Ripamonti *et al.,* 1992c).

Advances in molecular techniques have resulted in the identification of several other gene-related products of BMP family which have been cloned

and expressed (Özkaynak *et al.,* 1990; 1992; Celeste *et al.,* 1990). There was also a construction of a synthetic consensus gene on basis of structural information from Drosophila *dpp*, Xenopus *Vg-1* and amino acid sequences of native bovine osteogenic protein (Sampath *et al.,* 1990). This resulted in identification of a novel human gene, related to murine gene *Vgr-1*, encoding a member of the BMP family, the *osteogenic protein-1* (*OP-1*) (Özkaynak *et al.,* 1990). It is now clear that BMP-7, identified subsequently together with BMP-5 and BMP-6 (Celeste *et al.,* 1990) is identical to OP-1.

To activate BMP response genes, the signal is sent *via* specific Smads proteins to the cell nucleus. When reaching the nucleus the signaling cascade activates expression of the response genes leading to the synthesis of macromolecules involved in cartilage and bone formation by inducing the mesenchymal cells to become chondroblasts or osteoblasts (Massagué, 1996). Noggin and chordin were identified as BMP antagonists as they have some affinity to BMP like the BMP-receptors (Zimmerman *et al.,* 1996).

1.2.2 TGF-β Isoforms

TGF-β proteins are 25 kDa proteins comprising two 12.5 kDa subunits held together by disulphide linkage (Assoian *et al.,* 1983). The TGF-β family comprises five closely related isoforms called TGF-β1 to –5 (Sporn & Roberts, 1990).

As with BMPs, the carboxy terminal domain of the TGF-βs is highly conserved between species (Roberts *et al.,* 1991). TGF-β5 was identified in *Xenopus laevis*, while TGF-β4 was also discovered in human endometrium and described as an endometrial bleeding associated factor (EBAF) (Kothapalli *et al*., 1997; Tabibzadeh *et al.,* 1997). TGF-β1, TGF-β2 and TGF-β3 were found to be mammalian (Wataya-Kaneda *et al.,* 1994). TGF-β1 is identical in man, monkey, cow and chicken. TGF-β2 is 71% homologous to TGF-β1, while the other TGF-βs share homology in the range of 64% to 83% with TGF-β1 (Roberts *et al.,* 1991). The mature region of TGF-β3 has an approximately 80% identity to the mature regions of TGF-β1 and TGF-β2. The precursor regions of the first three isoforms share 27% sequence identity (ten Djike *et al.,* 1988; Derynck *et al.,* 1988).

The fact that TGF-βs are not osteoinductive in rodents does not exclude their requirement and interaction with other bone morphogens during the complex and co-coordinated molecular events that control cell proliferation and differentiation culminating in the organogenesis of bone complete with marrow (Centrella *et al.,* 1994; Reddi, 1994). This is supported by the fact that TGF-β1 and TGF-β2 are found in the extracellular bone matrix together with BMPs and other growth factors. However, the relationship between BMPs and other TGF-βs found in the extracellular bone matrix is poorly understood. Although TGF-β1 and TGF-β2 share a limited homology with BMP proteins, BMP4 and BMP-7 do not have overlapping receptor binding specificity with TGF-βs (ten Dijke *et al.,* 1994).

TGF-β family members play a role in repair and regeneration of tissues during post-fetal life (Sporn & Roberts, 1990; Derynck *et al*., 1994; Kingsley; 1994). While during embryogenesis the TGF-β proteins play a critical role in the migration, proliferation and differentiation of a variety of cells (Heine *et al.,* 1987; Pelton *et al.,* 1990).

1.2.3 Growth Differentiation Factors (GDFs)

Using procedure adapted from that used for the isolation of BMPs from the bone matrix (Wang *et al.,* 1988; Luyten *et al.,* 1989), other morphogenetic proteins were identified in cartilage matrix. These are the cartilage-derived morphogenetic proteins (CDMPs) also known as growth differentiation factors (GDFs). These proteins belong to the TGF-β superfamily and are closely related to BMP-5, -6 and –7 (Chang *et al.,* 1994). In contrast to the other members of the BMP family, expression of CDMP-1 and –2 is predominantly in cartilagenous tissues and particularly in condensing mesenchyme of developing limbs (Chang *et al.,* 1994). The mouse equivalent of CDMP-1 called GDF-5 is linked to a mouse disorder characterised by a distinct shortening of the limbs without other tissue abnormalities (Storm *et al.,* 1994).

GDF-10 was identified as a gene product closely related to BMP-3 and the two proteins are classified under their own subgroup in the TGF-β superfamily. The mature carboxy-terminal domain of GDF-10 and BMP-3 have 83% amino acid sequence identity and the pro-regions of these two proteins are also homologous having approximately 30% amino acid sequence identity (Cunningham *et al.,* 1995).

GDF-10 is also present in both neonatal and adult bone samples, with higher levels being detected in calvaria than in long bones. These findings indicate that GDF-10 may play a role in regulating cell differentiation events, including those involved in skeletal morphogenesis. GDF-10 was mapped to the proximal region of mouse chromosome 14 close to a region known to contain a spontaneous recessive mutation that is associated with craniofacial defects (Cunningham *et al.,* 1995).

1.3 Delivery Vehicles of Bone Forming Proteins.

In order to induce new bone formation, it is necessary to have both an osteoinductive protein and delivery vehicle. The proteins of the TGF-β superfamily are osteoinductive. However, there is a need to search for a biomaterial that could act as a suitable delivery vehicle for these osteoinductive proteins and be able to act as a scaffold supporting new bone that is being formed, while maintaining a space or volume in which bone formation can occur.

There are many biomaterials that could be used as delivery vehicles for bone forming proteins but not all meet the ideal. The ideal osteogenic biomaterial should be biocompatible *i.e.* not cytotoxic or able to elicit excessive inflammatory responses which interfere with bone induction (Shors *et al.,* 1989). To fulfill its function the biomaterial must be cell-compatible enabling it to support cell proliferation and provide a suitable attachment substratum. Also, it should be capable of directly influencing cellular differentiation and expression of the osteogenic phenotype (Anselme, 2000). Moreover, biomaterials should act by binding to BMPs and presenting the proteins to cell receptors directly (Reddi & Cunningham, 1993). The slow release of BMPs is critical as it provides physiological concentration of BMPs in the vicinity of the implant for a prolonged time.

The released BMPs are capable of attracting target cells by chemotaxis to a desired site for bone induction (Cunningham *et al.,* 1992).

Insoluble collagenous bone matrix (ICBM) has been commonly used as a delivery system for bone forming proteins. A clinical trial has been made on patients with tibial nonunions, treating patients by inserting an intramedullary rod and recombinant human (rh)-BMP-7 delivered by the insoluble collagenous bone matrix, a xenogeneic bovine matrix packed along the rod (Friedlander *et al.,* 2001). Results showed that rh-BMP-7 treated nonunions healed as well as nonunions treated by autografts. However, the patients treated with rhBMP-7 did not suffer from donor site pain as compared to patients treated with autografts (Friedlander *et al.,* 2001). In Baboons ICBM combined with TGF-β1 and BMP-7 showed that the two morphogens synergised to bring about rapid bone formation heterotopically (Ripamonti *et al.,* 1997).

The delivery systems that have also proved to be osteoconductive are polymer matrices which are made out of α-hydroxy acid polymers, poly (lactide) (PL), poly (gly-colide) (PG) and their copolymers (Whang *et al.,* 1998). In one study (Saito *et al.,* 2001) poly-D, L-lactic acid-polyethylene glycol block copolymers (PLA-PEG) were used as delivery systems for BMP-2. This study showed that heterotopic implantation of these polymers

carrying BMP-2 in mice would show new bone formation having hematopoietic marrow and osseous trabecular after three weeks of implantation. However there was no bone formed in polymers which had no BMP-2 (Saito *et al.,* 2001). Another study showed that PLA-PEG blend microparticles can serve as delivery vehicles for controlled release of TGF-β 1 also initiating bone formation (Lu *et al.,* 2001).

Calcium phosphate bioceramics have been studied and used as delivery vehicle for BMPs, hydroxyapatite (HA) and tricalcium phosphate (TCP) are one of these calcium phosphate ceramics. Hydroxyapatite is a natural component of bone tissue; it is non-resorbable and thus suitable for long term restorative clinical procedures (Jarcho, 1986). While TCP is chemically similar to HA it is partially resorbable and could be used in non-pathological sites where resorbability of the implant would be needed. It is worth noting that a highly resorbable implant could be resorbed before complete new bone formation has occurred (Shors *et al.,* 1989), thus failing to support the new forming bone.

A significant breakthrough was made in the search for osteoconductive delivery vehicles, in a study involving porous hydroxyapatite implants which were implanted heterotopically in rabbits, dogs and baboons (Ripamonti, 1996). After 90 days of implantation it was found that there was minimal

amount of bone that was formed in implants harvested from rabbits and dogs and a substantial amount of bone formed in baboons. This experiment showed that in primates, porous hydroxyapatite implants could induce bone formation without any exogenous addition of osteogenic proteins, i.e. without being used as a delivery system for osteogenic proteins (Ripamonti, 1996). This means that the porous hydroxyapatite implants can intrinsically induce bone formation. With these interesting findings it was clear that more studies should be conducted, in order to understand the mechanisms behind not just osteoconductive properties but also osteoinductive properties of porous hydroxyapatite implants.

1.3.1 Osteoinductive Porous Hydroxyapatite Implants

1.3.1.1 Manufacturing of Hydroxyapatite Implants

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] forms the main mineral component of the bone matrix. It is deposited as spindle shaped crystals along the collagen fibres during embryogenesis (Holmes & Hagler, 1988). Hydroxyapatite implants have been made in such a way that their infrastructure mimics that of the natural bone. Since hydroxyapatite is naturally found in bones it is biocompatible and would not evoke inflammatory response from the recipient thus making it a good delivery vehicle

Some of the hydroxyapatite implants used presently in pre-clinical and clinical studies are derived from corals, the marine invertebrates which secrete calcium carbonate exoskeletons. *Porites* and *Goniopora* mainly grow in groups of interconnected members and are the two coral species that are commonly used because their porous structure is similar to the architecture of bone (White & Shors, 1986). The similarity of these corals' architecture to bone, led to development of a hydrothermal phosphate exchange technique used to convert the calcium carbonate exoskeleton of corals into calcium phosphate (hydroxyapatite) (Roy & Linnehan, 1974).

The two coral species normally used in manufacturing hydroxyapatite are very similar architecturally, but their pore sizes differ. *Porites* species have an average pore diameter of 200 μm and hydroxyapatite implants derived from this coral use a trade name of Interpore 200 (Interpore International, Irvine, CA). *Goniopora* species have an average pore diameter of 600 μm and hydroxyapatite implants manufactured from these corals use a trade name of Interpore 500 (Interpore International, Irvine, CA) (Holmes *et al.,*1986).

1.3.1.2 Hydroxyapatite Implants Intrinsically Inducing New Bone

As already mentioned, it was found that intramuscular implantation of certain porous hydroxyapatite in baboons resulted in new bone formation within the porous spaces and in direct apposition to the hydroxyapatite (Ripamonti, 1991). What was striking about these results is that there was no exogenous application of BMPs on the implant. From a therapeutic perspective, an implant is required for local delivery of rhBMPs to evoke a desired osteogenic response, because it is the composite of a biomaterial carrier together with rhBMPs that triggers the bone induction cascade (Ripamonti & Reddi, 1994). These results (Ripamonti, 1991) indicate that porous hydroxyapatite biomaterials induced bone formation when implanted heterotopically in recipient animals, without any addition of exogenously applied BMPs was surprising. In addition the same results showed that porous hydroxyapatite could intrinsically induce bone formation at least in primate species.

1.3.1.3 Adsorption of Osteogenic Proteins onto the Hydroxyapatite Implants

It has been speculated that the intrinsic osteoinductive properties seen in porous hydroxyapatite, is a result of circulating and/or released extracellular matrix morphogens binding to hydroxyapatite until the

morphogens reach a certain concentration threshold that then triggers new bone morphogenesis as a secondary response (Ripamonti *et al.,*1999). Experiments have shown that BMPs have high affinity to hydroxyapatite, explaining why endogenously produced BMPs could be attracted to hydroxyapatite and bind to it (Ripamonti & Duneas, 1998; Ripamonti, 1996). This is supported by the *in vitro* binding of rhBMP-4 to porous hydroxyapatite, and by rapid generation of bone in porous hydroxyapatites pretreated with BMPs in heterotopic sites of rodents (Ripamonti *et al.,* 1992b) and orthotopic and heterotopic sites in baboons (Ripamonti *et al.,* 1992c; 1993).

Sequential time studies on porous hydroxyapatite harvested at one month (Ripamonti *et al.,* 1992b), two months (Ripamonti *et al.,* 1993), three months (Ripamonti, 1991; 1996), six months and nine months (Ripamonti, 1991) showed that bone differentiation in porous hydroxyapatite occurs by day 60 after implantation. This observation, combined with lack of bone differentiation in specimen harvested on day 30 after implantation, suggests that the initiation of bone formation may depend on a critical concentration of endogenously-produced morphogens adsorbed onto the hydroxyapatite.

1.3.1.4 Hydroxyapatite Implants and Animal Models

When studying osteoinductivity of porous hydroxyapatite, another critical parameter to be considered is the type of animal model used, as demonstrated in several studies (Goshima *et al*., 1991; Miller *et al*.1991; Ripamonti, 1992a; 1996). A study was conducted in which porous hydroxyapatite substrata were implanted in dogs and rabbits, this resulted in minimal bone formation (Ripamonti, 1996). In rats, subcutaneous or intramuscular implantation of porous hydroxyapatite does not initiate bone formation (Goshima *et al.,* 1991; Ripamonti *et al.,* 1989). Previously published experiments in rabbits have also shown lack of bone formation within the porous spaces of hydroxyapatite when implanted intramuscularly (Miller *et al.,* 1991). However, bone differentiation occurred when sintered or coral hydroxyapatites were complexed with BMPs (Ripamonti *et al.,* 1992a; Takaoka *et al.,* 1988).

Compared to dogs and rodents implantation of porous hydroxyapatite in baboons (*Papio ursinus*) intrinsically induced bone formation (Ripamonti, 1991; 1996; Ripamonti *et al.,* 1992a, 1993). The bone physiology of baboons is similar to those of humans, making baboons more suitable models for pre-clinical studies in bone formation (Schnitzler, *et al.,* 1993).

1.3.1.5 Pore Size and Geometry of Hydroxyapatite Implants

The physical parameters of porous hydroxyapatite play a significant role in bone induction. It has been shown that interconnectivity and pore size affect tissue infiltration in the implant. Lack of interconnectivity may lead to incomplete vascularisation of the bone tissue inside the implant, promoting infection and failure of the implant (Holmes *et al.,* 1987). It has also been demonstrated that pore sizes less than 10 μm result in no tissue infiltration, pore sizes between 15 μm – 50 μm result in fibrovascular invasion, pore sizes between 50 μm – 150 μm result in osteoid formation, and pore sizes greater than 150 μm result in mineralised bone ingrowth (Chiroff *et al.,* 1975).

Recently, studies conducted on porous hydroxyapatite physical parameters showed that the geometry and pore size of the implants are of great importance. One of the first studies on importance of hydroxyapatite geometry in new bone formation was conducted on Long-Evans rats. In this study, nonresorbable porous hydroxyapatite in granular and disc configurations of 200 and 500 μm pore sizes were pretreated with bovine osteogenin, and no osteogenin in controls (Ripamonti *et al.,* 1992a). The biomaterials were implanted subcutaneously and harvested after seven, 11 and 21 days. Bone differentiation was observed solely in osteogenin-treated discs as early as seven days after implantation. The osteogenin-

treated discs also showed high alkaline-phosphatase activity, a marker enzyme for osteoprogenitor and osteoblastic cells compared to granular implants. The control implants with no osteogenin did not induce bone differentiation. The most striking discovery in this study was that bone did not form in any of the osteogenin-treated granular hydroxyapatite implants in all pore sizes (Ripamonti *et al.,* 1992a), showing the importance of geometry in hydroxyapatite implants.

Another study (van Eeden & Ripamonti, 1994) was conducted comparing two distinct geometric configurations of porous hydroxyapatite implants having two different pore sizes. The substrata were blocks of hydroxyapatite in rod configuration of 200 μm and 500 μm pore size and granular hydroxyapatite implants (van Eeden & Ripamonti, 1994). The biomaterials were implanted intramuscularly in eight subadult male baboons. Specimens were harvested after 60 and 90 days and subjected to histological and histomorphometric analyses. Bone was only found in blocks of hydroxyapatite in rod configuration at both observation periods. The lack of bone formation in the granular hydroxyapatite indicates the critical role of geometry of the substratum in bone differentiation (van Eeden & Ripamonti, 1994). The convex geometry of granular hydroxyapatite implants did not induce bone formation, while concavities

found in the porous spaces of blocks of hydroxyapatite encourages rapid vascularisation and mesenchymal cell invasion.

In another experiment (Magan & Ripamonti, 1996) hydroxyapatite discs were prepared by cutting the implant either longitudinally or transversally according to the corallite geometry. Implants with different configurations were implanted in calvarial defects of subadult baboons and harvested on day 90 after surgery. Histomorphometric analysis on undecalcified and decalcified sections showed greater amounts of bone formed in porous hydroxyapatite cut in the longitudinal plane when compared with hydroxyapatites cut in transverse plane. These results indicated that the porous configuration of longitudinally prepared discs might be promoting superior osteoconductivity, possibly by enabling faster fibrovascular ingrowth (Magan & Ripamonti, 1996) since angiogenesis is a prerequisite for osteogenesis (Trueta, 1963).

Monolithic discs of sintered hydroxyapatite, fabricated with concavities of 800 μm and 1600 μm diameter in both planar surfaces were implanted in *rectus abdominis* muscles of the baboon (*Papio ursinus*). Histological results on days thirty and ninety revealed bone formation exclusively within the concavities of hydroxyapatite substrata. Subsequently, porous hydroxyapatites were fabricated with porous spaces formed by the

coalescence of repetitive sequence of concavities (Ripamonti *et al.,* 1999). These were sintered in rod and disc configurations for implantation in intramuscular and calvarial sites, respectively. Bone formed in concavities of the substratum 30 days after implantation in the *rectus abdominis* muscles and after 90 days of implantation there was bone morphogenesis associated with bone marrow. Calvarial specimen showed substantial bone formation, culminating in complete penetration of bone within the porous spaces. On day 30, immunolocalisation of BMP family members (BMP-3 and BMP-7) in cellular material at the hydroxyapatite interface suggested that the sintered substratum acts as a solid-state matrix for adsorption of endogenously produced BMPs. These experiments demonstrate intrinsic osteoinductivity of monolithic and porous hydroxyapatites. Furthermore it indicates that the geometry of the substratum profoundly regulates the expression of the osteogenic phenotype (Ripamonti *et al.,* 1999).

1.3.1.6 Chemical Composition of Hydroxyapatite Implants

The chemical composition of hydroxyapatites also plays a major role as it greatly affects its biodegradability (Shors *et al.,* 1989). Biodegradability is a desirable attribute because if biodegradable and resorbable the implant would be ideal for segmental defect repair, as the implant would decrease the effects of residual carrier on the biomechanical properties of repair. The

main challenge is that rapid biodegradation does not give the implant enough time to induce bone formation and support the newly formed bone. Calcium carbonate implants biodegrade rapidly thus segmental defects do not close when treated with these implants (Shors *et al.*1989). It is important that the rate of biodegradation should not exceed the rate of bone morphogenesis. Also the reduction of implant strength should correspond with the increase in new bone strength (Klein *et al.,* 1990).

As already mentioned the exoskeleton of *Goniopora* and *Porites* corals are made up of calcium carbonate, which biodegrades rapidly. Hydroxyapatite is manufactured from these corals by hydrothermal conversion of calcium carbonate to calcium phosphate the latter not degradable. It was found that biodegradable implants could be manufactured by partial conversion of calcium carbonate to hydroxyapatite, limiting the amount of phosphate used in the hydrothermal reaction (Klein *et al.,* 1990). Knowing that hydroxyapatite implants do induce new bone formation, and knowing that the chemical composition of porous hydroxyapatite influences bone induction, this study sheds further light on the initiation of bone induction by porous hydroxyapatite in relation to the implants chemical composition and the duration of the implant within the animal model.

1.4 Aim

To study the expression of *collagen type II, collagen type IV, BMP-3, BMP-7, GDF-10, TGF-β1* mRNAs and the process of new bone formation within the vicinity of hydroxyapatite implants, in relation to the type porous hydroxyapatite implant used and the implantation period.

2 MATERIALS AND METHODS

2.1 Porous Hydroxyapatite Implants

The studied hydroxyapatite implants were manufactured by Interpore International (Irvine, California). These implants were derived from *Goniopora*, a coral species which secretes a calcium carbonate exoskeleton and have an average porosity of 600 μm. The calcareous tissue of the exoskeleton consists of calcium carbonate fibers which can be converted to calcium phosphate (hydroxyapatite) by a hydrothermal reaction with a phosphate (White & Shors, 1986).

This study employed five different types of porous hydroxyapatite implants: 100% hydroxyapatite (100% HA) implants, 5% conversion hydroxyapatite/calcium carbonate (5% HACC), 13% conversion hydroxyapatite/calcium carbonate (13% HACC), sintered hydroxyapatite (SHA) and a peptide-coated hydroxyapatite (P15 HA). All implants used were rods of 20 mm in length and 7 mm in diameter with an average porosity of 600 μm.

100% HA implants:

The 100% HA implants were manufactured by full conversion of calcium carbonate to hydroxyapatite.

5% HACC implants:

When converting the calcium carbonate microstructure in these implants, phosphate was limited in the reaction such that only 5% of the calcium carbonate was converted to hydroxyapatite.

13% HACC implants:

While converting a calcium carbonate microstructure in these implants, phosphate was limited in the reaction such that only 13% of the calcium carbonate was converted to hydroxyapatite

SHA implants:

The SHA implants were prepared by heating up fully the converted hydroxyapatite material to 1100 ºC.

P15 HA implants:

The P15 HA implants were prepared by coating the fully converted hydroxyapatite implants with a synthetic peptide p15, a peptide known to

increase the adhesion of fibroblasts to bone mineral (Qian & Bhatnagar, 1996).

After manufacturing, all the implants were individually packed and sterilised using gamma irradiation.

2.2 Animal Selection and Care

Six clinically healthy female Chacma baboons (*Papio ursinus*), with a mean weight of 21.3 ± 3.5 kg were selected from the non-human primate colony of the University of the Witwatersrand, Johannesburg. The criteria for selection was skeletal maturity, shown radiographically by closure of the distal epiphyseal plates of the radius and ulna (Ripamonti, 1991) and normal haematological and biochemical profiles (Melton & Melton, 1982). Following standard quarantine procedures, the animals were housed individually in suspended wire-mesh cages in the non-human primate unit of the University of the Witwatersrand, 1800 meters above sea level. The rooms of the animals were kept under slight negative pressure (-25 kPa), with controlled ventilation (18 filtered air changes each hour), temperature kept at 22 ± 2 °C, humidity of 40 ± 10% and the photoperiod of 6 am to 6 pm. The baboons diet consisted of balanced protein, fat, carbohydrates with vitamins (thiamine, riboflavin and nicotinic acid) and mineral supplements (calcium: phosphorus = 2:1) and had free access to water. The experiment described in this dissertation have been approved and cleared by the Animals Ethics Screening Committee of the University of the Witwatersrand, Johannesburg (AESC NO. 96/95/5).

2.3 Surgical Procedures and Implantation Design

The baboons were starved the evening before surgery, with access to water *ad libitum*. Before surgery, the baboons were immobilised with an intramuscular injection of ketamine hydrochloride (8 mg/ kg body weight). The baboons were anaesthetised with intravenous sodium thiopentone (15 mg/ kg weight body weight) and maintained by halothane vapour in 100% oxygen after orotracheal intubation. The baboons' abdomens were then shaved and the surgical areas isolated with sterile surgical drapes. Midline incisions were made along the abdominal walls, the skins were reflected and intramuscular pouches were created bilaterally in the *rectus abdominis* muscles of the baboons. Implants were placed in pouches, with two rods of 100% HA, two rods of 13% HACC, two rods of 5% HACC, two rods of SHA, and only one rod of P15 HA substrata implanted heterotopically in each baboon (Figure 2.1). The pouches were then closed by repairing in layers the fasciae and the superficial tissues with atraumatic resorbable sutures (Vicryl, Ethicon NJ). The animals were then given a mixture of benethamine and procaine penicillin intramuscularly. To control pain, they were given buprenorphine hydrochloride (0.3 mg) intramuscularly. The animals were housed individually and were fed a soft diet until they healed. Thereafter they returned to their normal diet as described earlier. All animals healed well with no complications.

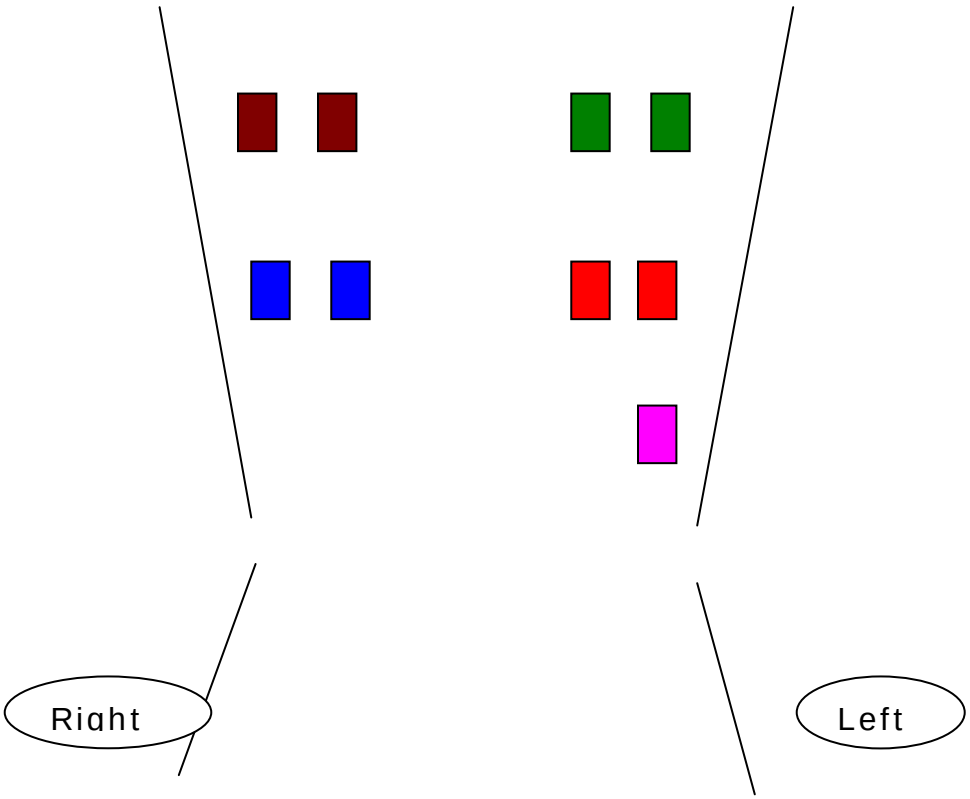


Figure 2-1 Implantation Protocol.

The implants were fabricated as rods, 20 mm in length and 7 mm in diameter. The types of hydroxyapatite implanted in the rectus abdominis muscles of the adult baboons were:

- 100% hydroxyapatite (100% HA).
- 13% conversion hydroxyapatite (13% HACC).
- Sintered hydroxyapatite (SHA).
- 5% conversion hydroxyapatite (5% HACC).
- Peptide-coated hydroxyapatite (P15 HA) (one implant per animal).

2.4 Tissue Harvest

Anaesthetised animals were killed with an intravenous overdose of sodium pentobarbitone on day 60, 90 and 365 post implantation, two animals per implantation period. Implants were harvested and subjected to biochemical, histological, and molecular analyses (Ripamonti, 1991). The post-operative harvesting times were chosen in order to study molecular mechanisms and histological developments within the implants in early stages prior to bone formation and during bone formation.

2.5 Probes Preparation

2.5.1 Amplification of Eschericia coli Constructs

METHOD:

The XL1 Blue strain of *Escherichia coli* (Stratagene) a gift from Professor de Wet, University of the Witwatersrand, Johannesburg) was used as a vector for transformation and cloning. An overnight culture was prepared in *Luria Bertani* (LB) medium [(0.5 % w/v Bacto-yeast extract; 1 % w/v NaCl; Bacto-tryptone (Life Technologies, UK) in deionised water which was autoclaved at 121ºC)]. The bacteria were then grown overnight on LB-agar plates. To make the bacteria competent, the $CaCl_2$ method was followed (Cohen *et al.,* 1972) by picking a single bacterial colony from the LB agar plate and inoculating it into 10 ml LB medium containing tube. This was incubated overnight at 37°C with vigorous aeration. From this overnight incubation 1 ml was taken out and transferred to a sterile 1 litre flask that contained 100 ml of LB medium and it was incubated again at 37°C with vigorous aeration until the OD_{600} was approximately 0.4. The cells were then transferred to a sterile falcon tube embedded on ice. The cells were cooled to 0°C on ice for 10 minutes and were centrifuged at 4000 rpm for 5

minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 30 minutes. The cells were centrifuged again at 4000 rpm for 5 minutes at 4°C. The pellet was resuspended on 2 ml ice-cold 0.1 M $CaCl_2$ for each 50 ml of the original culture, then mixed with glycerol (0.15 ml glycerol: 0.85 ml cells) and stored at -70 ºC prior to use.

2.5.2 Transformation of Eschericia coli.

PROBES USED:

The following probes were used:

Type II collagen cDNA: a 2.2 kb fragment of the gene coding for amino acids 343 of the triple helix to 70 amino acids inside the C-propeptide (Baldwin *et al.,* 1989) cloned into AR5960.

Type IV Collagen-alpha cDNA: the most collagenous domain (Hostikka & Tryggvason., 1988) cloned into Ac2.

BMP-7 cDNA: comprises of 679 bp cloned in p0320 and encodes amino acids 63 to 262 of the pro-region and the first 25 amino acids of the mature polypeptide (Özkaynak *et al*., 1990)

TGF-β1 cDNA: the unmodified wild type of the TGF-β1 precursor cloned into pRK5 (Derynck *et al*., 1985).

BMP-3 cDNA: has 1508 bp EcoRI fragment insert including the full coding region of hBMP-3 (Wozney *et al.,* 1988).

GDF-10 cDNA : cloned into p5k (Cunningham *et al.,* 1995)

Gamma actin cDNA

cDNAs for gamma-actin, collagen type II and collagen type IV were gifts from Professor de Wet, University of the Witwatersrand, Johannesburg.

cDNAs for BMP-7 and TGF-β1 were gifts from Professor Vukicevic, University of Zagreb, Croatia.

The cDNAs for BMP-3 and cDNAs for GDF-10 were gifts from Professor A. Hari Reddi, John Hopkins Maryland, USA.

METHOD:

For transformation, 200 μl of *Eschericia coli* bacterial suspension was transferred to a sterile polypropylene tube. Super coiled plasmid DNA (100 ng) was added to the tube, mixed and stored on ice for 30 minutes. A control was made with competent bacteria that received no plasmid. The cells were then transferred to a water bath at 42ºC for 90 seconds, and back on ice for 2 minutes. LB medium (800 μl) was added to the cells and with gentle aeration they were incubated at 37°C for 45 minutes, allowing the bacteria to express the antibiotic resistance markers encoded by the plasmid DNAs. 100 μl of transformed competent cells were plated onto LB agar plates which contained an antibiotic. The cells were then inverted and incubated at 37°C overnight.

2.5.3 Preparation of Plasmid DNA

A High Pure Plasmid kit Isolation Reagent, (Boehringer Mannheim) was used for this purpose. Plasmid DNA was isolated from *E.coli* (XL1 Blue) by lysing the bacterial cells according to the alkaline lysis method (Birnboim and Dolly, 1979). An overnight culture of transformed bacterial cells was grown in 4 ml of LB at 37°C. Cells were collected by centrifugation at 4300*g* for 5 minutes in a GS3 rotor. The cells were lysed and bacterial RNA

was removed by the addition of RNAse A supplied with the kit. The lysate was neutralised and adjusted to high salt binding conditions after which chromosomal DNA was precipitated and the supernatant, which contained the plasmid was purified from the salts, proteins and other cellular impurities in 10 mM Tris-HCl buffer.

2.5.4 Preparation of Probe Labeled with α-^{32}P

METHOD:

The vector DNA was cleaved using restriction enzymes. Restriction digests of plasmid vectors and molecular weight markers were separated by electrophoresis through 1% (w/v) agarose gel in 1X Tris borate (TBE) buffer at 5 V/cm. The DNA was visualised by staining with ethidium bromide and viewing on a UV transilluminator. The concentration of DNA was determined by the spectrophotometric analysis: an absorbance reading of 1 unit at 260 nm corresponds to a DNA concentration of 50 μg/ml.

DNA, 25 ng, was labeled with α-^{32}P dCTP using a random prime labeling kit (DNA Megaprime labeling kit RPN 1606, Amersham Life Science, UK). The DNA purification kit (QIA quick, QIAGEN Germany, supplied by White

Head Scientific South Africa) was used to remove unincorporated label and the purified probe was used for hybridisation.

2.6 Northern Blot Analysis

2.6.1 Preparation of mRNA

METHOD:

Total RNA was extracted from pooled tissues of replicate 100 mg specimens. All glassware was baked at 250ºC over night and plastic ware autoclaved at 121ºC for 20 minutes. All solutions were prepared with deionised water treated with 0.1% DEPC (ICN Biochemicals Inc. Ohio). The harvested samples which were stored at -70ºC were crushed to fine powder with sterilised utensils pre-cooled at -70ºC. TriPure™ Isolation Reagent (Boehringer Mannheim Biochemicals, Germany) was added to the crushed samples and homogenised with an IKA Ultra-Turrax T025 (Janke and Kunkel, Staifen, Germany) tissue homogeniser at 20000 rpm, the samples were then centrifuged at 4300*g* for 10 minutes at 4ºC in a GS3 rotor. The supernatants were aliquoted in microcentrifuge tubes. 0.2 ml of chloroform was added and the tubes were shaken to allow mixture of the contents, and then incubated at room temperature for 15 minutes. This procedure was followed by centrifugation of the samples at 4300*g* at 4°C for 20 minutes in a GS3 rotor to separate the protein and RNA phases. The upper layer that contained RNA was transferred to new microcentrifuge tubes and 0.5 ml of 100 % isopropanol was added to 1 ml of RNA layer.

Following incubation at room temperature for 15 minutes, the samples were centrifuged again at 4300*g* at 4°C for 20 minutes in a GS3 rotor to pellet the RNA. The supernatant was discarded and the RNA pellet washed with cold 75% ethanol to reduce salt concentration. The ethanol was discarded after 15 minutes using a small-tipped pipette and the RNA pellets were air dried for about 10 minutes. The RNA pellets were resuspended in 20 μl of 1X resuspension buffer (0.5% lauryl sarcosine, 5mM EDTA, pH 8.0). RNA concentration was determined spectrophotometrically at 260 nm by using an extinction coefficient of 1 representing a concentration of 40 μg/ml. The quality of the RNA was judged by the A_{260}/A_{280} ratio of absorbance of the samples, visualization of the RNA bands on agarose gels and by the quality of gamma actin signals on the Northern blots.

2.6.2 Formaldehyde Agarose Gel Electrophoresis

GEL PREPARATION:

A 1.2% (w/v) agarose gel was prepared by mixing agarose powder with 0.1% DEPC-treated water. The solution was heated up to allow homogenization. Concentrated electrophoresis buffer (0.1M MOPS, 40 mM Sodium Acetate, 5 mM EDTA) was added to this solution. Formaldehyde

was added when the solution had cooled down making the final gel concentration of 1.2% (w/v) agarose. The contents were poured onto gel trays and allowed to set for at least 45 minutes at room temperature prior to electrophoresis.

ELECTROPHORESIS:

Preceding electrophoresis, samples of RNA were denatured at 65°C in formamide, formaldehyde and 5 X electrophoresis buffer (0.1M MOPS, 40 mM Sodium Acetate, 5 mM EDTA). Samples were mixed with the gel loading buffer and loaded in the wells of the agarose gel. The 5 X electrophoresis buffer was poured onto gel trays submerging electrodes and the gel, electrophoresis was carried out at 5 V/cm. After electrophoresis the gel was stained with ethidium bromide (Boehringer Mannheim Biochemicals) 0.5 μg/ml in 0.1 sodium acetate for 1hour then de-stained overnight with three changes of DEPC treated water. The gel was placed on an UV transilluminator to visually assess the RNA quality.

2.6.3 Transfer of RNA

RNA TRANSFER PROCEDURE:

RNA was transferred to a nylon membrane (Hybond N+, Amersham, UK) by capillary blotting using Semi-Dry transfer Cell (Biorad) apparatus

specific for Northern blot analysis (Life Science Technologies, UK). The transfer was allowed to proceed for overnight in the presence of 20X SSC (175.3 g Sodium Chloride, 88.2 g Tri-Sodium Citrate, in 1litre of milli Q water treated with 0.1 % diethylpyrocarbonate (DEPC)) (Saambrook *et al.,* 1989). The membrane was baked at 80ºC to effect the crosslinking of RNA to the nylon membrane.

2.6.4 Hybridisation

PRE-HYBRIDISATION AND HYBRIDISATION:

The membranes were prehybridised by immersion into a hybridisation solution (QuickHYB, White Head Scientific) and placed inside the hybridisation oven (Hybridisation oven, Hybaid Corporation, UK) set at 68°C for 40 minutes. In the mean time probes were mixed with sonicated fish sperm DNA (10 mg/ml) and boiled for two minutes to denature the DNA. Then prehybridised membranes, together with the probes were put in 50 ml falcon tubes, with a mesh between the tube and the membrane. Hybridisation was allowed to proceed for two hours in the hybridisation oven at 68°C. The membranes were washed twice for 15 minutes at room temperature with 150 ml of 2 X SSC, 0.1 % SDS followed by the stringency wash at 68°C with 0.1 X SSC, 0.1% SDS. The membranes were dried on the blotting paper and covered with a Saran wrap. The hybridised

membranes were exposed to Kodak film (Biomax MS) with intensified screens 15 to 75 hours at -70˚C. Signals were quantified relative to gamma actin blots by densitometric analysis (Gel documentation and Analysis system, Syngene, U.K) (Matsaba *et al.,* 2001).

2.7 Histology

Harvested tissues were fixed in 10% phosphate buffered formaldehyde (pH 7.4) and demineralised in formic acid-hydrochloric acid solution (Molnar, 1975). The end of decalcification was marked by the absence of a precipitate when 1ml of ammonium oxalate was added to 5ml of the decalcifying solution. Decalcified specimens were processed using a celloidin paraffin wax double embedding procedure (Bancroft & Stevens, 1996). Specimen blocks were sectioned on a Microm HM360 (Microm Laborgerate GmbH, Walldorf, Germany) rotary microtome with a section transfer system. Serial sections cut at 5µm were picked up onto formaldehyde/gelatin coated glass slides to minimise section detachment during staining. Sections were stained using a modified Goldner's Trichome method outlined below (Ripamonti *et al*., 1997; Duneas *et al*., 1998; Ripamonti *et al*., 1999; Ripamonti *et al*., 2000). Sections were analysed in an Olympus Provis AX70 research light microscope (Olympus, Tokyo, Japan) at a 10X magnification.

2.7.1 Method (Modified Goldner's Trichome)

Sections were stained in stable iron haematoxylin for 20 minutes, Fuschin Ponceau for 45 minutes, differentiated in Orange G for 20 minutes then stained in methyl blue for 10 minutes. In between the stains, sections were washed in running tap water, differentiated in acid alcohol and immersed in 1% acetic acid. The sections were then dehydrated in series of alcohol and mounted with entellan (Merck, Germany).

The difference between the original Goldner's stain and the modified method is the counterstaing. The original method counterstains with a green stain while the modified method counterstains with a blue stain so everything that is green representing mineralized bone in the original method will appear blue in the modified method. The blue stain representing mineralised bone was chosen for photographic purposes. Bone is differentiated from the dense connective tissue and any other tissue that make the blue stain by the fact that bone has osteocytes; this is a characteristic feature that all the other tissues in the study do not have. Morphologically, bone stains blue on decalcified sections (with modified Goldner's Trichome) as matrix filled with osteocytes and osteoblastic cells is often highly vascularised.

2.7.2 Histomorphometric Analysis

Specimen sections were subjected to histomorphometric analysis as described by Ripamonti (1991). A square Zeiss Intergration Plate II (Zeiss, Orberkochen,Germany) with 100 lattice points and a total area of 7.84 mm^2 was used to calculate the volume fraction composition of tissue components with the point counting technique (Parfitt, 1983). The technique computes fraction volumes in percent of histological components including bone, soft tissue and the hydroxyapatite substratum.

2.8 Statistical Analysis

The data were analysed using the GraphPad Prism ™ Version 2.0 (GraphPad Software Inc, San Diego, CA). A two-way analysis of variances test was used to determine the differences in expression of selected genes in respect to time and type of porous hydroxyapatite used. The level of significance used was $p < 0.05$.

3 RESULTS

Unlike *Collagen type IV, BMP-7* and *GDF-10* mRNAs, It was found that *Collagen type II, BMP-3* and *TGF-β1* mRNAs were not expressed at detectable levels in all the samples at all time periods. The intensity of the expression of *Collagen type IV* mRNA, *BMP-7* mRNA and *GDF-10* mRNA were normalized as percentages against gamma-actin intensity levels (in Densitometric Units), the Relative Densitometric Units (RDU) of these mRNAs in all implants were as follows:

Collagen Type IV mRNA expression

There was similarity in expression patterns of *Collagen type IV* mRNA (Appendix A) in all five types of hydroxyapatite implants. At two months *Collagen type IV* was highly expressed in all of the hydroxyapatite implants: range 0.74 to 0.98 relative densitometric units (RDU) (Figure 3-1). These expression levels decreased at three months (range 0.32 – 0.94 RDU). The levels of *Collagen type IV* mRNA at three months in P15 HA were lower than the levels detected in other implants. However, this difference was not statistically significant. There was a further statistically significant reduction in *Collagen type IV* expression at 12 months in all the implants (ranging from 0.05-0.20 RDU).

BMP-7 mRNA expression

All of the five different types of hydroxyapatite studied showed relatively high expression levels of *BMP-7* mRNA at two months (range 0.94-0.99 RDU) (Figure 3-2) . These expression levels decreased at three months (range 0.14-0.36 RDU) and at 12 months there was a further reduction of *BMP-7* mRNA levels (range 0.06-0.12 RDU) (Appendix B).

GDF-10 mRNA expression

The levels of *GDF-10* mRNA at two months (range 0.17- 0.3 RDU) were low relative to the mRNA levels of *Collagen type IV* and *BMP-7* at two months (Figure 3-3; compare Figures 3-1 and 3-2). The levels of *GDF-10* mRNA (Appendix C) decreased at three months (range 0.04- 0.18 RDU) (Figure 3-3) and were decreased even more at 12 months (range 0-0.12 RDU). The SHA expressed no detectable *GDF-10* mRNA at 12 months. It was surprising to find that the *GDF-10* mRNA levels at 12 months in 13% HACC implants were higher than expression levels at three months. However, the *GDF-10* message was lower at three months than at two months showing a fluctuating pattern. However, there was no significant difference in the expression of *GDF-10* mRNA between all studied implants.

The ANOVA test indicates that there is no significant difference in the variability of the levels of mRNA (*Collagen type IV, BMP-7 and GDF-10*) expressed in the five different types of hydroxyapatite implants studied ($p < 0.05$). A significant difference was found in the level of mRNA expressed and the duration of sample implantation ($p << 0.01$). The mRNA expression levels generally decreased with time.

Histology

The decalcified sections shown from Figure 3-4 to Figure 3-8 illustrating that at two months (A) there was invasion of dense, highly vascularised connective tissue within the porous spaces of the hydroxyapatite implants, with fibrous connective tissue aligned at the hydroxyapatite interface. At three months (B) the figures show that there was a loose cellular connective tissue which was highly vascularised with denser fibres at the implant interface. Most importantly there was no new bone formed at two and three months in all five types of porous hydroxyapatite implants.

The results of the specimen harvested at 12 months are shown in panel C in figures 3-4 through to figure 3-8. These specimens showed substantial amounts of new bone formed (indicated by arrows in figures) within all types of porous hydroxyapatite used. The new bone was formed

peripherally, at the hydroxyapatite implant interface as profoundly shown in figure 3-6 (c).

Histomorphometry

Histomorphometric results of the specimen are summarized in Table 3-1 (Crooks, 2001). The results showed that at two and three months there was no bone in all implants and no significant difference between the implants. Substantial amount of bone was formed at 12 months. There was no significant difference in the amount of bone formed between the different implants that were studied.

Figure 3.1 A graphical representation of normalised levels of Collagen type IV mRNA as measured by densitometry and Northern blots in the lower panel.

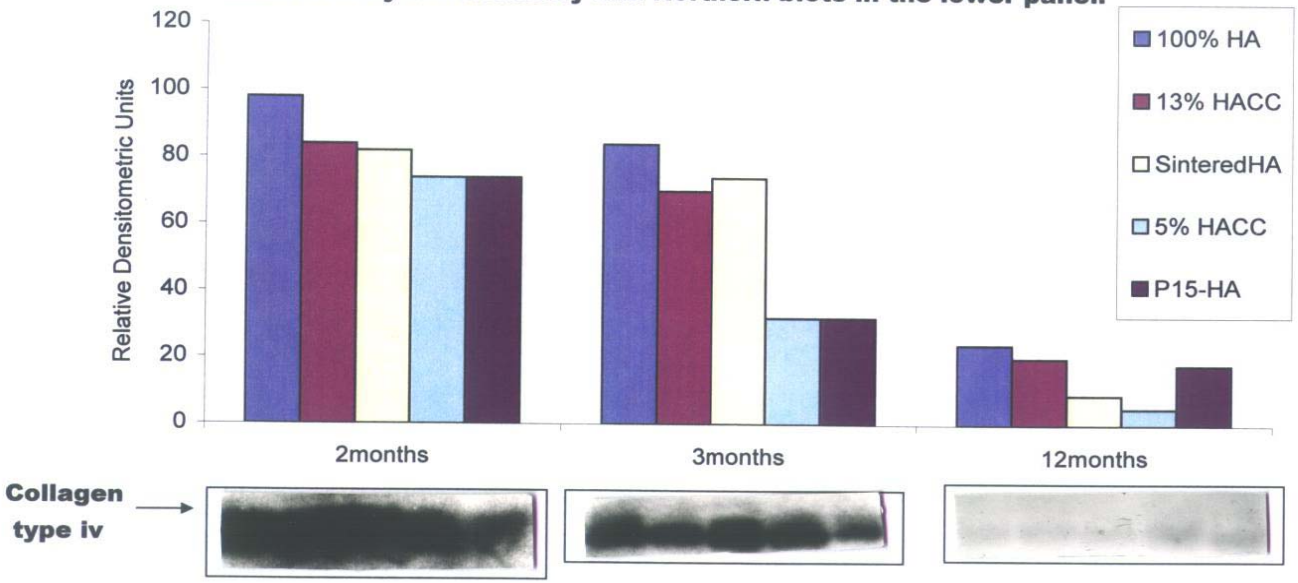


Figure 3.2 A graphical representation of normalised levels of BMP-7 mRNA transcripts as measured by densitometry and the Northen blots in the lower panel

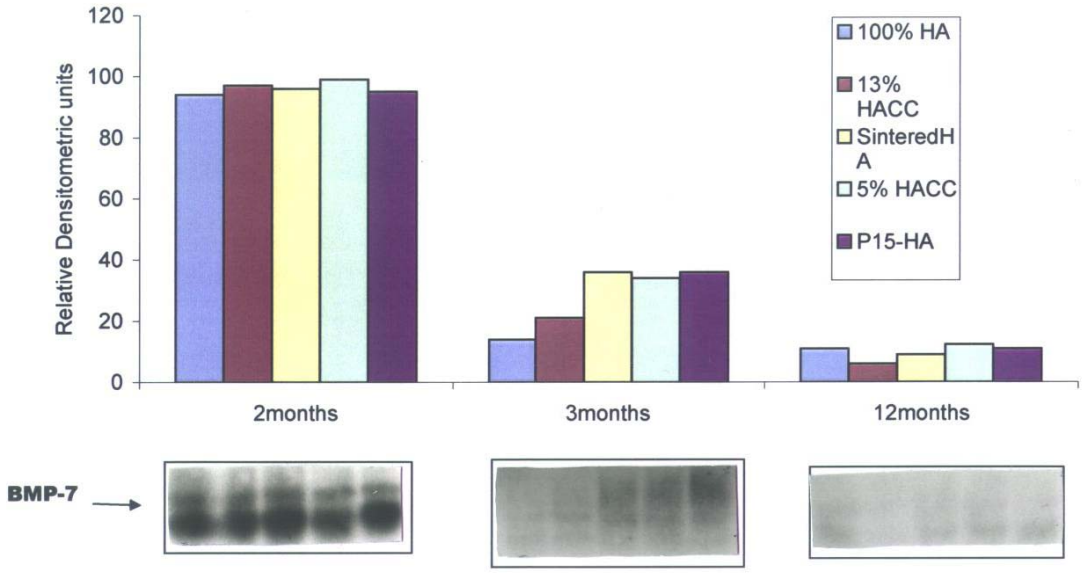


Figure 3.3 A graphical representation of normalised levels levels of GDF-10 mRNA transcripts as measured by densitometry and the northern blots in the lower panel

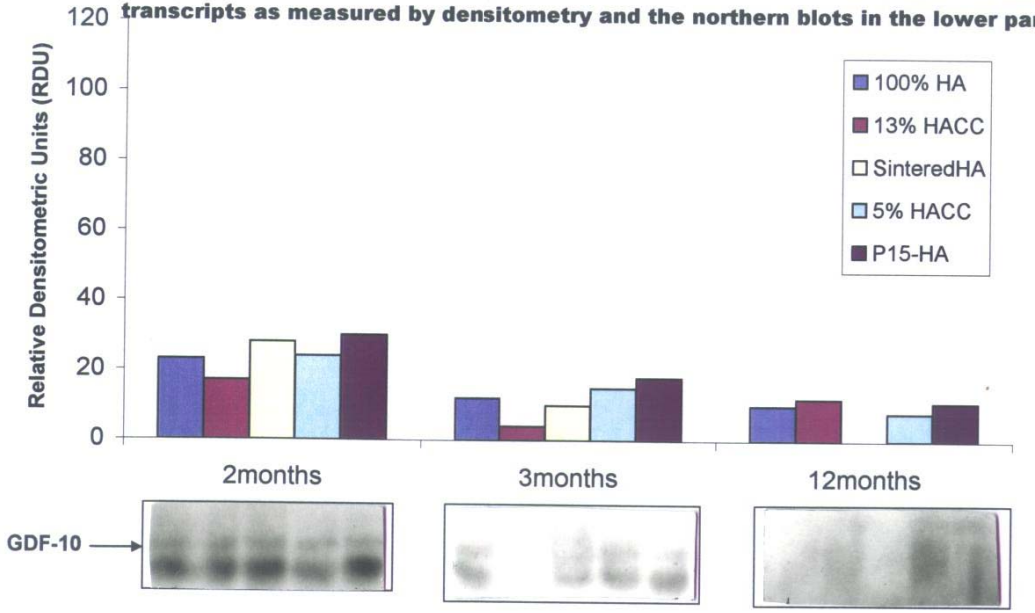
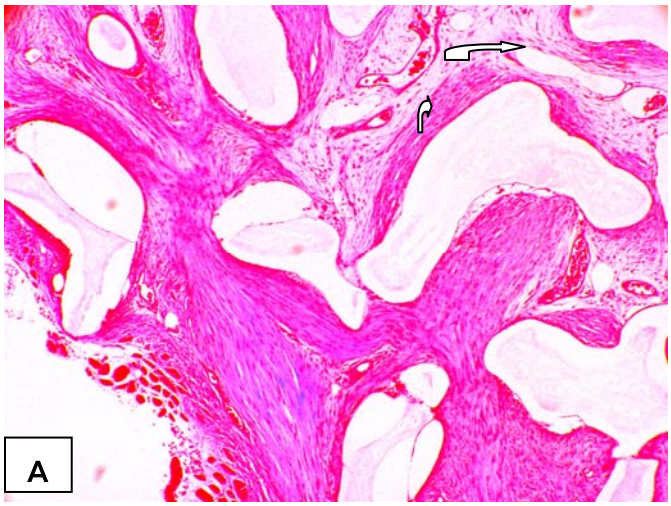


Figure 3-1 Photomicrographs of various stages of bone induction within fully converted hydroxyapatite (100 % HA) implants.

The 5 μm thick decalcified sections were stained with modified Goldner's Trichome (x10 magnification). (A and B) are sections from implants harvested at two and three months respectively, showing blood vessels (bent open arrows). (C) Sections from implants harvested at 12 months, mineralised bone is stained blue (arrows).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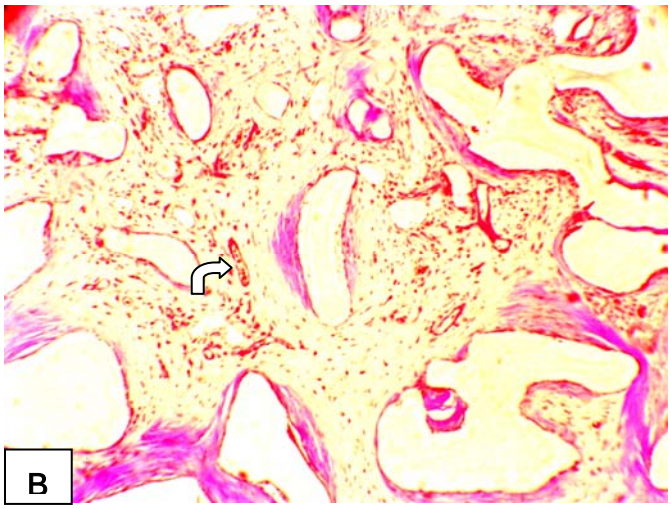

B

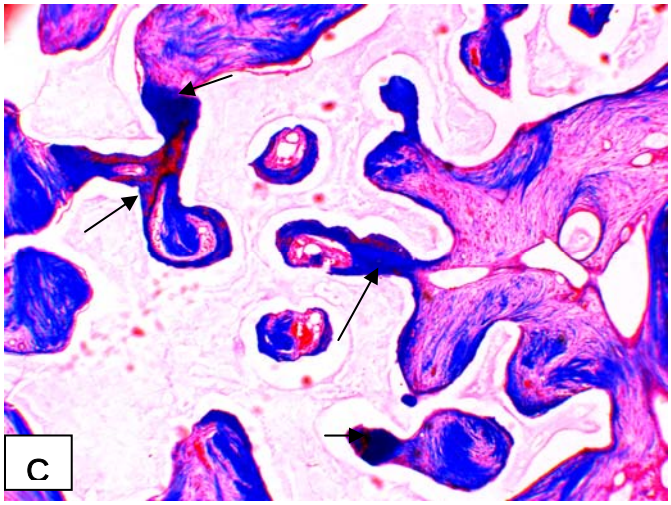
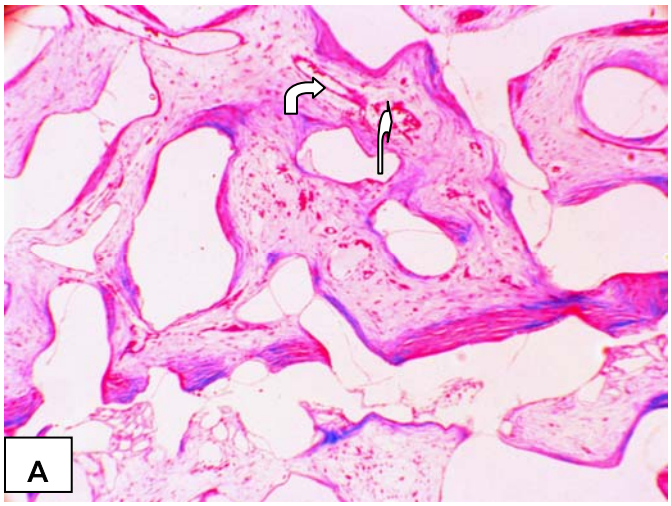

C

Figure 3-2 Photomicrographs of various stages of bone induction within partially converted (5 % HA) implants.

The 5 μm thick decalcified sections were stained with modified Goldner's Trichome (x 10 magnifications). (A and B) are sections from implants harvested at two and three months respectively, showing blood vessels (bent open arrows). (C) Sections from implants harvested at 12 months, mineralised bone is stained blue (arrows).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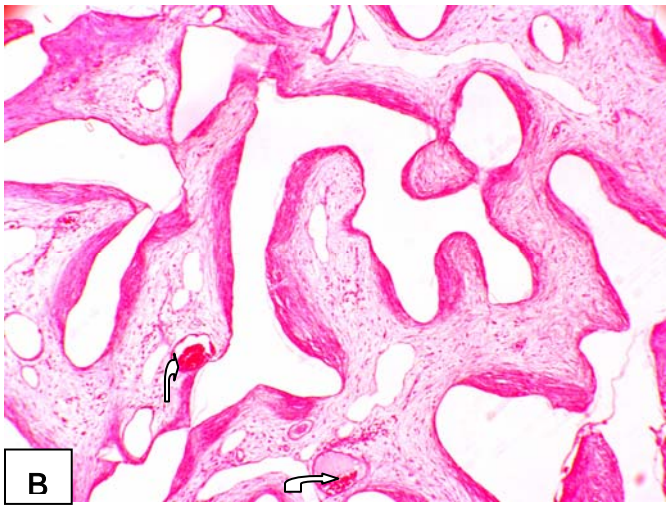
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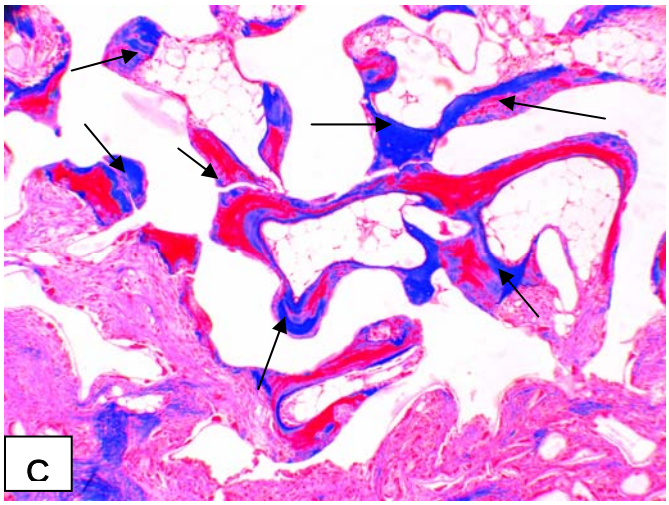
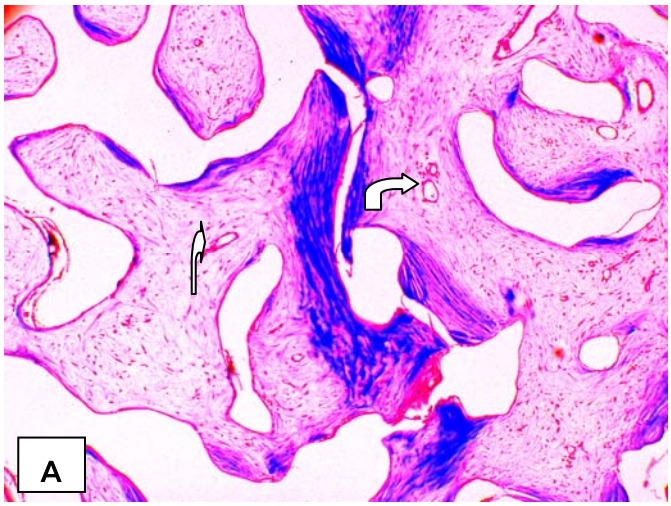

C

Figure 3-3 Photomicrographs of various stages of bone induction within partially converted 13 % HACC implants.

The 5 μm thick decalcified sections were stained with modified Goldner's Trichome (x 10 magnifications). (A and B) are sections from implants harvested at two and three months respectively, showing blood vessels (bent open arrows). (C) Sections from implants harvested at 12 months, mineralised bone is stained blue (arrows).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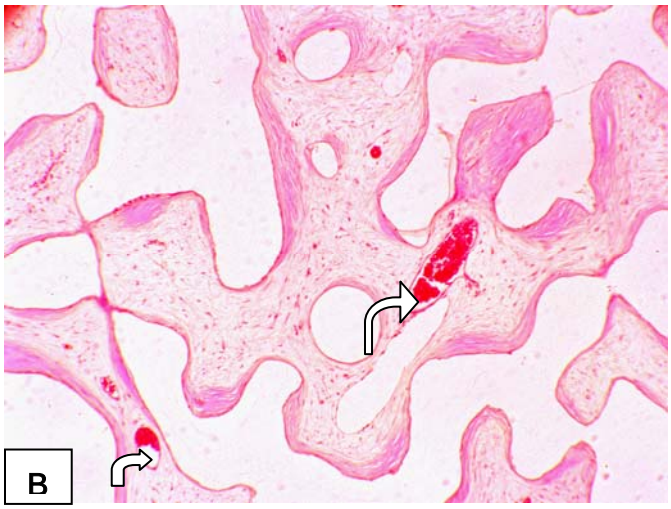
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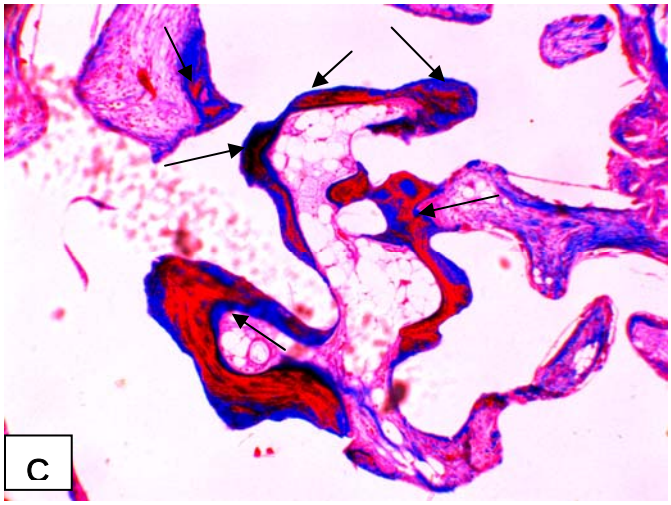
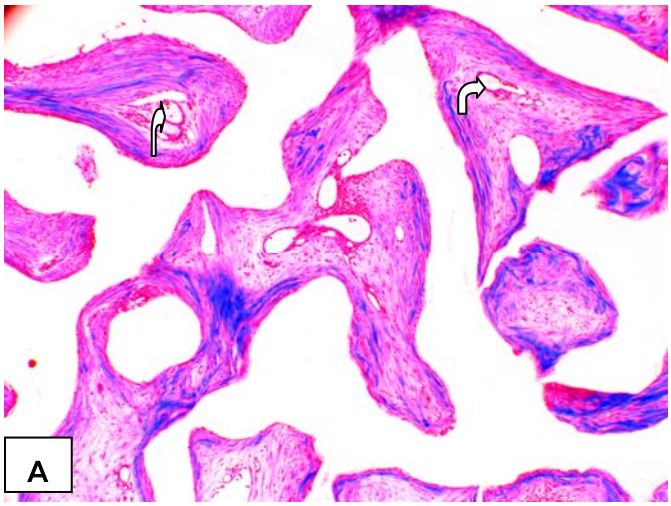

C

Figure 3-4 Photomicrographs of various stages of bone induction within sintered hydroxyapatite (SHA) implants.

The 5 μm thick decalcified sections were stained with modified Goldner's Trichome (x 10 magnifications). (A and B) are sections from implants harvested at two and three months respectively, showing blood vessels (bent open arrows). (C) Sections from implants harvested at 12 months, mineralised bone is stained blue (arrows).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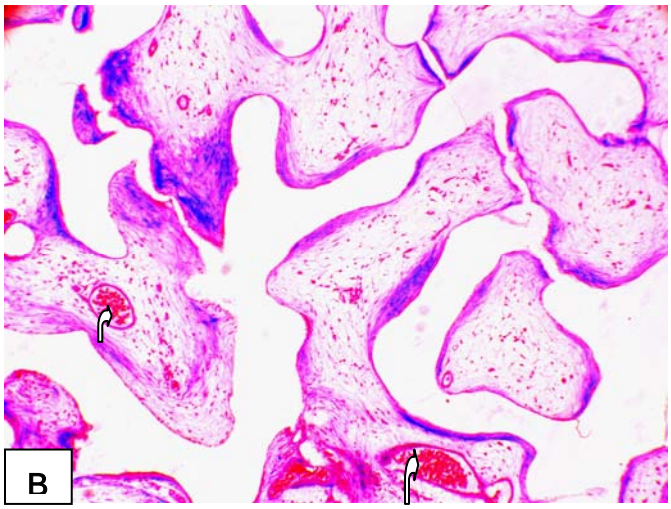
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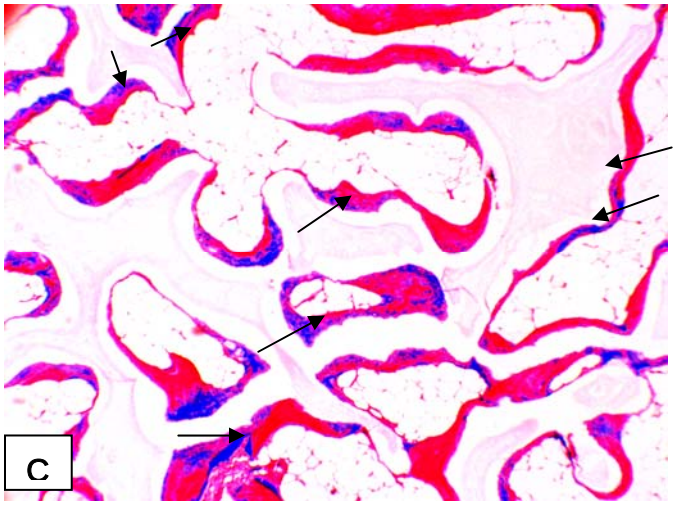
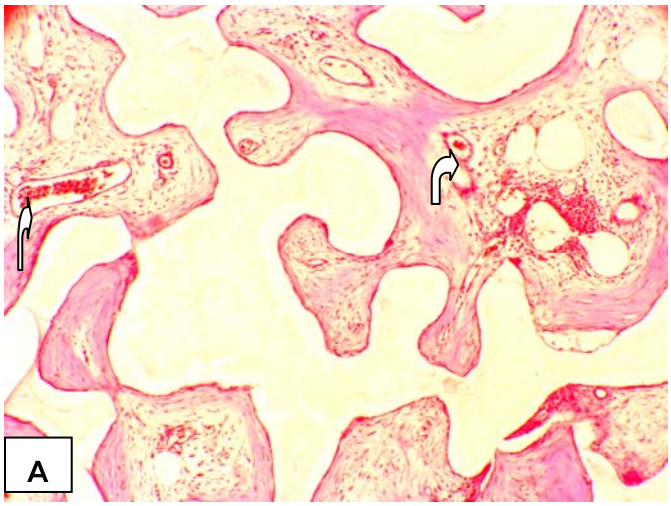
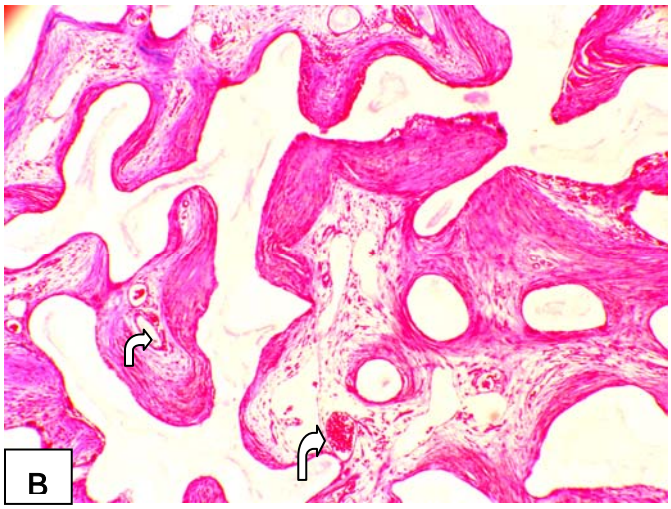

C

Figure 3-5 Photomicrographs of various stages of bone induction within hydroxyapatite implants coated with p-15 (P15 HA).

The 5 μm thick decalcified sections were stained with modified Goldner's Trichome (x 10 magnifications). (A and B) are sections from implants harvested at two and three months respectively, showing blood vessels (bent open arrows). (C) Sections from implants harvested at 12 months, mineralised bone is stained blue (arrows).


A


B

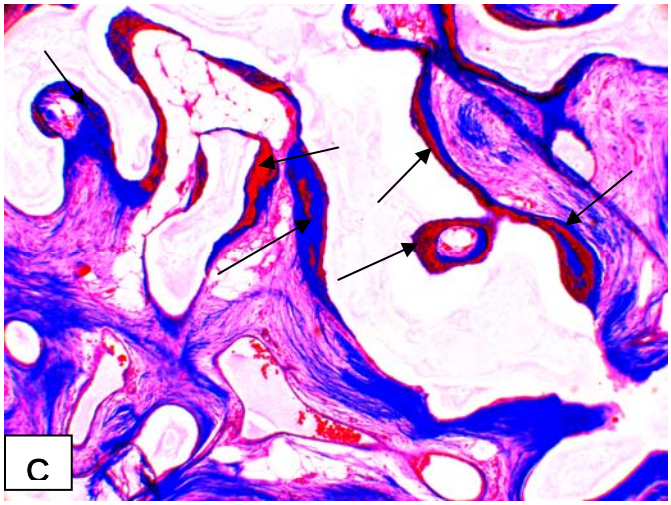

C

Table 3-1 Volume fraction composition (%) of decalcified heterotopic specimens of types of hydroxyapatite harvested at two, three and 12 months.

Treatment	Bone	Fibrovascular Tissue	Substratum
2 months			
100% HA	0.88	65.63 ± 1.10	36.50 ± 1.21
SHA	0.00	68.25 ± 1.53	33.75 ± 1.54
5% HACC	0.00	69.25 ± 1.87	27.50 ± 0.95
13% HACC	0.00	62.75 ± 3.03	31.25 ± 0.86
P15 HA	0.00	68.00 ± 2.15	29.00 ± 1.79
3 months			
100% HA	0.00	64.63 ± 2.16	35.38 ± 2.16
SHA	0.00	70.50 ± 1.35	29.50 ± 1.35
5% HACC	0.00	69.25 ± 2.11	30.75 ± 2.11
13% HACC	0.00	64.13 ± 1.36	35.87 ± 1.36
P15 HA	0.00	63.25 ± 1.47	36.75 ± 1.47
12 months			
100% HA	23.50 ± 4.29	43.00 ± 2.17	33.50 ± 2.31
SHA	17.75 ± 6.82	48.75 ± 5.82	33.50 ± 2.00
5% HACC	8.88 ± 3.55	51.75 ± 4.58	31.25 ± 6.04
13% HACC	25.13 ± 2.41	42.37 ± 3.17	32.50 ± 2.92
P15 HA	29.50 ± 1.54	43.38 ± 2.11	27.13 ± 1.74

Volume fractions of tissue components were calculated using Zeiss Intergrated Platte II with 100 lattice points superimposed over 4 sections per specimen. Substratum indicates the implanted framework of types of hydroxyapatite, having n=4 for all the substratum except p15 HA where n=2. Values (in percentage) are means ± standard error of the mean (Crooks, 2001).

4 DISCUSSION

To shed further light on the molecular signals that trigger intrinsic osteoinduction by heterotopically implanted porous hydroxyapatite (HA), the expression of osteogenic markers was studied. Different types of HA were implanted in the *rectus abdominis* muscles of the baboons and harvested at different time periods. To relate the different expression patterns of the osteogenic markers and morphological changes within the implants histological sections of the specimens were also studied.

Collagen type IV is a major constituent of vascular basement membranes. Previous studies have shown that one of the prominent features of osteoinduction at one and two months is the angiogenic response surrounding and penetrating the porous hydroxyapatite substrata (Ripamonti *et al.,* 1993). The study of laminin and collagen type IV during angiogenesis in endochondral bone formation indicates that there is a close relationship between vascular invasion and subsequent bone formation (Foidart & Reddi, 1980). Trueta (1963) has shown that angiogenesis and vascular invasion are prerequisites for osteogenesis, since blood supply is important for delivery of oxygen, nutrients and bone forming morphogens to the induction site (Reddi, 2000; Geber & Ferrare, 2000; Colnot & Helms, 2001). The high expression of *collagen type IV* message, a marker of angiogenesis (Foidart & Reddi, 1980; Ramoshebi &

Ripamonti *et al*., 2000) at two and three months in this study suggests high angiogenic response, these results corresponds with histology showing blood vessels within the porous spaces of HA implants. The down regulation of *Collagen type IV* message at 12 months corresponds with maximum bone formation, suggesting a biofeedback mechanism that regulates the amount of blood vessels formed during osteogenesis.

There was no detectable expression of *Collagen type II* message, a marker of chondrogenic phenotype. This is consistent with the observation that bone formation occurring in porous hydroxyapatite implants does not proceed *via* chondrogenic phase (Ripamonti, 1991; Ripamonti *et al*.,1999; 2000;and the histological results in this study), possibly because of the implants geometry which allow rapid vascularisation that occurs at early stages of bone formation (Ripamonti *et al.,* 1999). Cartilage is an avascular tissue and vascularisation marks the onset of its degradation (Trueta, 1963). The histological results in this study show that from as early as two months there was substantial vascularisation, and no chondrogenic phase as cartilage is avascular.

The expression of *BMP-7* mRNA in all HA types were high at two months, dropping by three months and further reduced at 12 months. The osteoinductive properties of BMP-7 have been observed in several

experiments. It has been reported that implantation of recombinant BMP-7 along the lamina and facets of canines induced a solid interlaminar fusions by 12 weeks, as compared to 22 weeks when using autologous bone grafts (Cook *et al.,* 1994). Implantation of high doses of BMP-7 in calvarial defects in another study induced massive bone differentiation at day 30 (Ripamonti & Reddi, 1994), showing the importance of BMP-7 in new bone formation and how exogenous application of BMP-7 within implants accelerates new bone formation. BMP-7 also has angiogenic properties (Ramoshebi & Ripamonti, 2000). Supporting this statement are ossicles which when induced with BMP-7 and TGF-β1 expressed several fold increase in *type IV Collagen* mRNA expression, showing a synergistic interaction of TGF-β1 and BMP-7 to induce vascular invasion (Duneas *et al.,* 1998). In the present study expression of *BMP-7* message in the cells invading the implants shows that in early stages it is highly expressed and its expression fades with time. The expression of *BMP-7* message is critical in angiogenesis and osteoinduction mechanism and it is important that these mechanisms are regulated temporally.

The *BMP-3* transcripts were not expressed in the cells invading the porous spaces of hydroxyapatite substrata, suggesting adsorption of the protein from the circulatory system. Previous studies conducted on BMP-3 shows that this morphogen has high affinity for Collagen type IV (Paralkar *et al.,*

1990). Also, adsorption of BMP-3 onto hydroxyapatite resulted in osteogenesis within porous spaces of the substrata when implanted heterotopically in rodents (Ripamonti *et al.,* 1992b). When purified BMP-3 was adsorbed to porous hydroxyapatite and implanted orthotopically in baboons, extensive bone induction was reported (Ripamonti *et al.,* 1992c). The absence of *BMP-3* mRNA expression in the present results can be explained by the possibility that *BMP-3* are not being locally expressed but binding to the implants from the circulation.

The *GDF-10* transcripts in this study were expressed at low levels compared to expression of *BMP-7* and *Collagen type IV* transcripts (figure 3-3). Even though the *GDF-10* mRNA transcript levels were low at two months there was still a further decrease observed at three months and even a greater decrease at 12 months indicating that it is temporally regulated. However in 13% HACC implants at 12 months, the *GDF-10* transcription level was higher than that detected at three months but lower than the *GDF-10* transcription level at two months. As this rise at 12 months was not detected in other studied implants, it is suspected that these fluctuating transcription levels are related to the type of implant used, perhaps a longer study would show significant difference in the amount of bone formed between the other HA implants and 13% HACC. The fluctuating transcription levels may also be related to other growth factors

that are expressed within the 13% HACC implant or adsorbed to this particular substratum.

GDF-10 was found to be closely related to BMP-3, and strongly expressed in both neonatal and adult flat bones, with lower levels detected in bones of endochondral origin such as the femur (Cunningham *et al.,* 1995). A recent study showed that although *GDF-10* transcripts were expressed in ossicles with chondrogenic phase, its expression was greater in ossicles in which osteogenesis was not *via* chondrogenic phase but was intramembranous (Ripamonti *et al.,* 2000). In the present study, osteogenesis in all five different types of porous implants was intramembraneous and thus the expression of *GDF-10* clearly seen. Temporal regulation of *GDF-10* is also demonstrated in this study.

TGF-β1 transcripts were not detected in the studied porous substrata. TGF-β1 protein is known to have high affinity to collagen type IV (Paralkar *et al.,* 1991). In previous studies, TGF-β1 induced endochondral bone formation when implanted extraskeletally in baboons (Ripamonti *et al.,* 1997; Duneas *et al.,* 1998). Endochondral bone formation could also be accelerated by binary application of TGF-β1 with BMP-7 heterotopically and orthotopically in baboons (Duneas *et al.,* 1998). The lack of *TGF-β1* expression in the cells that invade the porous spaces of the implants does

not dismiss the evidence that TGF-β1 plays a major role in osteogenesis. However, the lack of detection of TGF-β1 message expression suggests that it is not locally expressed but may bind to the implant from the circulation, considering the affinity of TGF-β1 to collagen type IV.

There was a high inverse correlation between the levels of mRNA expressed and time of harvest. It is possible that the decrease in mRNA transcripts of *BMP-7*, *collagen type IV* and *GDF-10* as the time period increases is caused by synthesis of the protein these transcripts encodes. As more protein is synthesised to initiate bone formation as seen in histological sections there could be a negative feedback mechanism that inhibits production of more mRNA transcripts. Another possibility could be the activation of the morphogens inhibitors such as Smads, in response to the synthesised proteins and new bone formed. This could also be triggered by progression of osteogenesis into marrow formation.

The histological results of these specimens (Figure 3-4, Figure 3-5, Figure 3-6, Figure 3-7, Figure 3-8) indicates that at two and three months there was no bone formed in all the implants. However, at 12 months there was substantial bone formed in all forms of hydroxyapatite implants studied (Table 3-1). These histological results support the lack of difference in the expression of osteogenic markers between substrata, and also support the

significant difference that the period of implantation makes in the expression of these markers. It is also interesting that the expression of the osteogenic markers within the implants is reduced with time, suggesting that new bone will not grow *ad infinitum* but will be regulated with time. It should be noted that this study employed only a small number of specimens which could affect statistical interpretation of the results.

5 CONCLUSION

The results of this study show that the expression of *Collagen type IV*, *BMP-7* and *GDF-10* mRNAs are temporally regulated in all osteoinductive hydroxyapatite implants, having the highest expression at two months and the lowest expression levels at 12 months when there is substantial amount of bone formed within the implants. This temporal regulation may be in response to the amount of bone formed within the implants, triggering some negative feedback mechanism which works through inhibitory Smads proteins so as to control the amount of new bone to be formed. It is possible that osteogenic markers *BMP-3* and *TGF-β1* were not expressed within the implanted HA but that proteins are recruited and bind to the porous HA from the circulatory system. Further analysis such as western blot technique are needed to shed further light on the molecular mechanisms involved in the intrinsic osteoinductive properties of the porous hydroxyapatite implants.

6 APPENDIX A

Table A. Mean Values and Standard Deviations from normalized Relative Densitometric Units of Collagen type IV signal levels expressed within different types of hydroxyapatite harvested at different periods.

Type of implant		2 Months	3 Months	12 Months
100% HA	Mean	**98.025**	84.800	24.150
	SD	**8.108**	**18.780**	**3.515**
	N	**4**	**4**	**4**
13% HACC	Mean	84.350	70.300	20.350
	SD	2.914	12.465	1.136
	N	4	4	4
SH	Mean	82.950	74.825	9.550
	SD	3.511	4.421	3.979
	N	4	4	4
5% HACC	Mean	74.125	32.800	5.400
	SD	3.966	13.965	3.405
	N	4	4	4
P15-HA	Mean	74.300	32.000	18.150
	SD	0.141	1.414	1.626
	N	2	2	2

7 APPENDIX B

Table B. Mean Values and Standard Deviations from normalized Relative Densitometric Units of BMP-7 signal levels expressed within different types of hydroxyapatite harvested at different periods

Type of implant		2 Months	3 Months	12 Months
100% HA	Mean	94.500	14.750	11.725
	SD	6.758	7.095	4.903
	N	4	4	4
13% HACC	Mean	97.850	21.150	6.900
	SD	9.774	4.122	1.378
	N	4	4	4
SH	Mean	96.500	36.650	9.000
	SD	5.745	12.891	7.703
	N	4	4	4
5% HACC	Mean	99.375	34.700	12.825
	SD	9.446	15.200	1.558
	N	4	4	4
P15-HA	Mean	95.150	36.800	11.050
	SD	11.452	16.380	2.557
	N	2	2	2

8 APPENDIX C

Table C. Mean Values and Standard Deviations from normalized Relative Densitometric Units of GDF-10 signal levels expressed within different types of hydroxyapatite harvested at different periods.

Type of implant		2 Months	3 Months	12 Months
100% HA	Mean	23.425	12.850	10.750
	SD	2.741	1.134	1.210
	N	4	4	4
13% HACC	Mean	17.250	4.875	12.250
	SD	2.646	1.625	3.500
	N	4	4	4
SH	Mean	28.975	10.550	0.025
	SD	1.401	2.822	0.009
	N	4	4	4
5% HACC	Mean	24.475	15.150	8.000
	SD	4.818	1.617	1.826
	N	4	4	4
P15-HA	Mean	30.700	18.200	11.250
	SD	0.990	1.697	3.182
	N	2	2	2

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