On-Demand Autograft:

A Novel Method of Biomaterial-Induced Bone Growth

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Dedicated to my loving wife, Mai, and my darling daughter, Jena

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Abstract

Background

Reconstruction of craniofacial skeletal defects represents a challenge to plastic surgeons. Current craniofacial surgery techniques include autogenous, allogeneic and prosthetic materials. While reconstruction with the use of autologous bone graft remains the preferred approach, autografts are clinically limited by donor supply and are associated with significant donor site morbidities. Synthetic implants avoid these issues, but their efficacy is limited by the risk of growth restriction, infection, and unsatisfactory aesthetic outcomes. This study investigates the use of the calcium-phosphate phase monetite, to induce and augment bone growth on the skull as an onlay graft, which can then be excised and transplanted into a defect.

Methods

A porous monetite implant was prepared by autoclaving brushite. Twenty-five adult male Wistar rats were divided into 5 groups (N=5/group). In the group of interest, named the Transplanted Onlay (TO) group, the implant was placed directly on to area overlying the lambda, and kept in place for 8 weeks. After that, it was excised and transplanted into a 8mm defect anteriorly (overlying the bregma), which was created immediately prior to implant excision. It was then kept in the defect for 8 weeks. In the second group, named the Direct Placement (DP) group, an 8mm diameter defect was created in the superior portion of the skull, between the Lambda and Bregma cranial sutures. The implant was placed directly into this defect and kept in place for 8 weeks. In the 3rd group, named the Subcutaneous Transplant (ST) group, the implants were

placed for 8 weeks in subcutaneous pockets, following which they were excised and placed into newly created skull defects. This group served as a control for the previous group, as they were not attached to bone, and were only exposed to the subcutaneous environment. The remaining 2 groups consisted of negative and positive controls respectively, using animals that had empty (untreated) skull defects, and animals with skull defects that had been treated with calvarial bone autografts. New bone growth as well as implant-host bone integration was studied with scanning electron microscopy (SEM), micro CT, and histology. Mechanical testing was performed on the explanted grafts to assess load-bearing strength.

Results

Micro CT imaging with new bone volume calculation confirmed that the negative controls did not heal (8.8 ± 1.3% new bone formation), thereby demonstrating the 8mm defect to be a true "critical size defect". The autograft samples displayed minimal healing (34 ± 5.7%). Transplanted onlay (TO) samples yielded the highest rates of new bone formation (74 ± 3.1%), while direct placement (DP) implants and subcutaneous transplants (ST) showed comparable rates of new bone formation (61 ± 2.7% and 60 ± 2.8%, respectively). The formation of new bone was further confirmed by histomorphometric analysis, which confirmed that the radio-opaque graft and new bone could indeed be distinguished reliably, and that the highest amount of new bone growth had occurred in the Transplanted Onlay group (6.2 ± 0.3 mm^2), compared to lower rates in the autograft (AG), direct placement (DP), and subcutaneous transplant (ST) groups (3.1 ± 0.5 mm^2, 4.1 ± 0.4 mm^2, and 4.2 ± 0.3mm^2, respectively). SEM confirmed the presence of new bone in this group. Furthermore, preliminary work indicated that samples in this

group were able to withstand greater load forces upon mechanical testing and functional assessment. All results were statistically significant ($P < 0.05$).

Conclusion

Despite advances in contemporary treatment strategies, donor site morbidity and suboptimal restoration of form and function continues to drive the development of novel approaches to treat calvarial defects. This study proposes novel methods of inducing native bone to locally produce excess bone in a controlled manner. Our data show that bone was successfully grown within a micro-porous monetite onlay without the use of growth factors or genetic manipulation. This excess bone could be excised and used as "on demand" autologous transplants to treat critical-size skull defects. This technique has the potential to eliminate or reduce the need for autograft harvesting. The capacity to 3D print the graft offers the potential to fit the onlay recipient surface anatomy, and match the defect in size and shape.

Résumé

Contexte

La reconstruction des défauts squelettiques craniofaciaux représente un fardeau biomédical important. Les techniques actuelles de chirurgie craniofacial comprennent les matériaux autogènes, allogéniques et prothétiques. Bien que la reconstruction avec l'utilisation d'un greffon osseux autologue reste l'approche préférée, les autogreffages sont cliniquement limités par l'approvisionnement des donneurs et sont associés à des morbidités significatives du site du donneur. Les implants synthétiques évitent ces problèmes, mais leur efficacité est limitée par le risque de restriction de la croissance, d'infection et de résultats esthétiques insatisfaisants. Cette étude étudie l'utilisation de la monetite, une biocéramique récemment développée, pour induire et augmenter la croissance osseuse ailleurs dans le crâne, qui peut ensuite être excisée et transplantée dans le défaut.

Méthodes

Un implant mononetite poreux a été préparé par balayage à l'autoclave. Vingt-cinq rats Wistar mâles adultes ont été divisés en 5 groupes (N = 5 / groupe). Dans le groupe d'intérêt, appelé le groupe Transplant-Onlay (TO), l'implant a été placé directement sur une zone recouvrant le lambda et maintenu en place pendant 8 semaines. Après cela, il a été excisé et transplanté dans un défaut de 8 mm antérieurement (recouvrant le bregma), qui a été créé immédiatement avant l'excision de l'implant. Il a ensuite été gardé dans le défaut pendant 8 semaines. Dans le deuxième groupe, appelé groupe de placement direct (DP), un défaut de 8 mm de diamètre a été

créé dans la partie supérieure du crâne, entre les sutures crâniennes Lambda et Bregma. L'implant a été placé directement dans ce défaut et maintenu en place pendant 8 semaines. Dans le troisième groupe, appelé groupe de transplantation sous-cutanée (ST), les implants ont été placés pendant 8 semaines dans des poches sous-cutanées, après quoi ils ont été excisés et placés dans des défauts de crâne nouvellement créés. Ce groupe a servi de témoin pour le groupe précédent, car ils n'étaient pas attachés à l'os et n'étaient exposés qu'à l'environnement sous-cutané. Les 2 groupes restants se composaient respectivement de contrôles négatifs et positifs, en utilisant des animaux ayant des défauts de crâne vides (non traités) et des animaux présentant des anomalies du crâne qui avaient été traitées avec des auto-greffes d'os calvariaux. La nouvelle croissance osseuse ainsi que l'intégration osseuse implant-hôte ont été étudiées avec microscopie électronique à balayage (SEM), micro-CT et histologie. Des essais mécaniques ont été effectués sur les greffes explantées pour évaluer la résistance au chargement.

Résultats

L'imagerie micro-tomodensitométrique avec un nouveau calcul du volume osseux a montré que les témoins négatifs ne se guérissaient pas (8,8 ± 1,3% de la nouvelle formation osseuse), démontrant ainsi que notre défaut de 8 mm était un véritable "défaut de taille critique". Les échantillons d'autogreffe ont montré une guérison minimale (34 ± 5,7%). Les échantillons d'onlay transplantés (TO) ont donné les taux les plus élevés de nouvelle formation osseuse (74 ± 3,1%), tandis que les implants de placement direct (DP) et les transplantations sous-cutanées (ST) ont montré des taux comparables de nouvelle formation osseuse (61 ± 2,7% et 60 ± 2,8 %, respectivement). La formation de l'os nouveau a été encore confirmée par une analyse

histomorphométrique, ce qui a confirmé que le greffon radio-opaque et l'os nouveau pourrait bien être distingué de manière fiable et que la plus grande quantité de croissance osseuse avait eu lieu dans le groupe Transplanté Onlay (6,2 ± 0,3 mm2) Par rapport aux taux plus bas dans les groupes autogreffe (AG), placement direct (DP) et transplantation sous-cutanée (ST) (3,1 ± 0,5 mm2, 4,1 ± 0,4 mm2 et 4,2 ± 0,3 mm2, respectivement). SEM a confirmé la présence d'os nouveaux dans ce groupe. En outre, les échantillons de ce groupe ont pu résister à des forces de charge plus importantes lors d'essais mécaniques et d'évaluation fonctionnelle. Tous les résultats étaient statistiquement significatifs (P <0,05).

Conclusion

Malgré les progrès dans les stratégies de traitement contemporaines, la morbidité du site du donneur et la restauration sous-optimale de la forme et de la fonction continuent à favoriser le développement de nouvelles approches pour traiter les défauts calvariés. Notre projet propose de nouvelles méthodes pour induire l'os indigène à produire localement l'excès d'os de manière contrôlée. Nos données montrent que l'os a été cultivé avec succès à l'intérieur d'un micro-poreux monone onlay sans utiliser de facteurs de croissance ou de manipulation génétique. Cet excès d'os pourrait être excisé, fabriqué au besoin et utilisé comme transplantation autologue "à la demande" pour traiter les défauts de crâne de taille critique. Cette technique a le potentiel d'éliminer ou de réduire le besoin de récolte d'autogreffe. La capacité de l'impression 3D du greffon offre le potentiel d'adapter l'anatomie de la surface du destinataire de l'onlay et correspond au défaut de taille et de forme.

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Author contributions and statements of originality

The work described in this project was performed by the author, and is novel and original. This is the first report that describes the use of monetite-induced bone growth as a potential method for treating critical-size skull defects.

Chapter 1 - Thesis Introduction

Current surgical techniques for repairing craniofacial defects include autogenous, allogeneic and prosthetic materials. Despite advances made in these therapeutic modalities, large skull defects continue to pose a challenging problem to the plastic surgeon. Such cranial defects may result from trauma, infection, birth defects, or resection of tumors.[1] With more than 2.2 million bone grafting procedures carried out annually worldwide with the aim of repairing orthopedic, dental, congenital and neurosurgical defects,[2,3] bone grafting continues to be one of the commonest surgical procedures carried out in multiple clinical domains.[4] While reconstruction of skull defects with autologous bone graft remains the preferred approach [5], autografts are clinically limited by donor supply, are invasive to obtain, and are associated with significant donor site morbidities. Synthetic implants avoid these issues, but their efficacy is limited by the risk of growth restriction, in which the implanted material creates an unyielding environment that does not permit growth of the brain.[1] Other potential adverse effects include infection and unsatisfactory aesthetic outcomes.[6] Thus, there is currently a critical gap in knowledge that is hindering the identification of the optimal cranioplasty technique.

The work described in this thesis investigates the application of bone formed in an onlay graft as a source of non-vascularized autograft. The primary objectives of this research were the following:

1. Assess and compare bone growth induction by means of both cranial orthotopic onlays and subcutaneous implantation, using implants synthesized from calcium-phosphate composites.

2. Test the capability of these implants to treat critical-size cranial defects in adult rat models, and compare with healing of defects treated with bone autografts.

The driving force behind this research was the desire to eventually create a surgical technique that may eliminate the need for creating a bone defect at the harvest site. The ability to nduce the patient's own body to produce bone "on-demand" could in some indications be a solution to the morbidity caused by bone autograft harvest.

This thesis consists of an introduction to basic bone biology and remodeling. In the subsequent chapter, various methods of bone repair are discussed. This chapter also describes certain specialized techniques utilized for inducing bone growth with the aim of treating defects or repairing certain deformities. In the next chapter, my project is presented and my data are discussed. The final chapter consists of a general discussion of my results within the context of other current experimental studies, and potential future directions for further work.

The work was performed by the author between September 2015 and March 2017 in the McGill University campus laboratories, and the Montreal General Hospital surgical facilities. The project was supervised by Dr. Jake Barralet, Department of Surgery, and co-supervised by Dr. Mirko Gilardino, Division of Plastic and Reconstructive Surgery.

Chapter 2 - Introduction: Bone Biology and Remodeling

The normal mature human skeleton has 213 bones[7], with each bone undergoing constant remodeling and repair in response to injury and changing biomechanical forces.[8]

Bone can be categorized into four general groups according to shape: long bones (eg: clavicles, humeri, and radii), short bones (eg: carpals, tarsals, and sesamoid bones), flat bones (eg: skull, mandible, and sternum), and irregular bones (eg: sacrum, coccyx, and hyoid bone).[7] Flat bones form via intramembranous ossification, whereas long bones form by a combination of intramembranous and endochondral ossification.[8]

In addition to providing a framework of structural support for the rest of the body, the human skeleton serves a wide variety of functions by protecting the internal organs, maintaining homeostasis, and providing a source of growth factors and cytokines. Furthermore, it creates an optimal environment within the bone marrow spaces for hematopoiesis,[9]

2.1 Bone Structure and Composition:

Bone is composed of a mixture of different kinds of cells (Figure 2.1). Organic components make up roughly 30% of the entire dry weight composition of bone, and consist of collageneous (Type-1 collagen) and non-collagenous structural proteins. Inorganic components make up the remaining 70% of inorganic composition, which mainly consists of hydroxyapatite (HA).[10] The following three cell types play a crucial role in the development, remodeling, and hemostasis of the human skeleton:

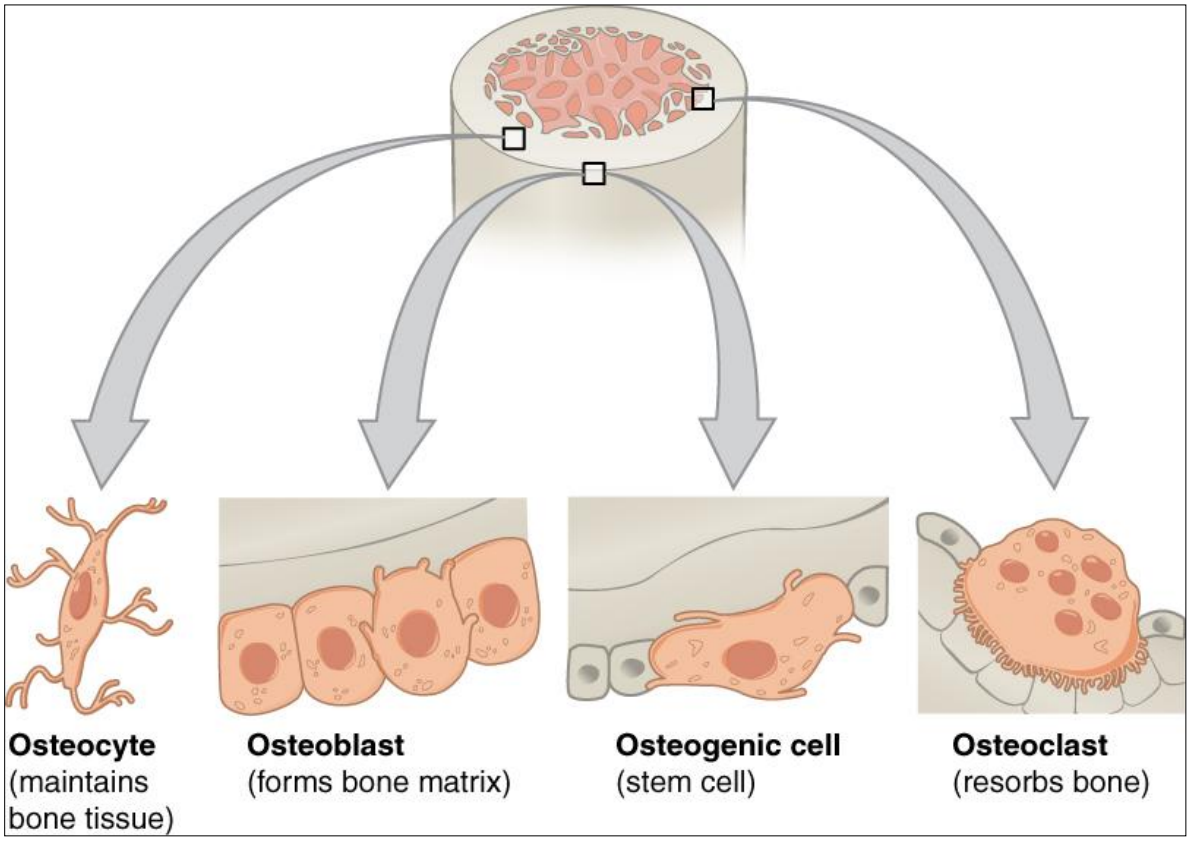

Figure 2.1 *Types of cells found within bone tissue. (Openly licensed content adapted from OpenStax College, Anatomy & Physiology. December 1, 2013)*

- Osteocytes:

The most abundant cell type in bone, these cells communicate with each other and the surrounding bone matrix via extensions of the plasma membrane.[11] Furthermore, within their capacity as mechanosensors, osteocytes instruct osteoblasts and osteoclasts to synthesize and break down bone respectively[12] thereby creating a controlled environment in which the sensitive remodeling process is finely coordinated.[13]

- Osteoblasts:

Osteoblasts are formed in the periosteum and bone marrow, where they reside for the duration of their existence.[14] The principle function of the osteoblast is to synthesize and subsequently mineralize the bone matrix. They are also responsible for regulating osteoclast production, and possess receptors for a variety of endocrine factors through which they bring about their effects on bone.[11]

Osteoblasts have been shown to recruit osteoclast precursors to the resorption site by the release of certain chemokines such as CCR, which are integral membrane proteins that specifically bind and respond to cytokines of the CC chemokine family[15], allowing the receptor RANK (Receptor Activator of Nuclear Factor κ B), which is expressed by the osteoclast precursors, to bind its ligand, RANKL (Receptor activator of nuclear factor kappa-B ligand). As a result, these precursors start differentiating into osteoclasts, expressing key features such as TRAP (tartrate-resistant acid phosphatase) and eventually complete maturation and fusion.[16]

- Osteoclasts

Osteoclasts are multinucleated cells that develop from hematopoietic cells of the monocyte-macrophage lineage, and form as a result of the self-fusion of macrophages, requiring the presence of several biochemical factors such as RANKL (receptor activator of nuclear factor κβ ligand) and M-CSF (Macrophage colony-stimulating factor).[17] These cells are unique in their ability to resorb mineralized tissues like bone and resorb bone to from pits in the bone surface, known as resorption bays or Howship's lacunae.[7] Along with osteoblasts, osteoclasts are instrumental in controlling the amount of bone tissue which is in a state of constant turn-over,

and an imbalance may lead to one of several pathological conditions such as osteoporosis, osteopetrosis, and pathological fractures.[18] Given the importance of osteoclasts in bone development and repair, the following aspects are explored in more detail:

Role of Osteoclasts in Cranial Development and Repair.

Coordinated longitudinal growth of the calvarium is crucial for housing and protecting the developing sensory structures involved in vision, olfaction, and audition. Most importantly, proper bone growth is essential for normal growth of the brain.

Cranial sutures connect interfacing flat bones of the skull vault and face and arise during the prenatal developmental period as amorphous connective tissue ligaments[19]. Characteristically, these sutures become patterned as a waveform demonstrating interdigitating or interlocking bony projections when viewed from an ectocranial perspective. Appositional bone growth along these suture margins is primarily responsible for the lengthening of calvarial bones rather than their thickening, i.e., the cranial suture is to calvarial growth as the epiphyseal growth plate is to long bone growth.[20] Premature fusion of cranial sutures commonly manifests as craniosynostosis. As a result, cranial vault growth is restricted along a single or along multiple axes depending on the amount of affected skull sutures. Osteoclasts have been implicated in modeling cranial bone thickening (along the endoectocranial axis) as well as lengthening.[21,22]

Two main biochemical events influence suture bone growth[23,24] :

1. Molecular signaling from the dura mater - the dura secretes a variety of growth and transcription factors that regulate cell activity within the overlying suture, such as TGF 1-3, FGF-2, BMP-4, in addition to others.

2. Biomechanical loads dissipated throughout the craniofacial architecture - mainly masticatory loads (rather than brain growth as previously thought).

A 2006 study by Byron[25] investigated the role of bone resorption by osteoclasts in defining interdigitations characteristic of cranial suture waveform. Male mice were analyzed at six age groups in order to study the ontogenetic changes of osteoclast counts using tartrate-resistant acid phosphatase-stained histological sections of sagittal sutures. Additionally, the complexity of suture lines was measured ectocranially from the same age groups. The results suggest that osteoclast resorption is a contributor to suture patterning. Specifically, osteoclasts show the greatest activity along concave suture regions at 42 and 84 days. In addition, mice given osteoclast-depleting injections of the bisphosphonate alendronate show a decrease in sagittal suture complexity. Data from this experiment indicate a positive relationship between suture complexity and osteoclast count. Increases in suture complexity and osteoclast activity occur after peak rates of cranial width growth and coincide with weaning and the transition to a hard chow diet.

Differences between the action of osteoclasts in the calvaria and in long bones

As previously discussed, bones are formed by either endochondral ossification, characterized by gradual replacement of the cartilage model by bone; or by intramembranous ossification, which is a result of direct bone deposition in connective tissue.[18] Given these different methods of bone

formation, it is reasonable to assume that cells in different skeletal sites differ in function and quality, and by applying this concept to osteoclasts in relation to bone resorption, several important differences can be found.

A) Enzymatic degradation:

Proteolytic enzymes are essential for the digestion of bone matrix constituents by osteolclasts. These enzymes primarily belong to two proteinase families :

1. Cysteine proteinases - mainly, Cathepsin-K
2. Matrix Metalloproteinases (MMPs)

As reported by Everts et al [18], osteoclasts from different bone sites utilize different enzymes to degrade the collagenous bone matrix. Their findings can be summarized as follows:

- Osteoclastic resorption of skull bone depends on cysteine proteinases activity that differs from the activity needed by long bone osteoclasts. The use of low molecular weight cysteine proteinases inhibitors demonstrated that resorption of the different types of bone analyzed (long bone vs. skull bone) was differently affected. Two of the five inhibitors tested in the culture experiments proved to at least partially inhibit resorption of skull bone but had no effect on long bone resorption. Analyses of bone extracts for the activity of cathepsins by using different substrates revealed for that cysteine proteinases activity was much higher in long bones than in calvarial bone samples.

- Although in both flat and long bones, cathepsin K is expressed by osteoclasts, the cathepsin K–deficient mice showed an osteopetrotic effect in long bones, whereas the calvariae appear normal.

- This lack of effect may be explained by the level of this enzyme in the two types of bone. Skull osteoclasts were shown to express much lower levels of cathepsin K than long bone cells, thus suggesting that cathepsin K is more important for resorption of long bones than for resorption of skull bone. Furthermore, by using MMP inhibitors it was shown that resorption of bone matrix by osteoclasts was strongly inhibited. However, this effect was only seen in calvarial bone explants, and not (or far less) in long bones, indicating that participation of MMPs in bon resorption shows site-specific variations

- Since both types of osteoclasts seem similarly effective in resorption, the findings also suggest that skull osteoclasts utilize, in addition to cathepsin K, not only MMPs but also other cysteine proteinases.

B) Genetic Differences:

An interesting finding by Odgren et al in rescue experiments with CD-4 driven RANKL in RANKL knockout mice was that the teeth of these mice did not erupt in response to the rescue, whereas osteopetrosis in the long bones was resolved[26]. These findings suggest that osteoclasts at different bone sites respond differently to rescue by RANKL, and that site-specific differences in the ability of bone tissue to recruit and activate osteoclasts exist. Two possible explanations for the existence of these differences were proposed by Everts et al[19] as follows:

Firstly, because of structural differences among the various bones of the skeleton, different bones may require osteoclasts with different activity properties. In addition, osteoclasts at different skeletal sites may be intrinsically different, resulting in the presence of osteoclasts which are best suited for their role in their local environment. Other genes have also been implicated in the development of different bone types. Expression and signaling of the Indian hedgehog gene

(IHH) was demonstrated by both Chung et al,[27] and Long et al,[28] to critically affect endochondral bone formation, whereas lack of IHH had no effect on the presence or activity of osteoblasts at sites of intramembranous bone formation. Similarly, Hypoxia-inducible factor α (HIFα) was found to be essential for osteoblastic activity during endochondral bone formation but not during intramembranous bone formation[29].

C) Differences in rates of remodeling:

In addition to structural differences, data indicate differences in bone remodeling times, where remodeling of calvarial bone has been shown to be significantly slower than the remodeling of long bones.[30] Furthermore, systemic in vivo administration of PGE2 in rats resulted in an increased bone formation in long bones without affecting calvarial bones, as demonstrated by Suponitzky et al, suggesting bone-site-specific differences in hormonal responses.[31]

Role of Osteoblasts in Cranial Development and Repair

The osteoblast is a cell of mesenchymal origin. In cell culture, osteoblasts are nearly indistinguishable from fibroblasts. The only morphological feature specific to osteoblasts is located on the exterior of the cell, in the form of a mineralized extracellular matrix (ECM). Thus, from a biochemical and genetic perspective, the osteoblast can be viewed as a sophisticated fibroblast.[32]

During skeletal development and throughout life, cells from the osteoblast lineage synthesize and secrete molecules that in turn initiate and control osteoclast differentiation.[33] In contrast to osteoclasts, there are no major differences between the role of osteoblasts in the skull and

elsewhere in the human skeleton. During embryonic development, osteoblast differentiation occurs via two distinct pathways. Except for the clavicles, the mandibles and certain bones of the skull, a cartilage template that is surrounded by a bony rim prefigures each future bone.[32] Following vascular infiltration of the cartilage template, the chondrocytes undergo apoptosis and are gradually replaced by osteoblasts brought in from the bone rim. This process is called endochondral ossification. In contrast, in the clavicles, the mandibles, and certain bones of the skull, the mesenchymal progenitor cells differentiate directly into osteoblasts. This process, which does not include any cartilaginous templates, is called intramembranous ossification.[34] The two processes of endochondral and intramembranous ossification are described below.

2.2. General Concepts of Bone Development:

The two essential processes of skeletal development during embryogenesis are intramembranous and endochondral ossification. Generally speaking, flat bones form by intramembranous bone ossification, whereas long bones form by a combination of endochondral and intramembranous ossification.[8]

I. Intramembranous ossification

Also known as mesenchymal ossification, this is the process in which bone is laid down into the mesenchyme, resulting in the formation of flat bones, such as the skull. This process also occurs during fracture healing treated with open reduction and internal fixation.[35] An ossification center is formed followed by calcification, leading to formation of trabeculae which eventually becomes surrounded by periosteum.[36] At these ossification centers, mesenchymal stem cells (MSCs) divide and congregate around a profuse capillary network. These cells then differentiate

and convert to osteoblasts, and deposit osteoid in the center of the aggregated cell mass. The osteoid then becomes mineralized, thereby creating a nidus which contains mineralized osteoid around which osteoblasts become entrapped.[37]

II. Endochondral Ossification

Also known as intracartilagenous ossification, endochondral ossification is an essential process during the embryological formation of long bones[38], the longitudinal growth of long bones[39], and the natural healing of bone fractures, i.e., without the use of surgical interventions such as open reduction and internal fixation in an environment of absolute stability.[40] The main difference between intramembranous and endochondral ossification is that cartilage is present during endochondral ossification. Cartilage is initially formed, and is then followed by the endochondral sequence of bone synthesis.[41]

2.3 Embryology of Craniofacial Development

The bones of the skull are either part of the neurocranium; a protective case for the brain or the viscerocranium; the skeleton of the face.[19] Unlike the long bones, vertebrae, ribs and other catilagenous bones that form from a cartilaginous precursor the skull is formed via intramembranous ossification of cells derived from the neural crest and paraxial mesoderm. The neurocranium ossifies in utero from primary ossification centers as bone spicules (Figure 2.2).

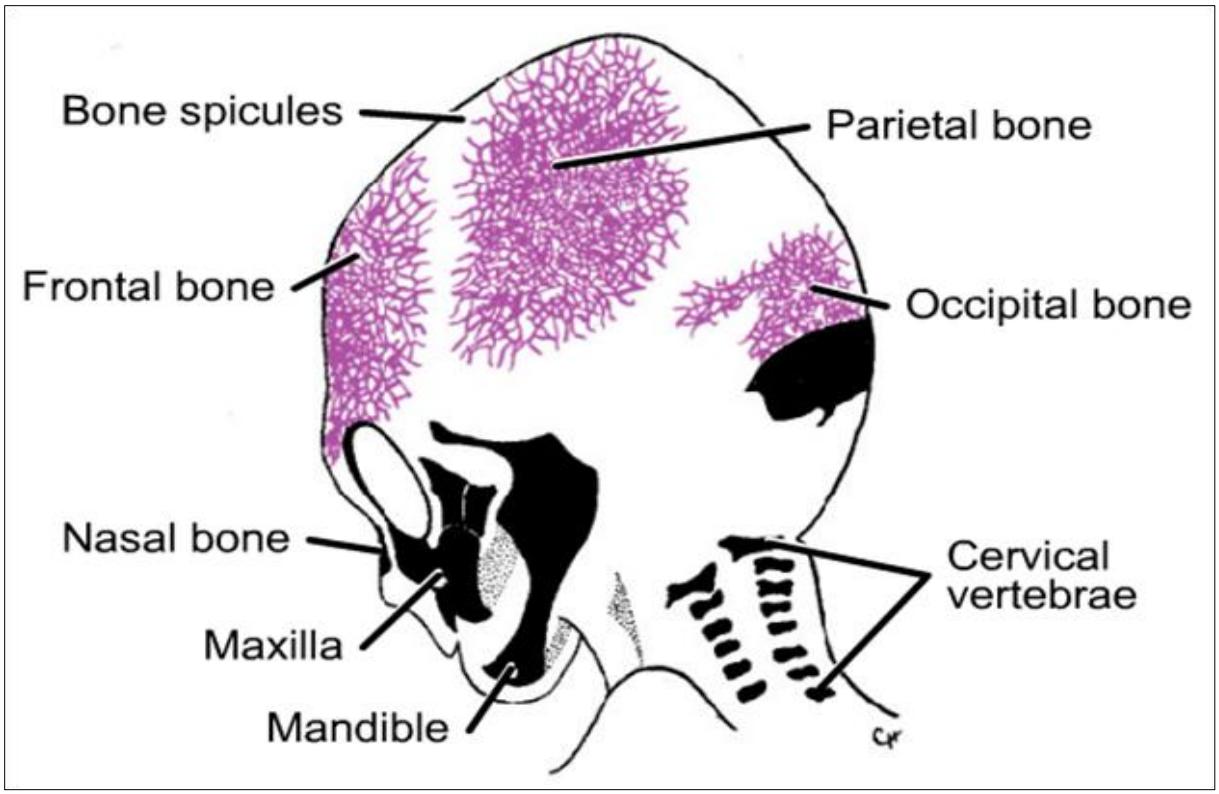

Figure 2.2 *Flat bones of the skull of a 3 month old fetus showing the spread of spicules from the primary ossification center (Adapted from Sadler, 2010)*

These ossification centers do not fuse by birth in order to aid childbirth and allow subsequent brain growth and development. The posterior fontanelle closes during the first year, and the anterior fontanelle closes during subsequent year.

The chondrocranium is formed by a combination of mesodermal sclerotome and neural crest cells. In the fetus, cartilage forms around the brain beginning at the notochord. The base of the skull is formed when the cartilage formed from these two cell types ossifies by endochondral ossification.[19]

Similar to the neurocranium, the viscerocranium is formed by both membranous and chondral bones. Membranous ossification gives rise to the maxilla, zygomatic bone, the vomer and the

palatine bone. The mandibular process contains the Meckel's cartilage. This becomes surrounded by mesenchymal cells that ossify by membranous ossification to form the mandible and the condyles undergo endochondral ossification.

Cartilage at the dorsal end of the Meckel's cartilage forms the malleus and incus while cartilage at the dorsal end of the second arch (Reichert's cartilage) forms the stapes and the styloid process. The ventral end of the second arch ossifies and forms the lesser cornu and the upper body of the hyoid bone. Arch three (ventral end) forms the greater cornu and lower body of the hyoid bone.

After birth, the developing skull differs from developing long bones in that it does not contain growth plates. Lengthening of the calvarial bones is achieved by appositional growth along the skull suture margins[25], matched by osteoclastic resorption on the inner surface

2.4 Bone Remodeling

Bone is in a constant state of resorption and formation, whereby mature bone is resorbed and new bone is synthesized. This continuous turnover state is a physiological response to the functional demands of mechanical loading that occur during life. During the first year of life, almost 100% of the entire skeleton is replaced, and the remodeling process proceeds at a rate of roughly 10% every subsequent year.[42]

The process of bone homeostasis involves multiple well coordinated events, both at the cellular and the molecular levels.[43] As previously mentioned, the two main types of cells that are responsible for bone metabolism are osteoblasts and osteoclasts. Intimate cooperation between

these two cell types and other cell populations present at the bone remodeling sites is crucial for proper homeostasis and maintenance.[44] Bone metabolism depends on complex signaling cascades and control mechanisms to achieve physiological growth and differentiation, and this in turn is reliant upon the action of several endocrine factors such as parathyroid hormone (PTH), vitamin D, growth hormone, steroids, and calcitonin, in addition to other bone marrow-derived cytokines and growth factors.

When exposed to excessive stresses, trauma, or certain disease conditions such as osteoporosis, the continuity of bone is lost, and a bone fracture occurs. A proliferative physiological process then takes place in which the body attempts to repair the fracture and restore the integrity of the bone. This process can be divided into three main phases[45,46], as follows:

1. Reactive (Inflammatory) Phase

The first and immediate reaction following a fracture is vascular constriction, whereby the blood vessels within the vicinity of the injured bone constrict in order to stop or reduce further bleeding.[40] Subsequently, the extravascular blood cells form a hematoma, resulting in the release of cytokines and an increase in the permeability of the surrounding capillaries. The fibroblasts adjacent to the hematoma replicate to form a loose aggregate of cells, interspersed with small blood vessels, known as granulation tissue[47], which serves to reduce the strain across the fracture site. Osteoclasts are then recruited to remove the resulting debris and other necrotic tissue, a process that takes up to 2-4 weeks post-injury. [47]

2. Reparative Phase

Within the subsequent week, the periosteal cells in closest proximity to the fracture spur the formation of chondroblasts, which in turn synthesize hyaline cartilage. In contrast, the periosteal

cells distal to the fracture gap develop into osteoblasts which synthesize woven bone. The resulting fibroblasts within the growing granulation tissue transform into chondroblasts.[40] These processes eventually result in a newly produced mass of heterogeneous tissue, known as the callus.[48] The fracture gap is eventually aligned and bridged by the hyaline cartilage and woven bone, partially restoring its strength. Through the process of endochondral ossification, the hyaline cartilage and woven bone are then replaced with lamellar bone. At this point, the mineralized bony matrix is gradually penetrated by channels containing a microvessel and multiple osteoblasts, which proceed to form new lamellar bone upon the recently exposed surface of the mineralized matrix in the form of trabecular bone[48],and the entire callus is eventually replaced by the new trabecular bone within 4-6 months, thereby restoring most of its original strength.

3. Remodeling Phase

After the fracture is repaired with trabecular bone, a process is required to replace the weak trabecular bone with stronger compact bone. This process is known as the remodeling process, and entails the resorption of trabecular bone by osteoclasts, resulting in shallow resorption pits known as a "Howship's lacunae". Osteoblasts then deposit compact bone within the resorption pit, resulting in a gradual remodeling of the fracture callus into its new shape, which closely resembles the original shape and strength. This process can take up to several years, and is influenced by several factors such as age, co-morbidities, and smoking.[49]

2.5 Conclusion

Bone is a dynamic biological tissue composed of metabolically active cells that are integrated into a rigid framework. The healing potential of bone is influenced by a variety mechanisms, including biochemical, biomechanical, cellular, hormonal, and pathological pathways.[50] Bone tissue repair is an intricate process that is set in motion as a response to injury.[51] A continuously occurring state of bone tissue deposition, resorption, and remodeling facilitates the healing process.[50] Regeneration commences with proliferation and migration of osteoprogenitor cells, and ceases with the reconstruction of bone with differentiation of osteoprogenitor cells and bone extra-cellular matrix (ECM) formation.[51]

Chapter 3 - Bone Repair and Induced Bone Growth - A Review of the Literature

3.1 Introduction

The search for the ideal cranioplasty technique poses a complex challenge to reconstructive surgeons. A layer of complexity is added in the repair of calvarial defects in the pediatric population, which requires various special considerations, such as the need to create a non growth-restrictive environment to allow enough space for the growing brain.[52,53] The evolution of cranioplasty techniques has spurred the development of allograft and autogenous materials that have greatly contributed to the advancement of cranioplasty[54], with recently developed techniques focusing on both restoring protection of the brain while simultaneously correcting esthetic deformities.[55] As the use of osteo-inductive materials in the clinical realm continues to gradually gain acceptance, current research is attempting to investigate not only the extent of skull integration into these grafts, but their mechanical strength and ability to provide a non growth-restricting environment.[52] In this chapter, the principles of bone repair are discussed, and a review of the available literature is discussed regarding certain specialized techniques of influencing bone to grow in a targeted fashion.

3.2 Bone Grafting

In order for a bone graft to successfully take, evidenced by the incorporation in the recipient bed, adequate coverage of the treated defect, and stability of the grafted bone, three main principles are involved[56] :

A. Osteoconduction:

The process of osteoconduction occurs when the grafted bone serves as a framework for subsequent new bone growth that is perpetuated by the native bone. Osteoblasts from the defect edges utilize the bone graft material as a scaffold upon which to spread and generate new bone. This process guides the reparative growth of the natural bone, and is considered the most essential of the three principles of bone grafting.

B. Osteoinduction

During the process of osteoinduction, osteoprogenitor cells are stimulated to differentiate into osteoblasts that initiate new bone synthesis. A bone graft material that is both osteoconductive and osteoinductive is highly advantageous, as it will serve the dual purpose of both acting as a scaffold for currently existing osteoblasts, in addition to spurring the formation of new osteoblasts, thereby promoting a more rapid graft-host integration.[56]

C. Osteogenesis

Osteogenesis occurs when osteoblasts from the bone graft material contribute to new bone remodeling along with bone growth generated via the other two mechanisms

3.3 Distraction Osteogenesis

Distraction osteogenesis (DO), is a technique employed in various surgical domains to repair certain skeletal deformities.[57] It was initially intended to treat conditions such as leg length discrepancy, however, it quickly evolved as an important management tool in the treatment of more delicate conditions such as hemifacial microsomias and craniosynostosis.[58] The first use in of mandibular distraction osteogenesis was pioneered by Wolfgang Rosenthal in 1930. However, due to skepticism and general lack of acceptance by the medical community, it lost favor for a few decades and was not further explored until the 1980s; which resulted in the first report of the modern era describing its clinical use published in 1992.[59]

The procedure of distraction osteogenesis involves creating controlled fractures in which multiple bone segments are slowly separated, thereby allowing the natural reparative bone healing process to occur and fill in the gap.[60] It consists of three phases as follows[58]:

I. Latency phase

In this phase, the bone is either partially or completely incised, and a distraction device is mounted to the bone on each side of the incision - no distraction or lengthening is performed during this phase.

II. Distraction phase

In a controlled manner, the distraction device is used to gradually separate (distract) the segments at specific, predetermined rate, thereby creating a space in which the new bone will

form. The rate is usually set at about 1mm/day, although this may vary according to the condition being treated and the desired length to be achieved.

II. Consolidation phase

Once the desired length is achieved, the distraction device is kept in place to provide stability and allow the healing process to occur. A subsequent surgical procedure is then performed to remove the device

The DO technique is frequently utilized in cranio-maxillofacial surgery.[61] The process has evolved dramatically since its inception, with mutliple clinical and experimental studies reported in the literature.[62] The technical aspects of the surgical procedure continue to evolve with the aim of providing the best functional and cosmetic results. An interesting recently reported development was that of developing devices and techniques that would allow DO in more directions simultaneously.[63]

3.4 Vertical Bone Augmentation

Vertical bone augmentation with the use of different techniques and biomaterials has shown promising results, however, failure rates and complications remain unacceptably high to recommend its introduction into routine clinical.[64] Vertical guided bone regeneration (GBR) is a technically challenging procedure that often fails due to wound dehiscence.[65-67]

The first reported applications of GBR were attempted in the early 1990s to treat mandibles that had atrophied due to various reasons.[65] Applying a titanium reinforced non-resorbable barrier membrane, severe vertical defects were treated in conjunction with dental titanium implants.

Soon after, mandibular distractors were reported to be used in patients with hemifacial microsomias.[59] Autologous bone grafting was also introduced in the early 1990s in an attempt to increase mandibular and maxillary vertical height by means of apposition.[68] These techniques however were only met with minimal success, with many authors reporting data on predictability, failure, and complications of these procedures.[69]

An interesting review by Esposito et al was conducted in 2010, in which the difference in success, function, morbidity and patient satisfaction between different augmentation techniques was studied. It was concluded that both GBR and distraction osteogenesis could augment bone vertically, however, the superiority of a certain technique over the others could not be proven.[64]

More recently, in a study by Okada et al (2017), the use of beta-TCP (beta-tricalcium phosphate) in sinus floor augmentation was investigated as an alternative to autogenous bone grafting. The osteoconduction potential of highly pure [beta]-TCP in sinus augmentation surgery treatment was evaluated, and was found to be a safe bone-grafting material with good osteoconductive properties. Histological and radiographic examinations showed that [beta]-TCP resorbed at a slow rate, which resulted in unresorbed graft material remaining as long as 6 months after the procedure, and that new bone replacement occurred slowly for approximately 1 year.[70]

3.5 Bone growth induction

Following tooth loss or dental extraction, irreversible alveolar bone resorption occurs at the rate of up to 2mm per year, leading to problems and challenges in providing dental treatments due to lack of bone tissue.[71]

In an attempt to promote adequate bone regeneration, numerous osteoconductive materials have been investigated with the aim of providing an appropriate matrix to allow and guide new bone formation.[72] Some of these materials, such as autologous bone grafts, demineralized bone matrices, and chitosan for example, are of biological origin, whereas others, such as polylactic-co-glycolic acid, tricalcium phosphate, calcium sulphate, synthetic hydroxyapatite are purely synthetic.[73-77] Synthetic materials have been rapidly gaining favor due to their cost-effectiveness and reduced risk of adverse effects such as immunological rejection and transmitted infections.[72]

Synthetic calcium phosphates that mimic the structure of normal bone have demonstrated their ability to be accepted by the human body and to integrate well into bone defects.[78,79] The biochemical structure of these materials is facilitates their use in preparing scaffolds with suitable pores in order to maximally enhance angiogenesis and cell proliferation during the bone regeneration process.[75] The following is a summary of some of the synthetic calcium phosphates described in the literature:

- Hydroxyapetite (HA):

Hydroxyapatite is a naturally occurring mineral form of calcium apatite, and is found in teeth and bones within the human body. Due to its similar composition to bone, it is frequently used as a filler to replace resected bone, or as a coating to promote bone ingrowth into prosthetic implants,[80] with many modern implants, such as hip replacements, dental implants and bone conduction implants, utilizing HA as a coating to promote osseointegration.[81]

- Tri-calcium phosphate (TCP):

Tricalcium phosphate is a calcium salt of phosphoric acid with the chemical formula $Ca_3(PO_4)_2$. It exists in the form of three crystalline polymorphs α, α', and β.[82] The use of TCP has been

explored for its potential in repairing skeletal defects as an alternative for autogenous bone graft, when use of the latter is not feasible or possible.[83,84] It has been used with varying degrees of success, either alone or in combination with a biodegradable polymer such as polyglycolic acid.[85]

- Dicalcium phosphates:

Also known as dibasic calcium phopshates, they exist in the three following forms[86]:

- Dihydrate form: $CaHPO_4 \bullet 2H_2O$ ('DPCD'), also known as bruhsite
- Hemihydrate form: $CaHPO_4 \bullet 0.5H_2O$
- Anhydrous form: $CaHPO_4$, ('DCPA'), also known as monetite.

These compounds have also been investigated as bioceramics with potential in bone tissue engineering[87-89], and their use in repairing cranio-maxillo-facial defects has been explored[72,88,90,91]

3.6 Conclusion

The need to replace bone that has been lost due to infection, trauma, tumor excision, or a multitude of other clinical problems continues to pose a challenge to surgeons in multiple domains. Autologous bone grafting remains the preferred approach, however, it is not always feasible, and poses the risk of multiple adverse effects.[1] Due to the pressing need to provide a stable base for tooth/implant placement in the mandible, numerous techniques have been employed. Chief among those has been the process of Guided Bone Regeneration (GBR). The concept of bone augmentation has been extensively researched and employed in the field of dental surgery and implantology. Calcium phosphate synthetic grafts have garnered considerable

attention due to their similar composition to natural bone. A study by Tamimi et al[72] investigated the use of granulated brushite cements to achieve vertical bone augmentation, and found that within four weeks of granule implantation, considerable cement resorption had occurred, in addition to an improvement in the bone mineral density and bone neoformation . In another study[72], the investigators were able to use monetite blocks to achieve vertical bone augmentations as high as 4mm. However, to date, there has been no attempt to explore the potential of these bone growth-inducing techniques to treat cranial defects outside the scope of oral and dental reconstruction in edentulous mandibles.[92] The work presented in the following chapter demonstrates the a novel attempt at inducing bone growth with the aim of treating large, critical sized calvarial defects.

Chapter 4 - Treatment of Critical-Size Calvarial Defects in Rats with a Novel Method of Monetite-Induced Bone Growth

4.1 Preface

The previous chapter discussed various techniques of bone repair, ranging from the "gold-standard" method of autologous bone grafting, to more specialized and recently developed techniques, such as guided bone regeneration and distraction osteogenesis. While there have been encouraging results reported in the literature with some of the more recent experimental approaches to vertical bone augmentation[72,88,93], a deficiency still remains that needs to be addressed. No single technique has been proven to sufficiently repair large skull defects in a reliable and predictable way, which explains why autologous bone grafting remains the preferred clinical approach, even though it is fraught with drawbacks and limitations.[5,6]

The work presented in this chapter involves the use of monetite, a recently developed calcium phosphate bioceramic[88,89], to augment native bone with the aim of treating critical-size calvarial defects, using a method that has not been reported in the literature to date.

4.2 Introduction

The search for the optimal cranioplasty technique continues to pose an arduous challenge for craniofacial surgeons. Despite refinements in bone grafting techniques and the use of semi-synthetic implants, major limitations to these techniques have spurred the search for alternative novel treatment strategies.[1] The current tissue engineering research paradigm involves investigating the use of bioactive growth factors and stem cells to regenerate damaged or deficient skeletal tissue.[6,94-96] While such treatment strategies may have significant future potential, they are not without drawbacks. The use of stem cells is hampered by complex ethical debates, lack of effectiveness, concerns about their tumorigenic propensity, and their high cost[94,95], while the use of osteo-inductive growth factors (such as bone morphogenetic proteins, vascular endothelial growth factor, and basic fibroblast growth factor) are associated with either proven inflammatory side effects or concerns, and the production of either insufficient or deleteriously excessive amounts of structurally abnormal bone.[96] Additionally the safety of stem cell-based techniques or those utilizing growth factors in the growing pediatric population has not been verified.[97]

In previous studies, monetite has demonstrated its ability to promote bone growth and regeneration when implanted directly onto the surface of a rabbit skull.[98] Other studies support these findings, and have clearly established the osteoinductive properties of 3d printed monetite and other similar calcium phosphate bone cements.[93] Furthermore, a recently published report highlighted the role of calcium phosphate bioceramics in inducing the production of the enzyme COX-2 (cyclooxegenase-2), which has been shown to be significantly involved in bone healing.[99]

Although many reports have described the use of calcium phosphate bone graft substitutes in the healing of long bone defects, there is a significant deficiency in published studies that objectively assess and compare the use of monetite-augmented bone in the treatment of calvarial critical-sized defects.[100-103] Furthermore, while bone tissue has been grown with varying degrees of success in subcutaneous pockets with the use of stem cells and osteogenic growth-factors [104,105], there are no studies that have investigated using the newly grown ectopic bone to heal skull or other bone defects by excising it from its subcutaneous location and transplanting it into a skull defect.

Drawing on the ability of monetite to induce or augment bone when placed directly onto normal native bone, in this study we sought to determine the quality of this newly grown bone, and whether or not it would ultimately fulfill the role of a clinically acceptable alternative option to autologous bone grafting.

4.3 Methods

4.3.1 Implant Materials:

4.3.1.2 Preparation

Brushite discs were prepared as described previously[89] by mixing monocalcium phosphate monohydrate (ABCR, GmbH & Co.KG) with beta-tricalcium phosphate (Merck), using a ratio of 1 to 1.2 respectively, which resulted in an optimal consistency and pH of the final product . The resulting cement-like paste was left to set in a pre-fabricated circular 8mm diameter polytetrafluoroethylene (PTFE) mold, forming hardened cement cylinders with a diameter of 8mm, and length of 4mm after being kept for 24 hours in a vacuum oven at 60° C. Monetite

cements grafts were then prepared by autoclaving the brushite disks, at a temperature of 120° C, and a humidity level of 100%.

4.3..1.3 Characterization

X-ray diffraction (Bruker Discover D8 diffractometer) and helium pycnometry (Accupyc 1330, Micromeritics) were used to determine the purity of the synthesized materials during both phases (as brushite and as monetite). Ni filtered CuKa radiation (k = 1.54 Å) was used with a two dimensional VANTEC area detector at 40 kV and 40 mA. A step size of 0.02° was used to measure from 10° to 50° 2h over 3 frames with a count time of 300 s per frame. The phase composition was compared and confirmed with the International Centre for Diffraction Data reference patterns for brushite (PDF Ref. 09-0077) and monetite (PDF Ref. 09-0080), JCPDS 2010 database.

4.3..2 Animal Surgical Protocol

The surgical protocols for animal research were approved by the McGill University Ethical Committee (AUP 7660, AUP 7756). Thirty adult male Wistar rats (35-40 days old, weighing 200-300g) were purchased from Charles River Laboratories, Montreal, Quebec, Canada. Animals were housed in light- and temperature- controlled facilities and given food and water ad libitum. The rats were divided into 5 groups (n=5), with 5 remaining rats kept as extras if needed. All animals were anesthetized in the same fashion, using inhalant isoflurane gas for both induction and maintenance. In the first group, an 8mm diameter calvarial defect was created in the superior portion of the skull, between the Lambda and Bregma cranial sutures. The implant was placed directly into the defect and kept in place for 8 weeks. This group was named the

Direct Placement (DP) group. In the second group, the implant was placed directly on to an area of the skull overlying the lambda, and kept in place as an onlay graft for 8 weeks. After that, it was excised and transplanted into a 8mm defect anteriorly (overlying the bregma), which was created immediately prior to implant excision. It was then kept in the defect for 8 weeks. This group was named the Onlay Transplant (OT) group (Figure 4.2). In order to see if simply being implanted for a few weeks had an effect on bone repair, implants were placed for 8 weeks in subcutaneous pockets, following which they were excised and placed into newly created skull defects (Figure 4.3). This group served as a control for the previous group, as in this group, the implants were not in contact with bone, and were only exposed to the subcutaneous environment. This group was named the Subcutaneous Transplant group (ST). The remaining 2 groups consisted of negative and positive controls respectively, using animals that had empty (untreated) skull defects, named the Empty Defect (ED) group, and animals with skull defects that had been treated with calvarial bone autografts, named the Autograft (AG) group. All wounds were closed in 3 layers using 4-0 resorbable monocryl sutures. All animals received a single dose of pre-operative antibiotic (penicillin, subcutaneous), and oral tramadol for post-operative pain control. Animal sacrifice was carried out using a combination of CO_2 and gas anesthetic (isoflurane) overdose, in addition to pneumothorax. The retrieved implants and explanted skulls were fixed in 4% paraformaldehyde for 48 hours, and were then washed twice and stored in phosphate-buffered solution.

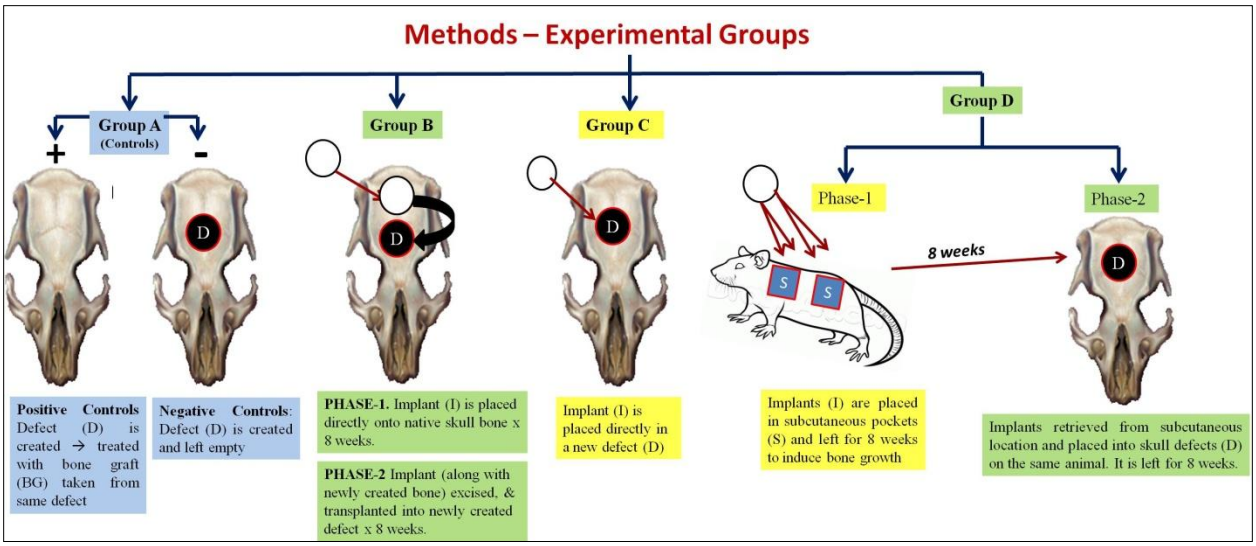

Figure 4.1 *Schematic depicting the experimental groups.. Group A depicts the control groups. The positive control group was a defect that was treated with a skull autograft, whereas the negative control group was a defect that was left empty. Group B (Transplanted Onlay group), consisted of an onlay that was excised and transplanted into a newly created 8mm defect. Group C consisted of a defect that was treated with direct placement of an implant that had not been kept as an onlay prior to implantation. This group was named Direct Placement (DP) group. Group D consisted of samples that were kept in subcutaneous pockets prior to being transplanted into a skull defect . This group was named Subcutaneous Transplant (ST) group.* ***BG****: bone graft,* ***D****: defect,* ***S****: subcutaneous pockets.*

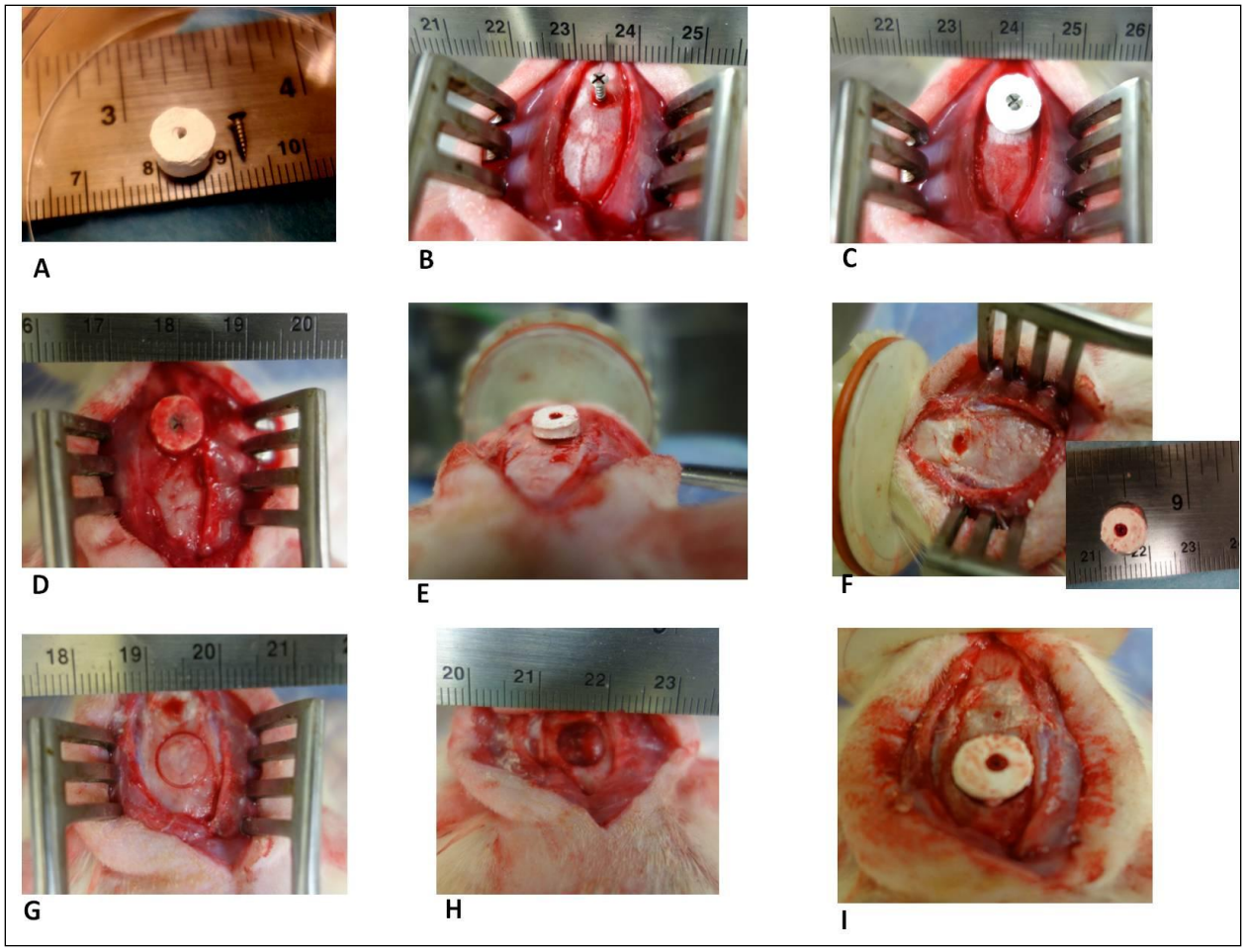

Figure 4.2 *Intra-operative pictures of Transplanted-Onlay (TO) group. A:Implant and fixation screw. B:Partial-thickness hole for screw created. C: Implant fixed in place using the screw. D:Appearance of the implant 8 weeks later. E:Implant in the process of excision. F. Appearance of the skull after implant excision. Note that the skull is intact (Inset: Excised implant). G:Full thickness 8mm circular skull defect created with trephine. H: Resulting defect prior to treatment with implant. I: Implant in place within the defect.*

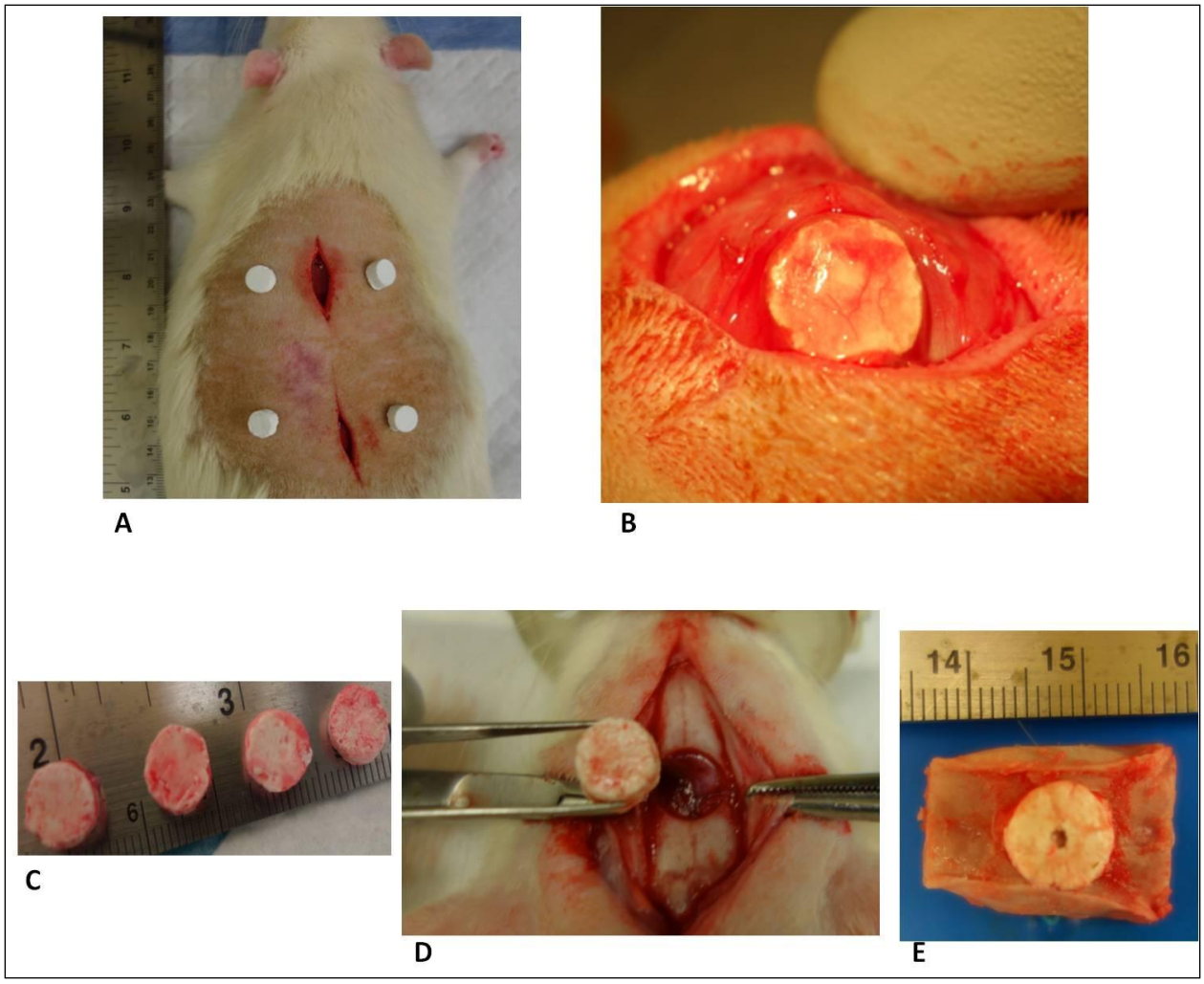

Figure 4.3 *Intra-operative pictures of the Subcutaneous Transplant (ST) group. A: Four implants positioned on the back to demonstrate where they will be located once implanted subcutaneously. B: Appearance of the implant in its subcutaneous location after 8 weeks. C: The four implants after excision from their subcutaneous location. One of the implants is selected based on appearance. D: Selected implant prior to transplantation into a newly created skull defect.. Appearance of the explanted skull with the implant in place.*

4.3.3 Post-implantation characterization

- Following explantation, the reconstructed defects were grossly examined to assess texture and stability of the construct, and to provide an overall general macroscopic comparison among the different groups.

- Micro-computed tomography (Micro-CT) (SkyScan 1172; SkyScan; Kontich, Belgium) was used to calculate bone volume and implant resorption at a resolution of 13 μm and 0.5 mm Al filter. Image reconstruction was performed using NRecon (Version 1.6.2.0; SkyScan). The CT analyzer (1.11.8.0; SkyScan) was used to measure static histomorphometric parameters of the region of interest. The bone density was expressed as a percentage of bone volume/ tissue volume % (BV/TV).

- The microstructure of the explant including the bone-implant interface was studied using scanning electron microscopy (SEM) (Hitachi S-4700 FE-SEM; Tokyo, Japan) in back-scattered electron (BSE) mode at an accelerating voltage of 2 kV.

- A customized push-out jig was fabricated and used with the Instron machine (Instron, 5544) to perform indentation testing by applying an increasing load force using a 100N load cell while measuring indentation in millimeters, at a rate of 1mm/minute. The load force was applied until either the first crack occurred or complete breakage took place.

- After explanting the samples and performing Micro CT, the samples were fixed in 4% paraformaldehyde (PFA) for 24 hours. Samples were then washed twice with phosphate-buffered solution (PBS) and dehydrated in ascending grades of ethanol, and were then cured with a polymethyl methacrylate (PMMA) histological resin (Technovit® 9100, Heraeus Kulzer, Wehrheim, Germany). The resin was then left to polymerize at -20° C for 5 days, and the

samples were then sectioned into ~40 μm histological slides with a diamond saw (SP1600, Leica Microsystems GmbH, Wetzlar, Germany) and dyed with basic fuchsine and methylene blue for histological analysis with optical microscopy. Three slides per sample (~40 μm each) were sectioned by cutting through the center of each sample. Histomorphometric analysis of the slides was performed using a Ziess microscope Axio Imager.M2 (Ziess® Gottingen, Germany) with a digital AxioCam IC camera (Ziess®160 Gottingen, Germany) and the image software ZenPro. Data quantification was then performed using Image J (Image J, NIH) to objectively calculate the amount of new bone ingrowth within the defect area. All histomorphometric measurements were calculated as mean percentage values ± the standard deviation.

4.3.4 Statistical Analysis

Data is presented as the mean ± the standard deviation. Statistical analysis was performed using the statistical software Microsoft Excel (2007). Statistical significance ($p < 0.05$) was determined by conducting Student's t-test (two-tailed). Further testing was done to determine difference between groups by conducting one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc analysis. Statistical significance was set at a value of $P < 0.05$.

4.4 Results

4.4.1 Characterization of the implant material

4.4.1.1 Helium pycnometry

Three batches of brushite were prepared, and were subsequently converted to monetite by autoclaving as previously described. There was good consistency between batches (Table 4.1),

with consistently similar densities nearing those of true brushite and monetite (2.328 and 2.929 respectively).

						Properties As Brushite *(True Density = 2.328)*					Properties As Monetite *(True Density = 2.929)*				
Batch #	Number of Samples	P/L Ratio	Diameter (cm)	Height (cm)	Volume (cm^3)	pH	Mass (g)	Measured Bulk Density (g/cm^3)	Pycno. Density (g/cm^3)	Porosity (%) *100 - (bulk/true x100)*	pH	Mass (g)	Measured Bulk Density (g/cm^3)	Pycno. Density (g/cm^3)	Porosity (%) *100 - (bulk/true x100)*
1	8	3:1	0.8	0.4	0.2	6.5 - 7	0.3078	1.539	2.3198	33.9	6.5 - 7	0.2699	1.3345	2.9226	54.2
2	7	3:1	0.8	0.4	0.2	6.5 - 7	0.2654	1.327	2.3255	43	6.5 - 7	0.2639	1.3198	2.8995	54.9
3	9	3:1	0.8	0.4	0.2	6.5 - 7	0.3028	1.5043	2.3301	35.4	6.5 - 7	0.2724	1.3621	2.9197	53.5

Table 4.1 *Physicochemical properties of the implants as brushite (before converting to monetite) and after conversion to monetite from brushite. Means ± standard deviations are as follows: Mass as brushite: 0.292 ± 0.02g. Measured bulk density as brushite: 1.456 ± 0.11g/cm^3. Pycnometry density as brushite: 2.325 ± 0.005g/cm^3. Porosity as brushite:37.45 ± 4.8%. Mass as monetite: 0.27 ± 0.02g. Measured bulk density as monetite: 1.33 ± 0.02g/cm^3. Pycnometry density as monetite: 2.91 ± 0.01g/cm^3. Porosity as monetite: 54.2 ± 0.7%. (**P/L**: powder to liquid ratio g/ml)*

4.4.1.2 X-ray diffraction

X-ray powder diffraction (XRD) confirmed that the as prepared implants were predominantly brushite. After autoclaving the brushite samples, XRD was repeated and the resulting patterns confirmed that the implants had converted to monetite (Figure 4.1)

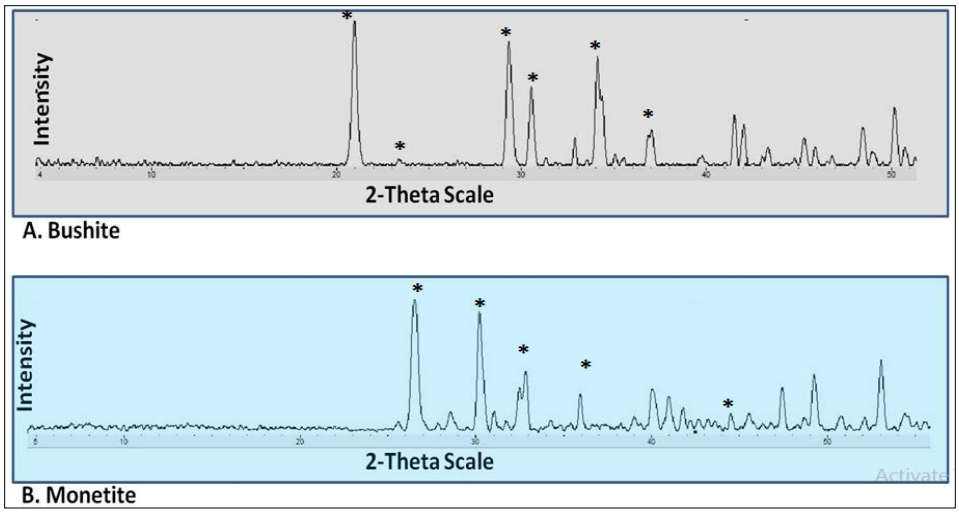

Figure 4.1 *X-ray powder diffraction (XRD) of the implants before (A), and after(B) autoclaving. Standard peak positions associated with di-calcium phosphate dihydrate (brushite) and di-calcium phosphate anhydrous (monetite) are marked with asterisks, indicating that a phase transformation from brushite to monetite had occurred as a result of autoclaving*

4.4.2 Gross (clinical) examination post implantation:

Macroscopic observation of the explanted skulls (Figure 4.2) revealed no significantly discernible differences among the groups, except for the empty defect (ED) group, which had not healed. Upon gentle palpation, the autografted bone in the autograft (AG) group appeared to be loosely adhered to the surrounding bony defect with what seemed to be fibrous tissue. Gentle palpation on samples in the transplanted onlay (TO), direct placement (DP), and subcutaneous (ST) groups all revealed a similar level of good, firm adherence between the implant and the defect edges.

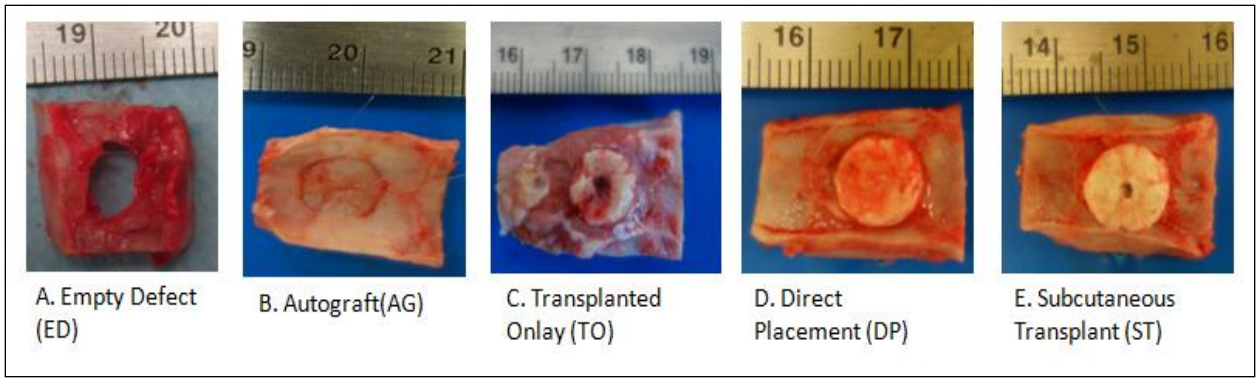

Figure 4.2 *Gross macroscopic appearances of explanted skull samples from each group (ED: empty defect group, AG: autografted group, TO: transplanted onlay group, DP: direct placement group, ST: subcutaneous transplant group.*

4.4.3 Micro-computed tomography (Micro-CT)

Micro CT imaging and quantification of the results are shown in figures 4.3 and 4.5 respectively. As a method of comparison, a micro-CT of a single Day-0 defect was obtained, to ascertain the extent of healing that had occurred in the 8-week empty defect (ED) group, which was found to be 8.8 ± 1.3%. In the autograft (AG) group (Figure 4.3 - C), all samples consistently failed to show a complete autograft-bone defect adherence, with an average of 34 ± 5.7% new bone formation. Transplanted onlay (TO) samples (Figure 4.3 - C) yielded the highest rates of new bone formation (74 ± 3.1%), with most samples showing complete bridging of the defect. Furthermore, the empty area in the center of the implant which was previously occupied by the screw showed new radio-opaque material (Figure 4.4) The direct placement (DP) and subcutaneous transplant (ST) groups (Figure 4.3 D and E respectively) both showed comparable

rates of new bone formation (61.5 ± 2.7% and 60.1 ± 2.8% respectively). All P-values were < 0.05.

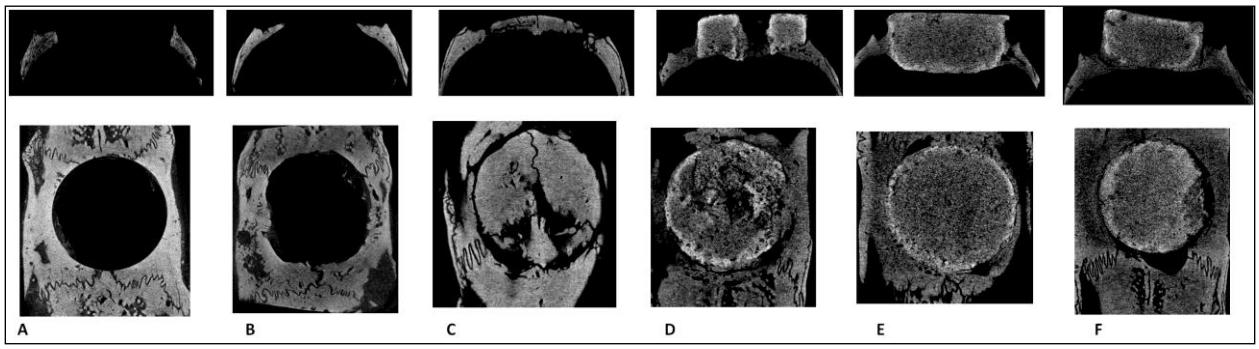

Figure 4.3 *Micro-computed tomography (Micro-CT) imaging of explanted samples. Top row: coronal view, bottom row: axial view. A: Day-0 empty defect, B: 8-week empty defect (ED) group, C: Autograft (AG) group, D: Transplanted Onlay (TO) group, E: Direct Placement (DP) group, F: Subcutaneous transplant (ST) group.*

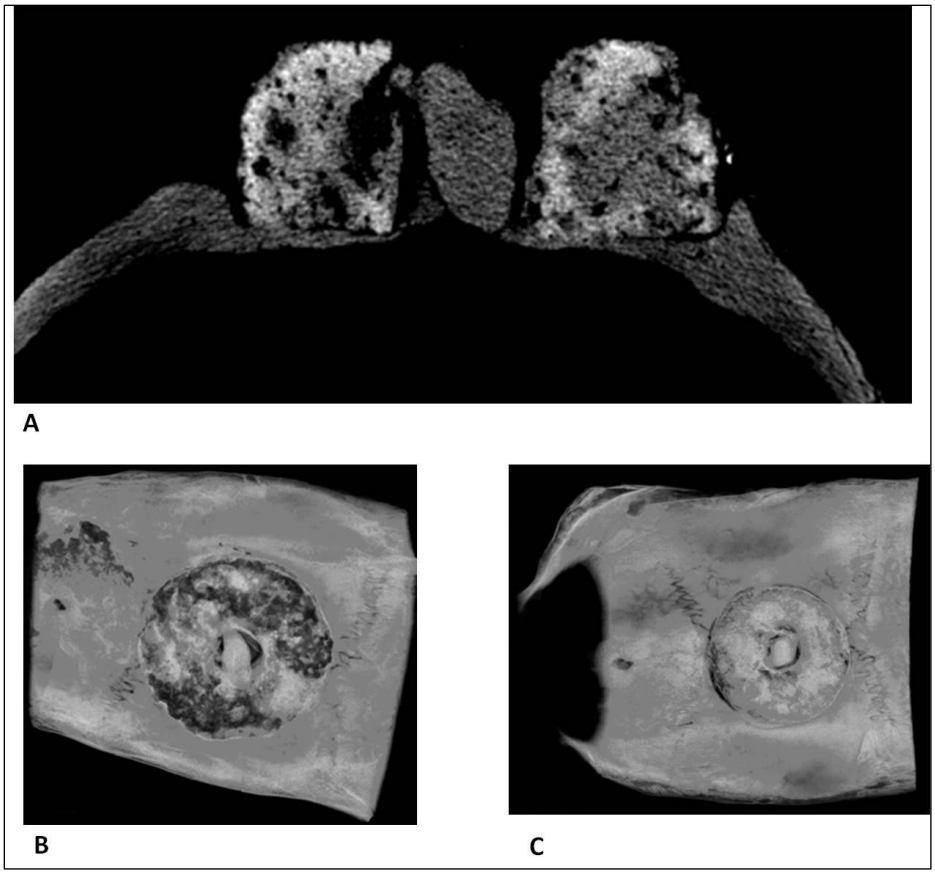

Figure 4.4 *Micro-computed tomography image of a sample from the transplanted onlay (TO) group. A: Coronal view, showing a large amount of radio-opaque material occupying the central screw-hole. B: Axial view taken superiorly of the same sample after 3-D reconstruction. C: Axial view taken inferiorly of the same sample after 3-D reconstruction.*

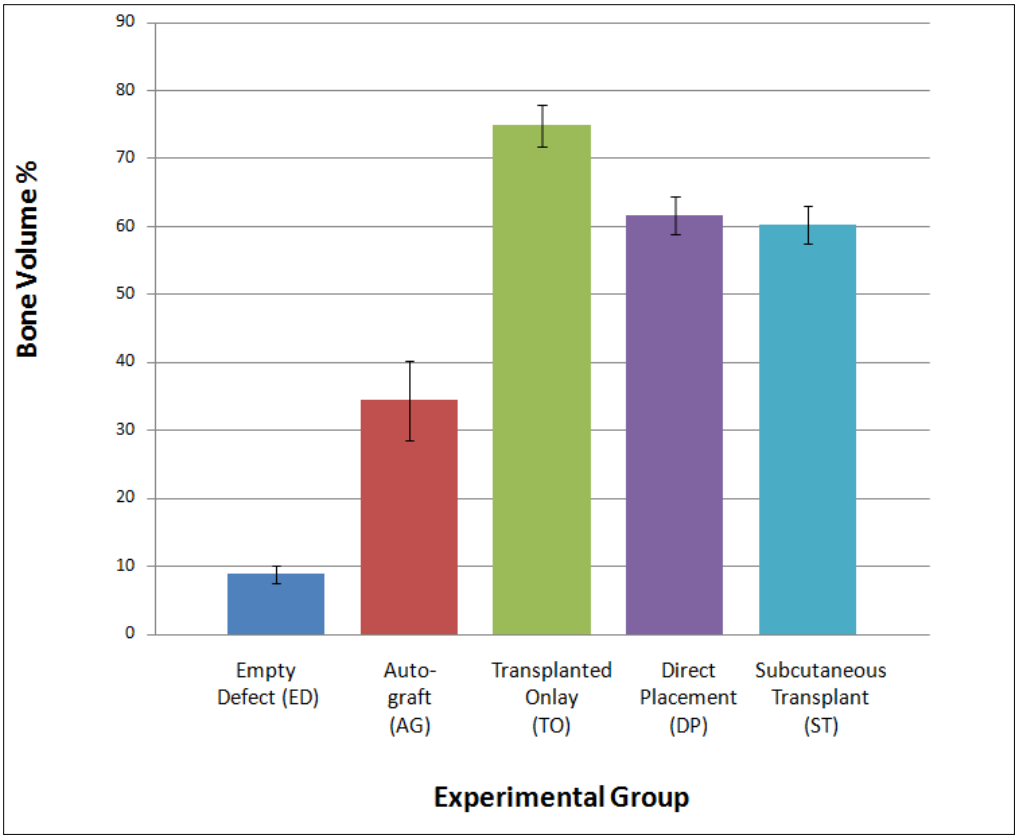

Figure 4.5 *Bone volume percentages quantified from images obtained by micro-CT. Columns represent the mean percentage of new bone formed in each group, while bars represent the standard error of the mean (SEM). A two-tailed t-test was conducted to compare the percentage of new bone volume in each group to the autograft group (AG), which served as the positive control. A P-value of < 0.05 was considered statistically significant. The results are as follows:: Empty defect (**ED**): 8.8 ± 1.3%, Autograft (**AG**): 34.3 ± 5.8%. Transplanted onlay (**TO**): 74.8 ± 3.1%, Direct placement (**DP**): 61.5± 2.7%, Subcutaneous transplant (**ST**): 60.1± 2.8%. A one-way analysis of variance (ANOVA) with Tukey's post-hoc HSD test was then conducted to determine whether any differences existed between the groups. A statistical significance (P-value <0.05) was found between all of the pairs except for DP and ST.*

4.4.4 Scanning Electron Microscopy (SEM)

SEM imaging (Figure 4.6) was able to differentiate between bone and implant. Low magnification imaging (Figure 4.6-A) confirmed bridging of the defect, and higher magnification (Figure 4.6-B) confirmed the presence of mineralized tissue within the implant.

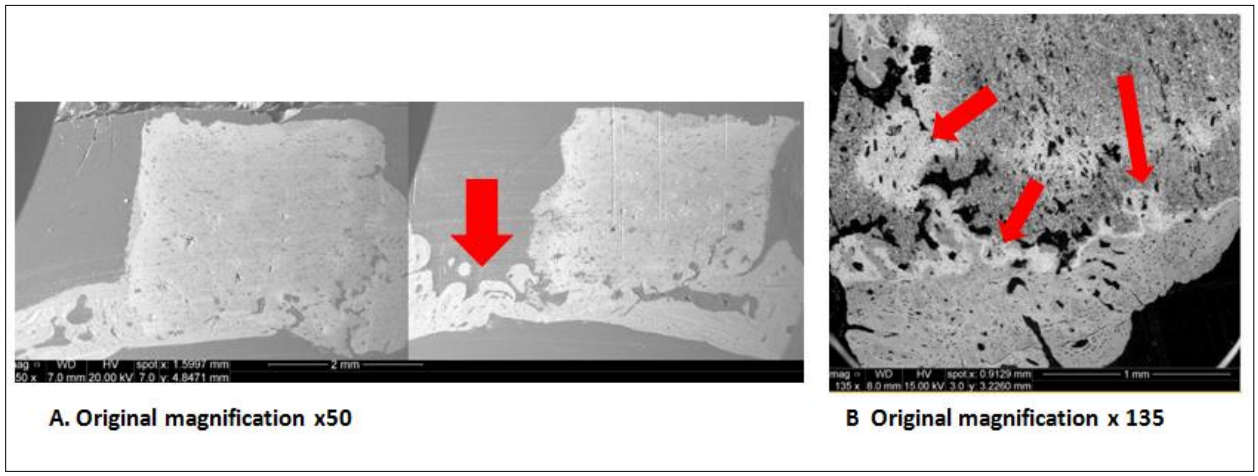

Figure 4.6 *Scanning electron microscopy (SEM) of a sample from the Transplant Onlay (TO) group.* ***A****. SEM low magnification of entire sample. Arrows indicate bridging of the defect.* ***B****. Bone-implant interface at the left side showing presence of mineralized tissue within the implant (arrows).*

4.4.5 Mechanical Testing

Mechanical testing (Figure 4.7) revealed that normal cranial bone (N=6) was able to withstand a mean of 93.6 ± 7.3 N of compressive force before breaking in our push-out testing jig, with the first crack appearing at 70.2 ± 6.6 N, which occurred at 1 ± 0.19mm extension. A single sample from each group was randomly selected to undergo the same testing procedure (N=1). The

autograft (AG) sample was only able to withstand 60N before breaking, with the first crack appearing at10 N, occurring at 0.75mm extension. Samples in the other groups were not tested until complete breakage in order to preserve them for further histological testing. Rather, they were partially tested until shortly after the first crack occurred, evidenced by the first sharp dip in the curve. In the transplanted onlay (TO) group, the first crack was seen at 20N with 0.8mm of extension, whereas 10N were required at an extension of 0.6mm to crack the direct placement (DP) group. In comparison, 15N were required at an extension 1.2mm to crack the subcutaneous (ST) group.

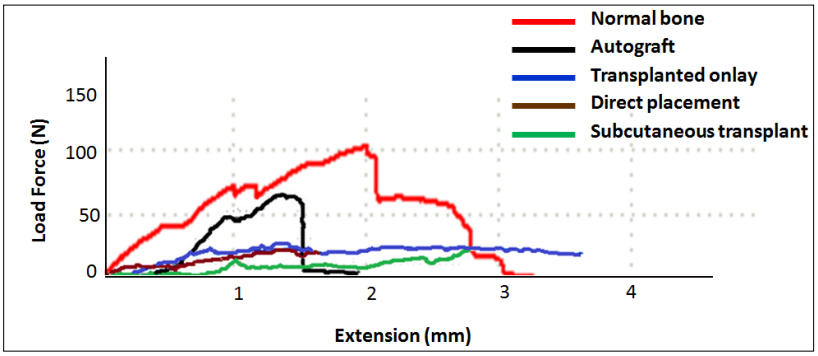

Figure 4.7 *Results of mechanical testing conducted on samples taken from each group. Samples from the empty defect (ED) group were not subjected to mechanical testing since in most samples there was either no tissue at all within the defect or a thin, incomplete layer of fibrous tissue. Normal bone taken from intact skulls was tested and included in the graph for comparison.*

4.4.6 Histology

4.4.6.1 Histological Observations

Upon observation of light microscopy images (Figure 4.8), the empty defect (ED) group showed minimal healing. The autograft (AG) group showed that tissue with a fibrous appearance had grown between the autografted bone and the defect rim.

The transplanted onlay (TO) group showed complete bridging of the defect, with new bone spanning the entire defect and occupying the area that was previously occupied by the fixation screw, with a thickness comparable to that of the native, uninjured bone beyond the defect. Upon further magnification (Figure 4.9), new tissue was seen to resemble mature bone, and further magnification revealed cells with morphologies resembling those of osteoblasts and osteoclasts

The direct placement (DP) showed growth of a thin, incomplete layer of new bone that did not span the entire defect, with multiple blocks of unresorbed monetite present, and an abundant ingrowth of fibrous tissue between the remaining monetite blocks.

The subcutaneous transplant (ST) group showed a similar pattern of thin, incomplete bone growth that was sparse and interrupted in nature, however, most of the monetite remained unresorbed in one large block. To determine the presence of vascular ingrowth in the subcutaneous transplant (ST) model, a sample was decalcified and embedded in paraffin wax. and was then stained with hematoxylin and eosin (H&E), and CD34 (Figure 4.10). Multiple blood vessels varying in size were seen to be dispersed along the sectioned surface, however, quantification of the blood vessels revealed that a higher number in the periphery compared to the center (Figure 4.11).

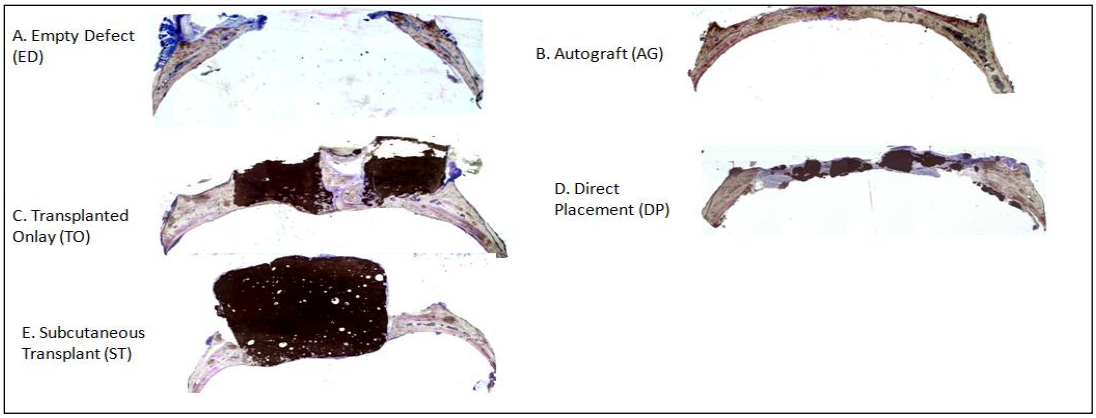

Figure 4.8 *Coronal histological sections from each group. Stain: Basic Fuschin and Methylene Blue. Magnification: x2.5*

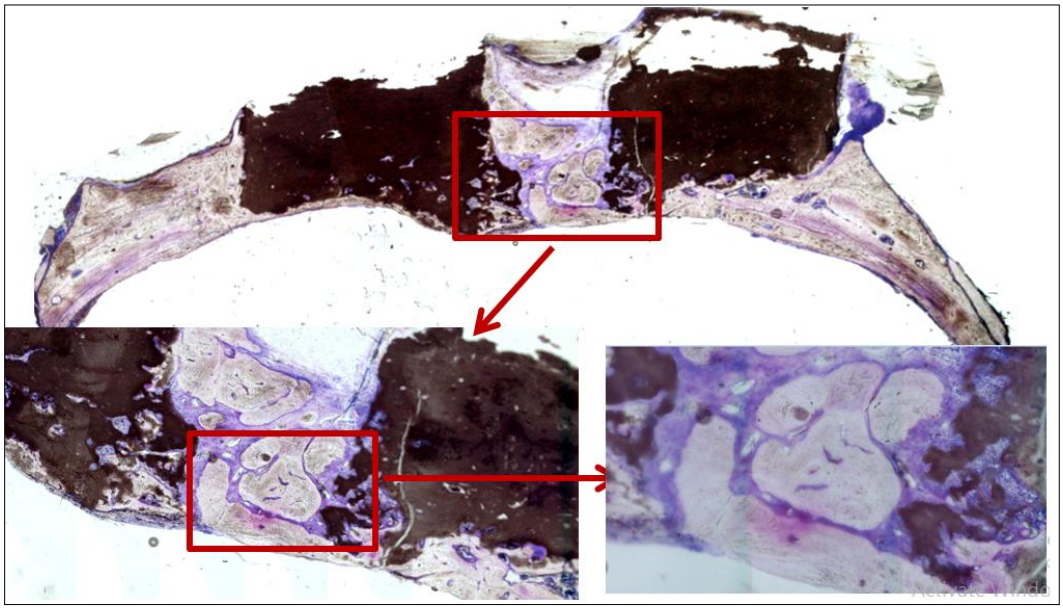

Figure 4.9 *Higher magnification histological images of an a sample from the transplanted onlay (TO) group. Top image: Original magnification x2.5. Area highlighted in the square is the empty space that was left behind by the screw, now seen to be occupied by new bone tissue. Bottom Left: Higher magnification (x5) of area within red square. Bottom Right: higher magnification of adjacent image (x10)*

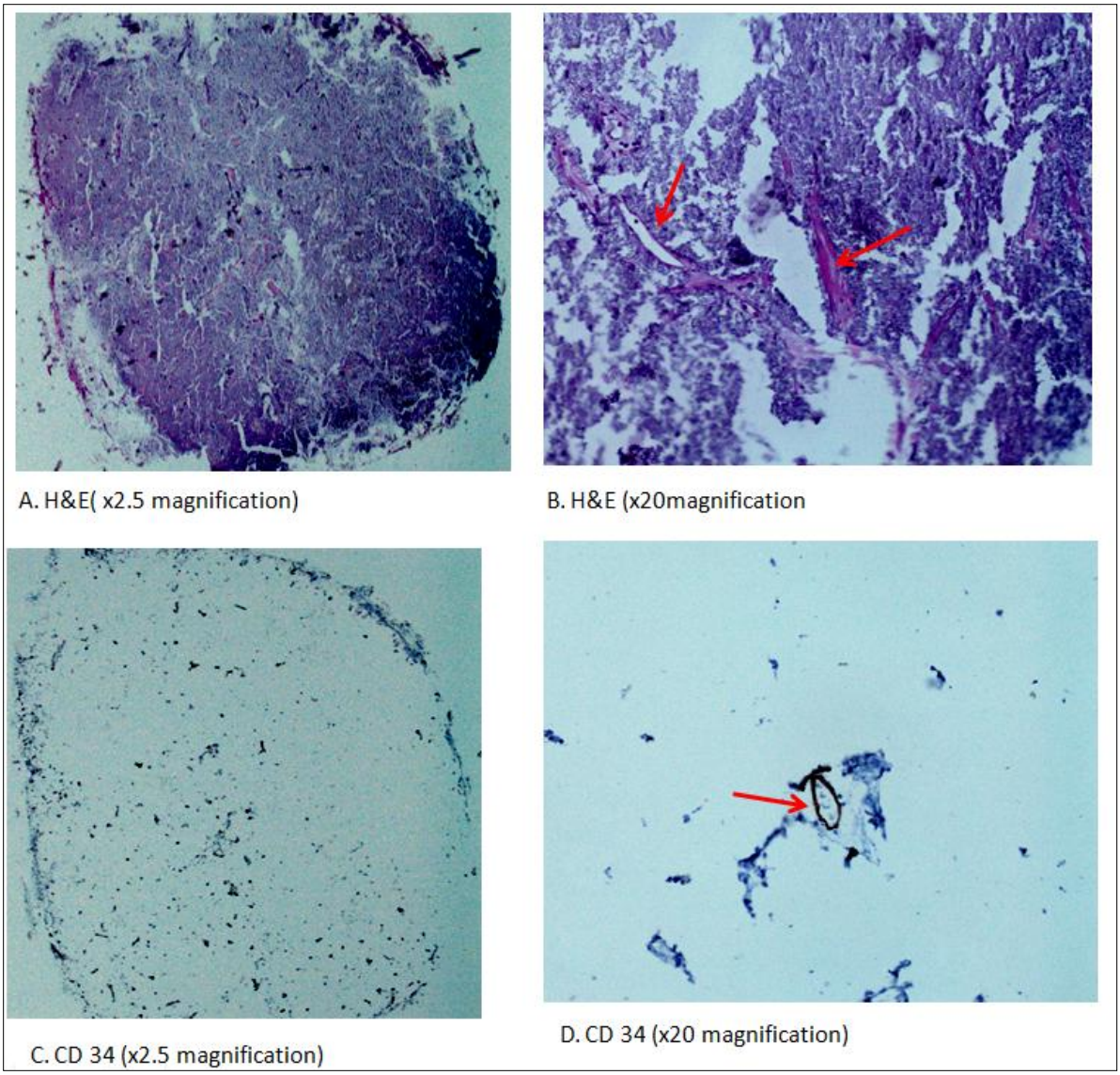

Figure 4.10 *Sample from the Subcutaneous Transplant (ST) group stained with H&E (top row), and CD34. Multiple blood vessels can be seen in both sections (arrows).*

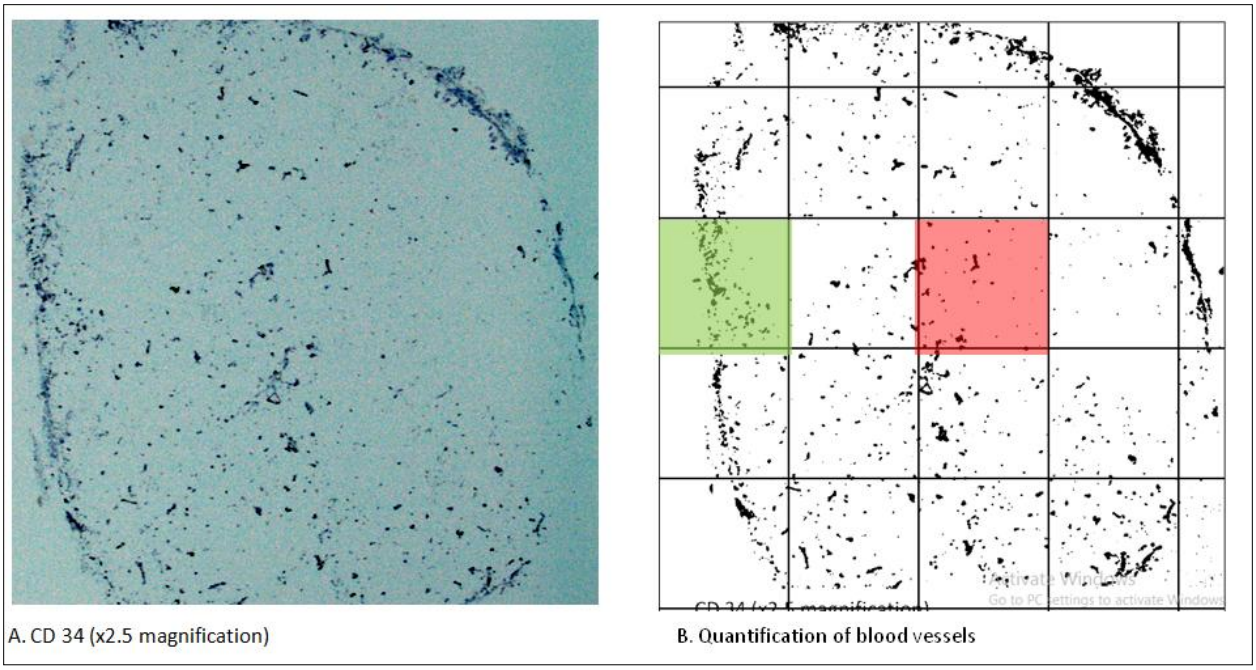

Figure 4.11 *Quantification of the number of blood vessels in a coronal section of a CD34-stained sample from the subcutaneous transplant (ST) group. A: Histological slide prior to image processing. B: Histological slide after alteration of color threshold to facilitate quantification of blood vessels. Vessels counted in the periphery (green) were found to be 118/square, and vessels counted in the center (pink) were found to be 52/square.*

4.4.6.2 Histomorphometry

Histomorphometric analysis (Figure 4.12) showed that the highest amount of new bone occurred in the transplanted onlay (TO) group (7.7 ± 0.3 mm^2), followed by the direct placement (DP) group (4.1 ± 0.4 mm^2). The autografted bone (AG) group had 1.9 ± 0.5 mm^2 of new bone, whereas the subcutaneous group had 3.8 ± 0.6mm^2 new bone. The empty defect (ED) group had the least amount of new bone growth (1.3 ± 0.3 mm^2).

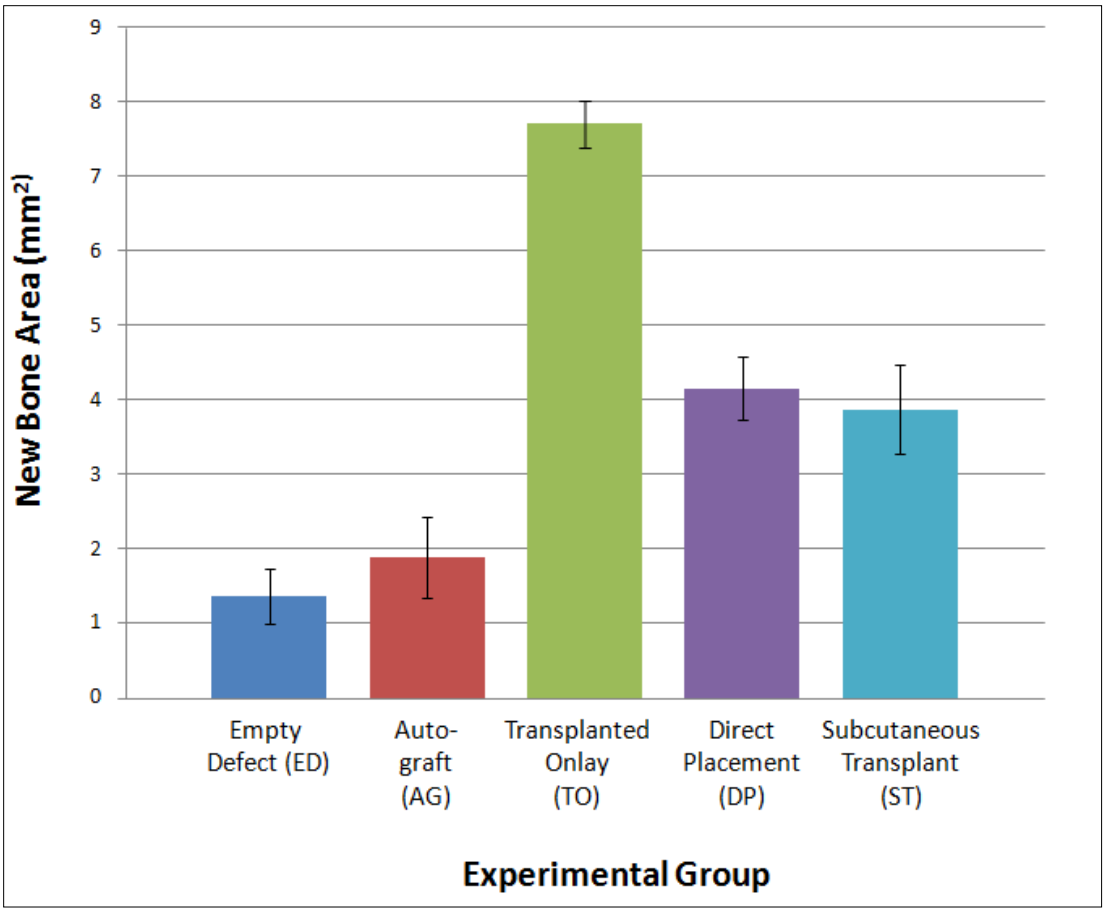

Figure 4.12 *Area of new bone ingrowth in* mm^2 *calculated by histopmorphometric analysis. Columns represent the mean area of new bone formed in each group, while bars represent the standard error of the mean (SEM). A two-tailed t-test was conducted to compare the area of new bone in each group to the empty defect (ED group), which served as the negative control. A P-value of < 0.05 was considered statistically significant. The results were as follows: Empty defect (**ED**): 1.3* ± 0.3 mm^2, *Autograft (**AG**): 1.8* ± 0.5 mm^2 *0.5, Transplanted onlay (**TO**): 7.7*± 0.3 mm^2, *Direct placement (**DP**): 4.1* ± 0.4 mm^2, *Subcutaneous transplant (**ST**): 3.8* ± 0.6 mm^2. A one-way analysis of variance (ANOVA) with Tukey's post-hoc HSD test was then conducted to determine whether any differences existed between the groups. A statistical significance (P-value <0.05) was found between all of the pairs except for two: ED and AG, and DP and ST.

4.5 Discussion

The technique of bone growth induction by vertical bone augmentation is often employed in dentistry,[71,72] with numerous studies reporting the use of calcium-phosphate biomaterials such as tricalcium phosphate, calcium sulphate, and synthetic hydroxyapatite. [73-77] However, to date, the applicability of this technique in treating larger defects, such as calvarial critical-size defects, remains largely unexplored, possibly in part due to skepticism that a technique meant for repairing small, mandibular defects will not be successful in producing an amount of bone sufficient in quantity and quality to treat larger calvarial defects. Since synthetic calcium phosphates that mimic the structure of normal bone have previously demonstrated their ability to be biocompatible in the human body and to integrate well into bone defects[78,79], it is therefore not surprising to observe the amount of past and ongoing research in utilizing these biomaterials to attempt treat various types of bone defects. [87-91] While there has been encouraging progress in using these materials to treat critical-size defects, the common denominator in some of the more successful techniques seems to be the inclusion of certain biological components with the biomaterial, such as bone marrow, bioactive growth factors, or stem-cells.[1,6,94-96] This adds a layer of complexity to the treatment of skull defects, as many of these biological components are associated with risks and drawbacks, such as tumorigenic potential, inflammatory side-effects, unpredictable bone formation, and lack of cost-effectiveness.[94-97] For these reasons, we sought to develop a technique that is simple, reliable and easily reproducible by avoiding any biological components that might yield unpredictable or inconsistent results.

Bone formation at the graft site is crucial for the success of bone defect reconstruction. While all of the experimental groups resulted in varying degrees of bone growth (Figure 4.8), the amount of bone shown in the transplanted onlay (TO) group was significantly larger than the other

groups. This could be appreciated both visually (Figure 4.8) and upon histomorphometric analysis and quantification (Figure 4.12). In this group (TO), it was interesting to observe on micro CT imaging a sizeable amount of radio-opaque material occupying what should have been an empty hole left by the screw (Figure 4.4). This was confirmed by histology to be bone tissue (Figure 4.8-C), and was found to be comparable in thickness to the normal, un-injured bone on either side of the defect. The rest of the experimental groups consistently showed only a thin layer of incomplete bone that did not always bridge the entire defect (Figure 4.6 D,E).

Given the importance of maintaining the structural integrity of the calvarial vault to ensure protection of the underlying brain, it was important to assess the mechanical ability of the newly grown bone to resist load forces that are normally applied to the skull. Using our custom0zed push-out testing apparatus with the Instron machine (Instron, 5544), we conducted preliminary testing of some our samples to determine the extent of load force they could withstand. While autografted bone seemed to withstand the most pressure (~ 55N), the first crack occurred at around 10N (Figure 4.7), whereas in the transplanted onlay (TO) sample, the first crack occurred at around 25N, Samples from the direct placement (DP) and subcutaneous transplant (ST) groups withstood the least amount of force before cracking. Due to the need to preserve the samples for histological testing, we elected to test one sample from each experimental group until occurrence of the first crack. This resulted in a small sample size (N=1) that did not meet statistical significance, in which samples were not tested until complete breakage took place. However, from a preliminary standpoint, these results could indicate the ability of the TO group to perform at least as well as the autografts, although further testing needs to be done to confirm this.

Although the subcutaneous model (ST) did not result in much bone formation, it was a group that yielded other interesting findings. In this group, we originally intended to determine the

effects of the subcutaneous environment on the implant. The purpose of having this group was to act as a control group to the transplanted onlay (TO) group. During the onlay phase of the TO model, the implants are exposed to both the overlying subcutaneous environment and the underlying osseous environment, whereas in the ST group, the implants are only exposed to the subcutaneous environment. While samples in this group resulted in the least amount of bone formation (Figure 4.8), they were observed to be "bloody" in appearance during harvesting *in vivo* from their subcutaneous pouches (prior to transplant in the skull) (Figure 4.3-B), therefore, we decided to stain a sample from this group to ascertain the presence of blood vessels. The resulting histological section showed complete infiltration of the implant with numerous blood vessels (Figure 4.10), which could be a possible explanation to their hemorrhagic appearance, If this finding is replicated in future work, it may reveal that the subcutaneous environment is optimal for the vascularization of calcium-phosphate implants. It could be worthwhile to test this effect on implants that have been kept as skull onlays, prior to transplanting into a skull defect, as vascularization may indicate resorption rather than bone formation, indicating the need for bone quantification. Alternatively, another way to further explore this finding would be to first place the implants subcutaneously to allow blood vessel infiltration, then proceed to the skull onlay phase, before finally transplanting into a skull defect. However, this finding first needs to be replicated to ensure that it is not incidental, and similar blood vessel staining must also be performed in the other groups.

The current standard of care in the treatment of cranial defects is the use of bone autografts, although many surgical techniques combine the use of autogenous, allogeneic, and synthetic materials.[1] While the preferred method continues to be autologous bone grafting, it is not always a feasible option, and may at times be associated with significant donor-site morbidity.[1,5]

Synthetic materials have the advantage of avoiding these issues by being theoretically unlimited in supply, and obtaining them does not involve donor-site morbidity.[6] However, their efficacy is often limited by infection, structural failure, graft migration, and unsatisfactory aesthetic outcomes. [106,107]

In our study, we sought to determine whether the technique of monetite-induced vertical bone augmentation might have clinical applicability in the realm of cranial reconstruction of critical-sized defects. We were able to demonstrate that our method of bone growth induction has the ability to regenerate bone in critical-sized defects in adult rats. A major advantage of our method is that it does not involve the use of any biological components (such as stem cells, growth factors, or bone marrow). Furthermore, it combines the most advantageous properties of all three current treatment strategies as follows:

- Bone autografts: By virtue of the identical composition of the induced bone to normal bone tissue, our technique generates autologous bone-grafts that do not intrinsically originate from the patient. At the end of the onlay-transplant procedure, new bone was grown in a controlled manner and in an easily accessible location of our choice. Therefore, harvesting this new bone once it has grown will cause considerably less morbidity than harvesting rib or iliac crest autografts.

- Allogeneic grafts: While the main advantage of such grafts is that they avoid donor-site morbidity in addition to being available in greater amounts than autografts, the issues of infection and graft rejection are two significant drawbacks that are difficult to overcome.[106] Our technique greatly minimizes the risks of infection, as our implant is prepared and handled in a

strictly sterile environment, and is autoclaved prior to use. Furthermore, because it does not contain any biological components, there is no risk of graft immune rejection.

- Synthetic grafts: Prosthetics have the major advantage of being unlimited in supply. However, similar to allogeneic grafts, their usage is sometimes hampered by infection and structural failure, which often necessitates their removal. Our implant starts out as a easily manufactured synthetic implant, but by the end of the onlay-transplant procedure, it undergoes a transformation that results in resorption of a large portion of the original implant, during which it is replaced by new, mature, stable bone. It therefore combines the advantages of synthetic implants with autografts, by starting out as a synthetic implant and then transforming into an "autograft".

4.6 Conclusion

Despite advances in contemporary treatment strategies, donor site morbidity and suboptimal restoration of form and function continues to drive the development of novel approaches to treat calvarial defects. Our project proposes a unique method of inducing native bone to locally produce excess bone in a controlled manner. We were able to successfully induce bone growth into a monetite implant, without the use of exogenous growth factors or genetic manipulation. This new bone had the appearance of normal, mature bone, and behaved as such. Using this technique, this excess bone was successfully excised and used as "customized" autologous transplants to treat critical-size skull defects, thereby circumventing the need for the other currently used techniques, and avoiding the risks and drawbacks associated with these invasive and often unsuccessful methods.

Chapter 5 - Final Discussion and Thesis Conclusion

Major challenges associated with craniofacial reconstruction and repair continue to drive the search for innovative treatment strategies that circumvent the need for autografting, which in addition to carrying the risk of morbidity, is also not always a feasible option, especially in the pediatric population and in the severely injured.

The work described in this thesis described a novel approach to the treatment of bone defects. Bone was successfully grown within a micro-porous monetite onlay without the use of growth factors or genetic manipulation. This excess bone could be excised, fashioned as needed, and used as "on demand" autologous transplants to treat critical-size skull defects. This technique has the potential to eliminate or reduce the need for autograft harvesting. The capacity to 3D print the graft offers the potential to fit the onlay recipient surface anatomy, and match the defect in size and shape.

The future of this project lies in exploring the applicability of this technique in treating defects, that are larger than critical-size, and that are irregular in shape, such as those arising from trauma.. Other variables can also be examined. An important variable of interest is implantation time, and to what extent this can be minimized. Furthermore, additional qualitative tests can be conducted on bone to determine the which properties lead to the most favorable outcome. For example, once excised, can the newly augmented bone be safely stored for a period of time prior to implantation into a defect? This would allow for prior growing and harvesting of the bone in situations where the physician knows in advance that the patient will have a skull defect in the

future (Eg: prior to a tumor removal), and will thus help in pre-operative planning. Our model will also allow for future testing in various simulated clinical scenarios, such as the following:

1. Use of young (growing) animals to simulate the pediatric patient.

2. Use of an irradiated skull to simulate a skull weakened by radiotherapy (Eg following tumor resection)

3. Creating multiple, large defects that are irregular in shape to simulate a trauma patient. Use of our 3-dimensional printers will facilitate creation of implants that match the size and shape of the defect (Figure 5.1)

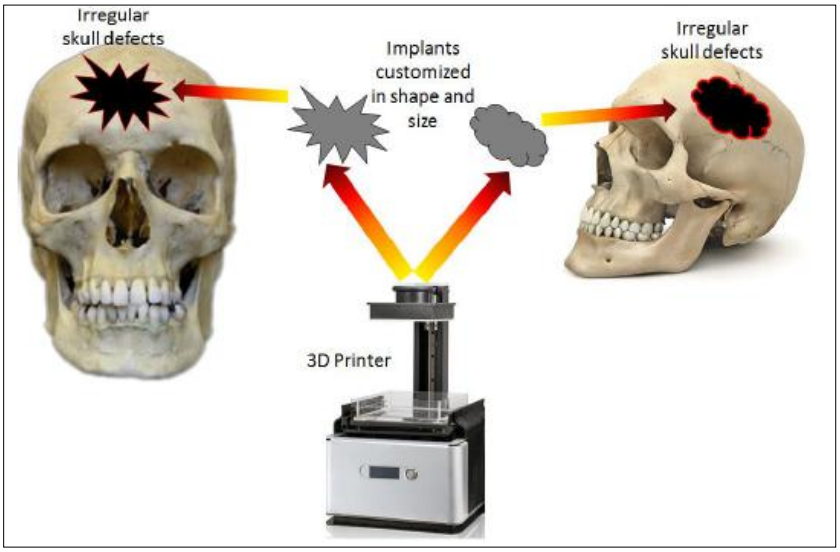

Figure 5.1 Creation of "customized" implants using our 3D printer

Chapter 6 - Bibliography

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