Effectiveness of Masquelet Technique with β-TCP Grafts in Critical Sized Bone Defects

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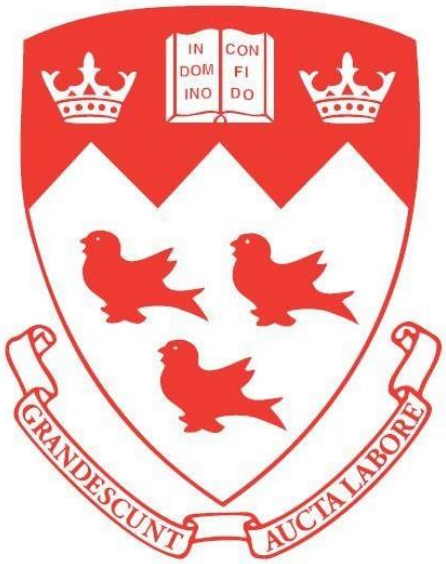

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Abstract

Introduction:

Massive bone defects are a common challenge facing orthopedic surgeons. These defects are unable to heal naturally despite having surgical stabilization and require further reconstruction. Massive defects are defined as having a defect measuring >6 cm in long bone and >4 cm in the mandible. Critical sized defects can be treated with non-vascularized grafts, while massive defects are best treated with vascularized grafts through procedures such as, vascularized fibular grafts, Ilizarov Technique and Masquelet Technique.

The Masquelet Technique is an important clinical method for management of massive defects due to osteogenic and angiogenic properties of the fibrous membrane created around the defect site. The technique involves two stages. A spacer is placed in a bone defect that will induce the formation of the fibrous membrane. The second stage of the procedure involves the removal of the spacer and placing a graft at the defect site.

The objectives of this project included, establishing that a 3.5-cm ulnar defect is a massive defect due to its inability to heal spontaneously, the ability of the Masquelet Technique to treat massive long bone defects and the efficacy of synthetic bone fillers combined with the Masquelet Technique in massive defects.

Methods:

Surgical procedures were performed on both ulnas of eight New Zealand rabbits aged six months weighing around 3.5 kg. The first stage involved creating a defect with placement of a spacer, which was left in for 4 weeks. This was followed by a second procedure that involved removing

the spacer and placing grafts at the defect site for an additional 6 weeks, or for other samples, that were left empty for the entirety of the experiment (10 weeks).

A pilot experiment was performed involving the creation of a 2-cm defect that included two ulnar samples (n=2) that contained autografts and six samples (n=6) that were empty defects.

The experimental group had 3.5 cm defects, a subset of ulnas had empty defects (n=4), another group had autografts (n=4) (harvested initially during the first step of the Masquelet Technique), with another group having a PTFE membrane added (n=3). Another subset of ulnar defects was filled with β-TCP blocks (n=6) and granulated β-TCP with PTFE (n=2) and one block with PTFE (n=1).

Results:

Micro-CT analysis of the samples has shown that two out of six of the 2-cm empty defects have shown a significant amount of bone regeneration. While the empty defects of the 3.5-cm samples have shown minimal amounts.

The 3.5-cm samples containing autografts and β-TCP without PTFE have shown comparable amounts of defect volumes, 593.36mm^3 (SD 147.35) and 616.93mm^3 (SD 86.04) respectively. When the PTFE membranes were added to other samples (autografts, β-TCP block and granules) it was shown that the amount of defect volumes where decreased, compared to the samples not having PTFE membranes.

Histological analyses have shown similar results. Samples without PTFE stained with TRAP and methylene blue showed an increase in TRAP activities and bone tissue formation in the autograft and β-TCP groups while showing significant decrease in the empty defect group. PTFE containing

samples have shown a slight decrease in TRAP activities but similar bone tissue formation on methylene blue staining compared with the PTFE samples.

Conclusion:

Although our results have shown new bone regeneration in the autograft and experimental controls overall, there was no evidence of complete osteo-integration and resorption. The literature has demonstrated that both the Masquelet Technique and the use of β-TCP showed promising results in various research studies and are useful tools and methods for surgeons clinically.

The most probable explanation is that a 6-week implantation time was not sufficient enough for the implanted materials to resorb and integrate in a massive defect spanning around 40% of the total length of the ulna.

Résumé

Introduction:

Les défauts osseux massifs ne peuvent pas guérir naturellement malgré une stabilisation chirurgicale et nécessitent une reconstruction ultérieure. Les défauts massifs sont définis comme ayant un défaut mesurant > 6 cm dans un os long et > 4cm dans la mandibule. Les défauts de taille critique peuvent être traités avec des greffes non vascularisées, alors que les défauts plus volumineux sont mieux traités avec des greffes vascularisées au moyen de procédures telles que des greffes fibulaires vascularisées, la technique Ilizarov et la technique Masquelet.

La technique Masquelet est une méthode clinique importante pour la gestion des défauts massifs dus aux propriétés ostéogéniques et angiogéniques de la membrane fibreuse créée autour du site du défaut. La technique comporte deux étapes. Un spacer est placé dans un défaut osseux qui induira la formation de la membrane fibreuse. La deuxième étape de la procédure consiste à retirer l'entretoise et à placer un greffon sur le site du défaut.

Méthodes:

Les chirurgies ont été effectuées sur les deux ulnes de huit lapins néo-zélandais âgés de six mois et pesant environ 3.5 kg. La première étape consistait à créer un défaut avec la mise en place d'une entretoise, qui a été laissée pendant 4 semaines. Cette opération a été suivie d'une seconde procédure consistant à retirer l'entretoise et à placer les greffons sur le site du défaut pendant 6 semaines supplémentaires, ou pour d'autres échantillons, qui ont été laissés vides pendant toute l'expérience.

Une expérience pilote a été réalisée, impliquant la création d'un défaut de 2 cm comprenant deux échantillons ulnaires (n = 2) contenant des autogreffes et six échantillons (n = 6) qui étaient des défauts vides.

Le groupe expérimental présentait des défauts de 3,5 cm, un sous-ensemble d'ulna présentait des défauts vides (n = 4), un autre groupe avait des autogreffes (n = 4) (récoltées initialement au cours de la première étape de la technique Masquelet), avec un autre groupe présentant une membrane en PTFE ajouté (n = 3). Un autre sous-ensemble de défauts ulnaires a été rempli de blocs β-TCP (n = 6) et de β-TCP granulé avec PTFE (n = 2) et un bloc avec PTFE (n = 1).

Résultats:

L'analyse micro-CT des échantillons a montré que deux des six défauts vides de 2 cm ont montré une régénération osseuse importante. Alors que les défauts vides des échantillons de 3.5 cm ont montré des quantités minimales.

Les échantillons de 3.5 cm contenant des autogreffes et du β-TCP sans PTFE ont montré des quantités comparables de volumes de défauts, respectivement 593,36 mm3 (SD 147,35) et 616,93 mm3 (SD 86.04). Lorsque les membranes PTFE ont été ajoutées à d'autres échantillons, il a été montré que le volume des défauts était réduit par rapport aux échantillons ne comportant pas de membranes PTFE.

Les analyses histologiques ont montré des résultats similaires. Les échantillons sans PTFE coloré au TRAP et au bleu de méthylène ont montré une augmentation des activités du TRAP et de la formation de tissu osseux dans les groupes autogreffe et β-TCP, tout en montrant une diminution significative du groupe de défauts vides. Les échantillons contenant du PTFE ont montré une légère diminution des activités TRAP mais une formation de tissu osseux similaire sur la coloration au bleu de méthylène par rapport aux échantillons de PTFE.

Conclusion:

Bien que nos résultats aient montré une nouvelle régénération osseuse dans l'autogreffe et dans l'ensemble des contrôles expérimentaux, il n'y avait aucune preuve d'ostéo-intégration ni de résorption complètes. La littérature a démontré que la technique de Masquelet et l'utilisation de β-TCP ont donné des résultats prometteurs dans diverses études de recherche et constituent des outils et méthodes utiles pour les chirurgiens sur le plan clinique.

L'explication la plus probable est que le temps d'implantation de six semaines n'était pas suffisant pour permettre aux matériaux implantés de se résorber et de s'intégrer dans un défaut massif.

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Contribution to Original Knowledge

The work described in this manuscript is original and performed by the author. This study described the use of β-TCP bone substitutes in treatment of large bone defects with the utilization of the Masquelet Technique.

Contribution of Authors

Dr. Mohamed Nazhat Al Yafi: protocol development, performing experiments, samples processing, results analysis and drafting manuscript.

Dr. Edward Harvey: supervisor and protocol development.

Dr. Jake Barralet: supervisor, protocol development and manuscript revision.

Chapter 1:

Bone Biology and Fracture Healing

Introduction:

To understand the process of bone healing and how it regenerates, it is essential to appreciate how a normal healthy bone would heal. Bone healing and its regeneration directs further research in developing new treatment strategies such as the induced membrane and distraction osteogenesis techniques. It is important to note that the practical application of these treatment strategies is not an easy task due to the complexity of the numerous variables involved, such as the size of the bone defect, the state of the surrounding periosteum and soft tissues and the overall health of the individual.[1]

Normal Structure and Cells of Bone:

The human skeleton provides several important functions, such as maintenance of its structural integrity and the protection of other organs and mobility.[1] The bone is a rigid structure, but it is vital for the maintenance of other physiological processes that include mineral homeostasis (due to calcium and phosphorus storage), acid base balance and hematopoiesis (due to it containing bone marrow).[1, 2] The adult skeleton is composed of lamellar bone which is composed of a harder outer layer of cortical and an inner spongy cancellous bones with various ratios depending on the site of the bone.[1]

Running along the axis of the bone is the cylindrical Haversian system, which is the principal unit in cortical bone. At the heart of this system is the Haversian canal that carries the nervous and vascular supply to the bone and is surrounded by multiple layers of concentric

lamellae, where between each layer, lacunae are found and contain osteocytes.[2, 3] The cortical bone progressively becomes thinner and decreases in mass due to ageing and to the remodelling process that becomes more prominent at this stage.[2] Cancellous bone (also named trabecular bone), due to the honeycomb like trabecular networks that it forms with bone marrow filling its spaces.[1]

Two similar and essential components to the normal maintenance of bones, includes the periosteum and endosteum. Periosteum covers the outer surface of cortical bone whilst endosteum covers the inner surfaces, where the new bone is formed from the outer surface and where the old bone is resorbed and broken down from the inner surface, to prevent bone thickening.[2]

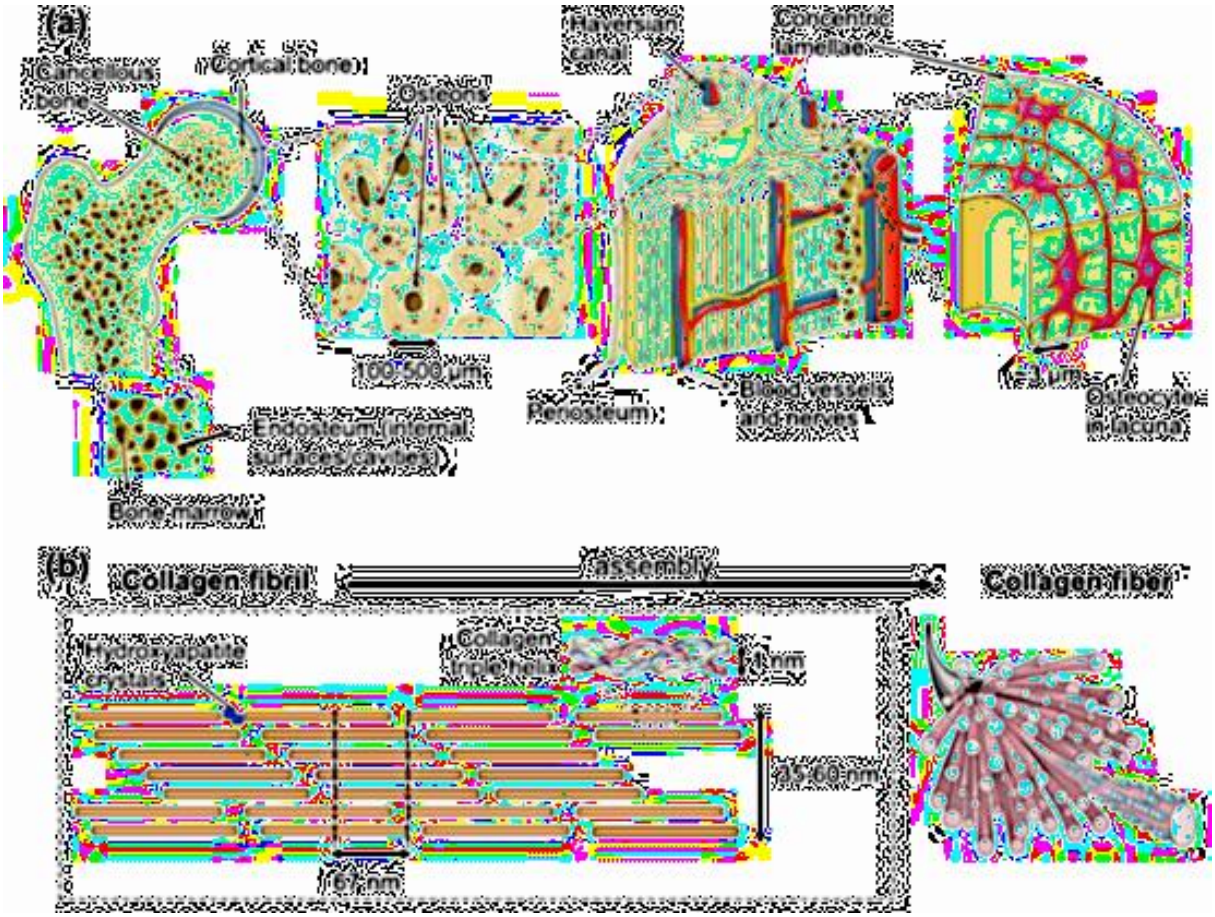

Figure 1. Interscale representation of bone. (a) A macroscopic-to-microscopic view of cancellous and cortical bone. Bone marrow lies in the cavities of cancellous bone, which are lined by the endosteum structure. Tightly packed osteons integrate cortical tissue, which is covered by the periosteum membrane. Osteons are formed by Harversian canals, which contain blood vessels and nerve tissue, surrounded by concentric lamellae that show thicknesses of circa 3 µm. Osteocytes reside in the osteon inside lacuna structures. (b) Bone tissue is constituted at the nanometric scale by collagen fibers that comprise assembled collagen triple helix structures that give rise to the collagen fibril, with a characteristic periodic spacing of 67 nm, and gaps of 40 nm where the mineral component of bone is located[1].

Osteoblasts:

Averaging around 4-6% of all bone cells[4], osteoblasts are one of the specialized cells that originate from osteoprogenitor cells which in turn originate from multilineage potential mesenchymal stem cells in the bone marrow.[5] In order for these cells to differentiate into the

osteoblastic lineage, certain pathways, proteins and transcription factors are essential.[2, 4] Fibroblast growth factors (FGFs) play a role in osteoblast differentiation. Interestingly, the disruption of FGF2 has not only been found to result in decreased osteogenesis and bone formation but also can change the lineage of mesenchymal stem cells to non-osteogenic cells.[6, 7]

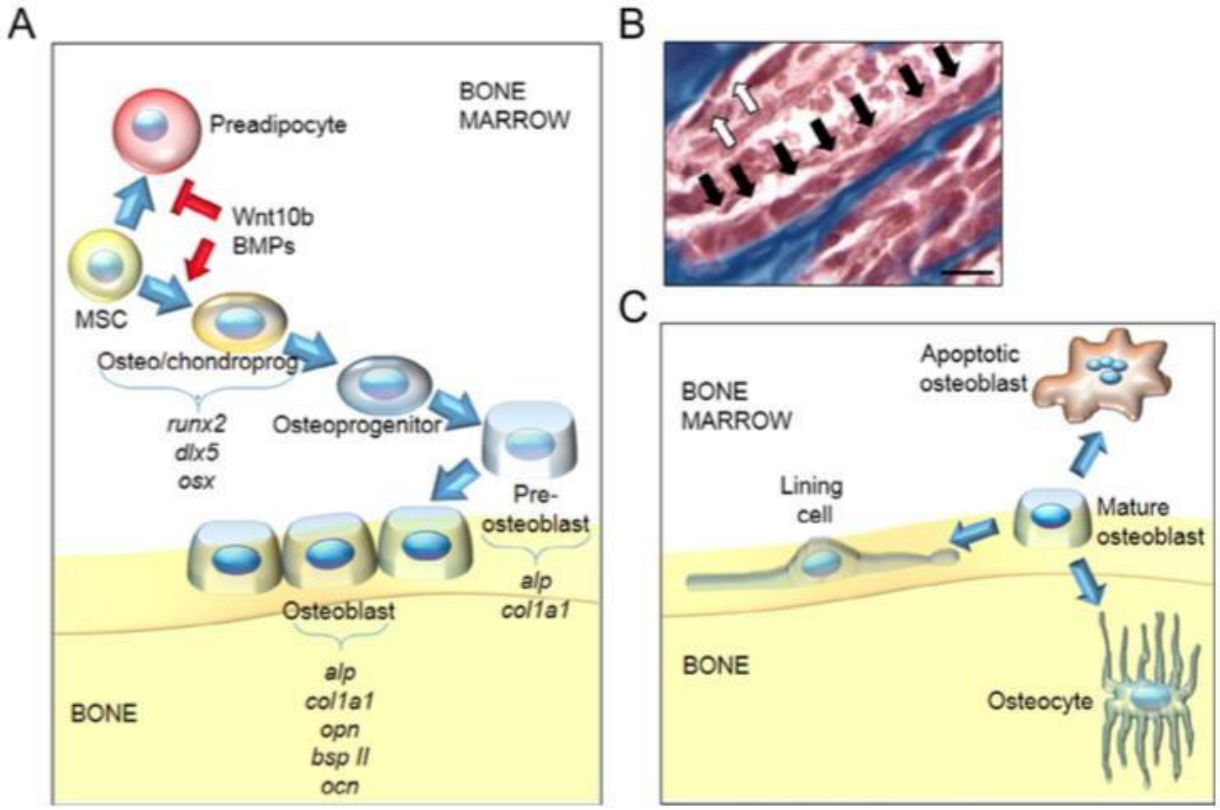

Figure 2. Osteoblast differentiation, morphology and fate. (A) Schematic representation of the multistep process of osteoblast differentiation (MSC = mesenchymal stem cell). (B) Histological section of a mouse tibia, stained with the Masson's trichrome. Black arrows indicate a row of osteoblasts on a bone trabecula (Blue staining), white arrows indicate bone-lining cells. Bar = 10 lm. (C) Schematic representation of the possible fate of a mature osteoblast[4].

The two main functions of osteoblasts include the production of bone matrix and balancing the rate of bone synthesis and resorption, by interfering and acting as a regulator of osteoclast production and differentiation.[4] Collagenous proteins are the most abundant proteins in bone, and bone matrix is mostly made up of type I collagen, while non-collagenous proteins and proteoglycans are found in lesser amounts. These materials that make up the osteoid (non-mineralized matrix) and produced by osteoblasts, undergo mineralization, consisting mostly of hydroxyapatite that is deposited between collagen fibrils extracellularly; which in turn results in providing strength and stiffness to bones.[2, 4]The second osteoblastic function is the maintenance of a balance between bone formation and resorption. This occurs due to several interactions between osteoblasts and osteoclast precursor cells. These include RANKL/RANK pathway and osteoblastic production of various factors such as, macrophage-colony stimulating factor (MCSF), osteoprotegerin, Il-6, TNF-alpha.[4, 8, 9]

Osteocytes:

These are the terminally differentiated cells in the osteoblast lineage that have the largest, longest life spanned and are the most populous cells in bone and serve to maintain its structure.[2, 4] They are found individually in lacunae surrounded by bone matrix and connected with other cells through canaliculi.[10]

Osteocyte differentiation journey takes osteoblasts into several stages before turning into old mature osteocytes when it becomes surrounded by mineralized bone tissue. The first transformation begins, when the bone matrix is still unmineralized, as type I then II preosteocytes where the cells shrink in size and starts developing cytoplasmic processes that will connect them to other cells. Further reduction in cellular size and during the start of the process of matrix

mineralization is when the cells become into type III preosteocyts. Finally, when matrix mineralization is complete the end result is a mature osteocyte.[4]

One of the roles of osteocytes is its involvement in bone formation and resorption by regulating osteoblast and osteoclast differentiation and functioning.[11] Osteocytic cell death or damage have been shown to increase bone resorption due to increase osteoclastic activity, as evidenced by numerous animal studies.[12, 13] This may be due to the loss of factors and signals that inhibit bone resorption, such as transforming growth factor-beta (TGF-β), a signaling protein. Another factor that plays a role is the resorption promoter, the TNF membrane protein, receptor activator of nuclear factor kappa-B ligand (RANKL), which is mainly produced by osteocytes and that its deletion results an imbalance in favor of bone formation resulting in osteopetrosis.[11]

Osteocytes have both stimulatory and inhibitory effects. Nitric oxide (NO) is one of the factors produced by osteocytes. Mancini et al[14], have showed that production of increased quantities of NO leads to osteoblastic cell death, while lower NO quantities enhances their production. Other animal studies have shown that the protein insulin-like growth factor-1 (IGF-1), produced by osteocytes in response to growth hormone (GH) and mechanical stimulation, acts on osteoblast resulting in increased bone formation and density[11, 15], while its deficiency negatively affects osteogenesis.[16]

On the other hand, two osteocyte-mediated factors that have osteoblastic inhibitory effects are sclerostin and dickkopf-related protein-1 (DKK-1), a protein produced during embryogenesis. Wnt signaling pathway (a signal transduction pathway involved in cellular functioning and differentiation), is a major pathway that leads to osteoblastic differentiation. But several studies have shown that sclerostin and DKK-1 have an inhibitory role, where they bind to lipoprotein

receptor-related proteins located on the cellular plasma membranes and therefore blocking the continuation of the Wnt pathway ultimately inhibiting osteoblast differentiation and bone formation.[17-19] It is also worthy to note that these two factors are sensitive to mechanical stimulation, as increased loading increases bone formation mediated by deficient production of sclerostin and DKK-1 and vice versa is correct when loading decreases.[11, 20, 21]

Osteoclasts:

Osteoclasts are large cells that contain multiple nuclei belonging to the monocyte/macrophage lineage.[22] The resorption functions are not only important locally for making way for the development of new bone but also systemically to maintain a normal range calcium blood levels.[23] The mechanism of bone resorption commences when osteoclasts isolate a specific area to dissolve. This allows targeted areas of resorption and confines the dissolving materials. Two changes occur at the cellular membrane level simultaneously, at the sealed zone side the membrane turns into the characteristic ruffled shape where the dissolving materials are secreted and on the opposite side the functional secretory domain (FSD) is formed, where the dissolved components are transported through.[24]

The bone matrix (mainly hydroxyapatite) and organic components (mainly collagen) are dissolved due to the secretion of hydrochloric acid, matrix-metalloproteinases-9 (MMP-9) and cathepsin K (CtsK).[22, 25] The acidic environment dissolves hydroxyapatite releasing calcium, phosphate and bicarbonate which are transported efficiently transcytotically through the FSD, as accumulation of these products hinder the efficacy of the resorption process.[22] In addition, this acidity acts as an activator for the lytic enzymes that dissolve organic components, namely MMP-

9 that is expressed in osteoclasts and CtsK against type I collagen.[25]

Fracture Healing:

Secondary fracture repair is the most common process of fracture healing. There are three main phases for fracture healing which includes, inflammatory, callus formation and remodeling phases. Immediately after a bone fractures, a hematoma is formed where immune cells, followed by mesenchymal stem cells, are recruited to the site of injury.[26] During the 7-day duration of this phase, numerous factors are secreted promoting the recruitment of the necessary pro-inflammatory and pro-angiogenesis cells. Factors expressed include the cellular signaling proteins, tumor necrosis factor-α (TNF-α), Interleukin-1, 6, 11 and 18, that usually peak during the first 24 hours post injury.[27] Simultaneously, fibrin is deposited at the hematoma site, which then undergoes endochondral ossification forming a cartilaginous callus initially that provides some stability to the fracture site.[26, 27]

As angiogenesis is underway in the callus, chondrocytes are terminally differentiated and synthesizes mineralized matrix, which then undergoes calcification. Mesenchymal stem cells are recruited locally and systemically to the fracture site and undergoes differentiation forming the osteoblastic cell lineage that deposits bone into the calcified callus. Furthermore, the already present macrophages differentiate into osteoclasts and starts resorbing the cartilaginous callus, making way for the new woven bone.[26]

Formation of new blood vessels is crucial as it prevents serious complications such as non-union. Once cartilaginous resorption occurs, this paves the way for two pathways to take place, angiopoietin pathway and the more crucial vascular endothelial growth factor (VEGF)

pathway.[27, 28] It has been shown by multiple studies that the expression of angiopoietin factors results in angiogenesis from the nearby periosteal vessels, whereas VEGF is capable of both angiogenesis and vasculogenesis.[27, 29, 30]

Finally, the remodeling phase is essential in restoring the previous physiological properties of bones. This occurs by replacing the previously deposited woven bone with lamellar bone. This process is regulated by factors such as TNF-α, IL-1 and BMPs.[27, 31] There are other systemic factors that play a role such as parathyroid hormone (PTH) which is an important regulator of calcium hemostasis and apparently is crucial for fracture healing, where PTH levels increase as early as 3 days post fracture and it has been noted by animal studies that it enhances the synthesis of a stronger lamellar bone to replace woven bone.[32]

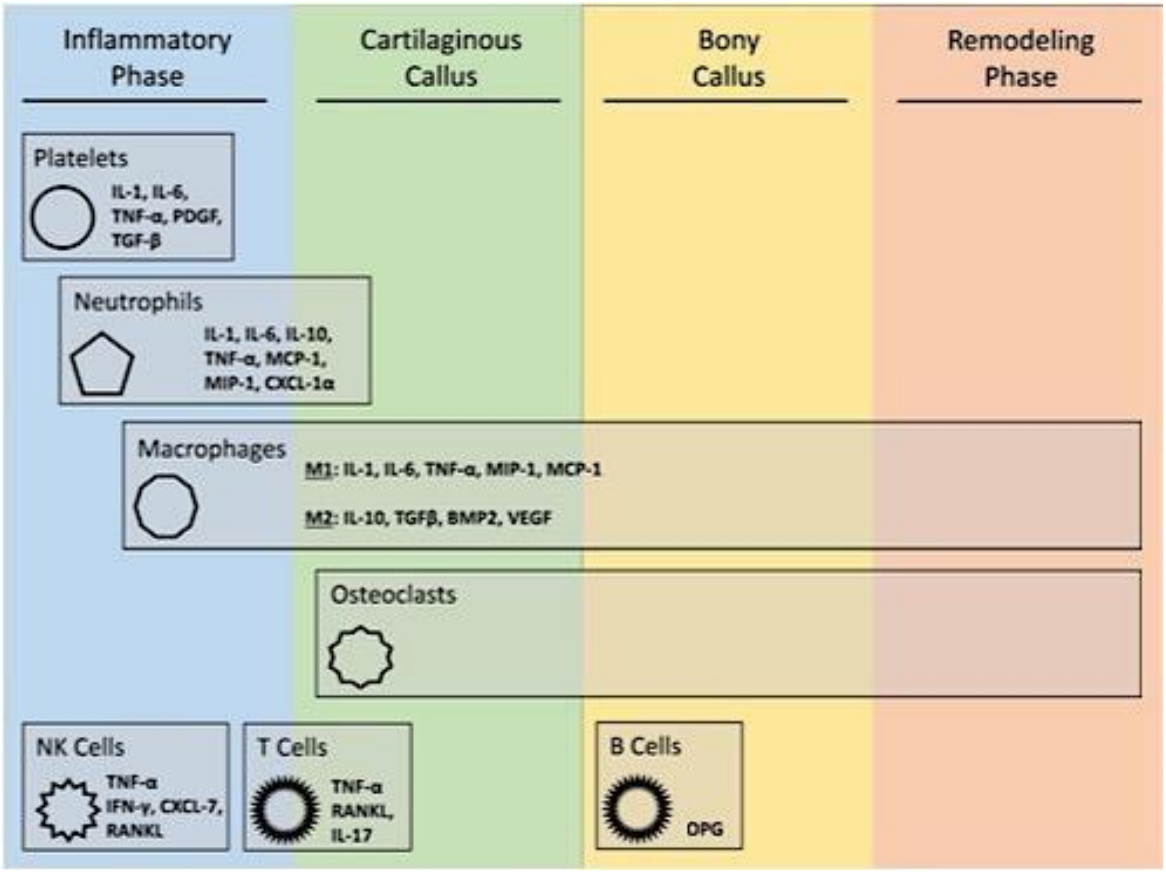

Figure 3. Role of immune cells during fracture repair. Bone fracture healing can be viewed as a four-stage process. Immune cells play important roles throughout this process; however, a majority of their activity occurs during early stages of fracture healing[26].

Mechanical stimulation is also vital as it leads to the buildup of strength of the newly formed bone. With proper physical activity and mechanical loading, improved bone production and remodeling occurs due to that the osteons are stimulated to form and grow in a parallel fashion to the long axis of the bone.[33] It is worthy to note that the remodeling phase is the longest phase in fracture healing, especially in humans, as proper strength maybe achieved after 6 months but it may well take several years depending the various factors involved, age and general health of the patient.[27, 33]

Chapter 2:

The Periosteum. A Review

Periosteum plays a significant role in bone regeneration, albeit the process is not entirely understood. Unfortunately, research has not focused on the periosteum and its osteogenic potential. In this review, we will shed light on this matter to provide a better understanding on the structure and function of the periosteum and its role in bone growth and healing of critical sized defects.

Structure of Periosteum:

Periosteum is a two-layered connective tissue, that envelopes all bony surfaces except for sites of articulation due to the presence of cartilage and sesamoid bones which are encased by tendons[34]. Sharpey's fibres are structures abundant with mainly collagen type III extending from the outer fibrous layer of the periosteum attaching it to bone. It is thought that the function of these fibres is not limited to attaching periosteum to bone but some studies have indicated that they are important factors for bone development and regeneration[35].

Two main layers compose the structure of periosteum which includes, the outer fibrous layer and the inner cellular cambium layer. The functional differences between the two layers were first established by Tang and Chai in 1986[36], and indicated that the cambium layer has significantly higher osteogenic cellular population compared to the poorly populated fibrous layer composed mainly of fibroblasts. The outer layer is further subdivided into two layers corresponding to zones II and III while zone I corresponds to the cambium layer, as described by Squier et al in 1990[37].

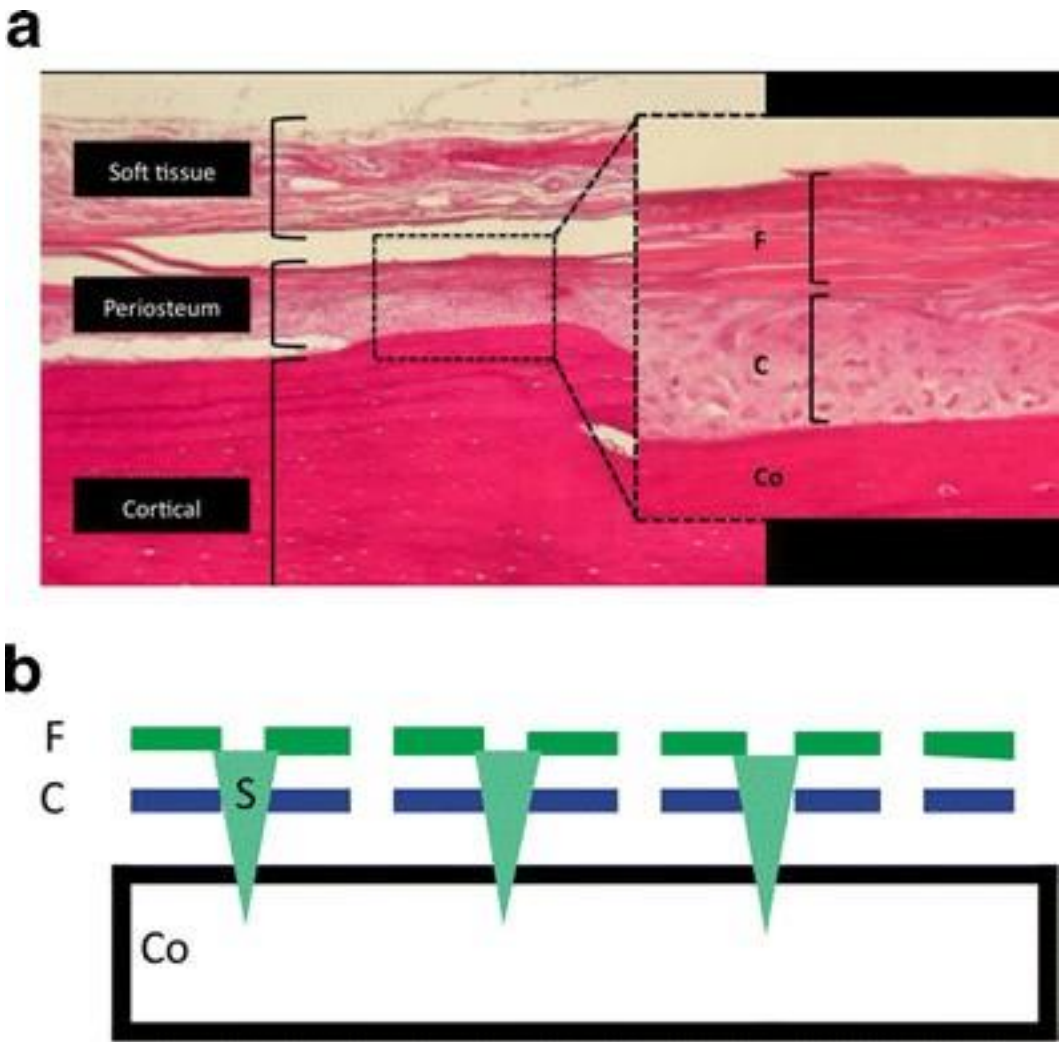

Figure 4. Normal adult periosteum, photomicrograph, and simplified diagram. (a) Low-power photomicrograph (hematoxylin-eosin-saffron stain) centered on the periosteum and showing the two layers of the normal periosteum: an outer, fibrous (F), firm layer, which can be fused with the epimysium, and an inner, proliferative, cambial layer (C). (b) Simplified diagram of the periosteocortical complex (cortex: Co) with Sharpey's fibers (S)[38].

The outermost and thickest layer (zone III) has been shown to be void of osteogenic cells and osteoblasts with scanty presence of blood vessels. The overwhelming majority of cells present in this poorly cellular zone are fibroblasts while the primary composition consists of collagen fibers and matrix to a lesser extent[37]. One of the functions of fibroblasts is the production of collagen and extracellular matrix, which are integral to the process of wound healing including bone.

Similarly, the deeper layer of zone II is void of osteogenic cells. Nearly a third of its volume is composed of matrix and equal amounts of fibroblasts and collagen with each consisting of a quarter of the total volume of the zone[37]. Zone II also contains sizable quantities of blood vessels in the form of capillaries that play a prominent role during bone healing and regeneration in cases of fractures. A study in 1989 by Diaz-Flores et al[39] showed that periosteal vascular supply is considered an additional source for osteoblasts that differentiated from pericytes located in venules and may probably have a role in angiogenesis alongside endothelial cells.

Zone I is in direct contact with the cortical surface and is quite thinner in comparison to zones II and III. The overwhelming majority of cell types present in this layer is osteoblasts and minute quantities of fibroblasts. A sub layer overlying osteoblasts are composed of osteoprogenitor cells that give rise to osteoblasts during appropriate stimuli and exposure to growth factors[37].

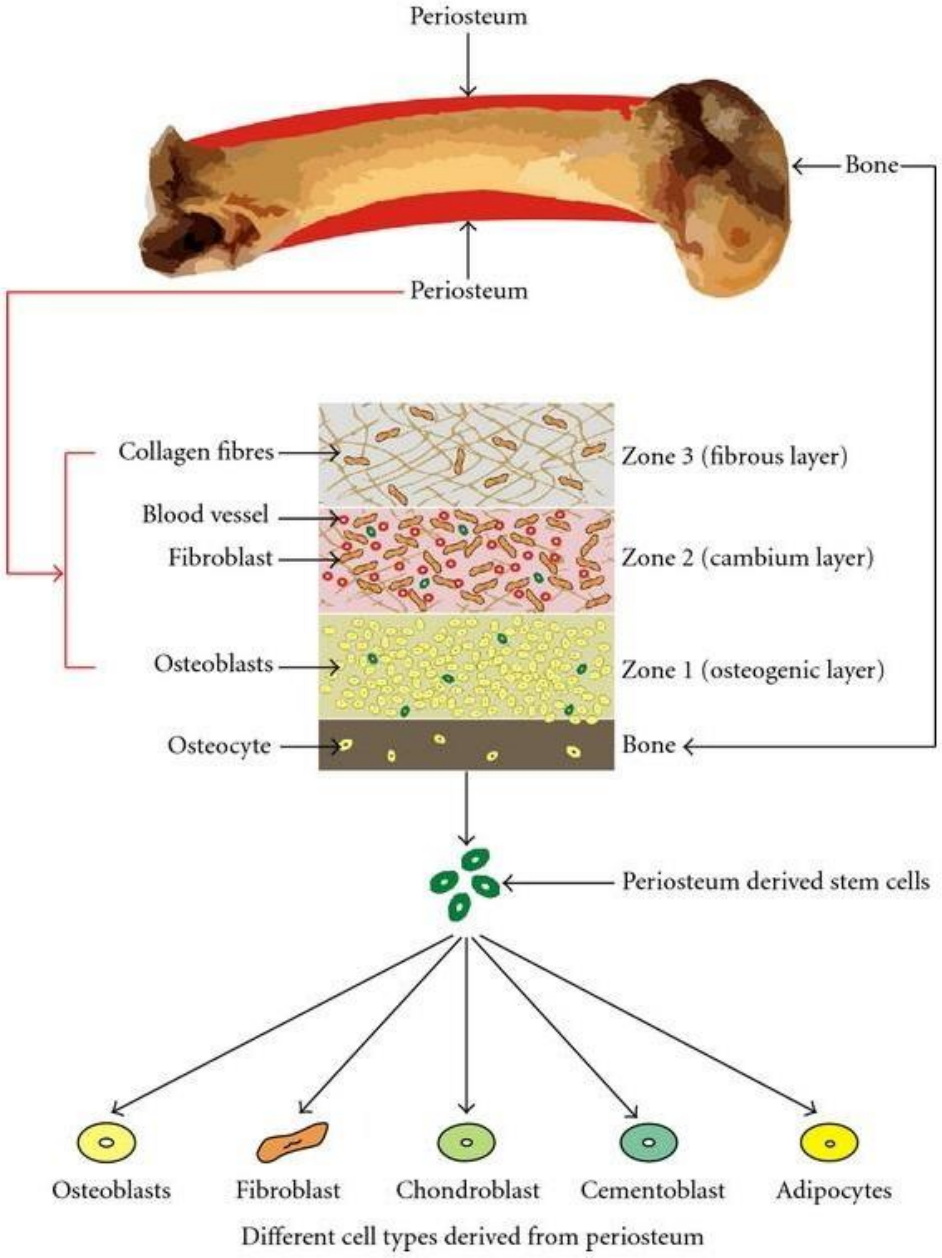

Figure 5. The three different Zones of periosteum; Zone 1 has an average thickness of 10–20 um consisting predominantly of osteoblasts; the majority of cells in Zone 2 are fibroblasts, with endothelial cells being most of the remainder. Zone 3 has the highest volume of collagen fibrils among all the three zones. The bottom of the figure shows regenerative capacity of the periosteum to form different cell types.[40]

Blood Supply of Periosteum:

Understanding the vascular networks supplying the periosteum is crucial due to its role in the process of osteogenesis, healing fractures[41] and its increase use surgically as grafts[42]. Simpson in his study[43] established the presence of four types of vascular networks found in periosteum.

Firstly, the intrinsic system runs in the fibrous layer of the periosteum as described previously. They can be found in several patterns, parallel to the long axis of the bone, circling the bone and a seemingly random layout of smaller vessels. Secondly, there is a musculoperiosteal system at the sites of muscular attachments to bone. This system provides considerably to the periosteal blood supply as muscular tissue and its covering layer (epimysium) that attaches to the fibrous layer of the periosteum are richly vascularized. Thirdly, the fascioperiosteal system varies depending on the bone and limb as the anatomical structures of fascial planes and muscles differs. The blood vessels of this system arise from the main artery supplying the limb. There is a venous network present where two veins usually follow an arterial branch. Finally, a system of capillaries lie perpendicular to the long axis of the bone and anastomose with the blood vessels running through the Haversian canal in the osteon. These periosteocortical capillaries become denser in the diaphysis compared to metaphysis of long bones.

Periosteum During Childhood:

There are two pathways for normal bone formation which includes, intramembranous and endochondral ossifications. In addition, there are multiple factors that play significant roles in bone formation such as gender and ethnicity[44]. A study by Turner et al[45], demonstrated how sex hormones in males and females contribute to bone formation, as estrogens have suppressive effects

in female rates while testosterones trigger larger bone formation in male rats. Environmental factors also have effects, as many acquired childhood diseases are caused by nutritional deficiencies such as nutritional rickets (Vitamin D deficiency)[46] and alcohol consumption during pregnancy that increases the risk of fetal alcohol spectrum disorder (FADS) which in turn negatively impacts embryonic bone growth and an affected child suffers growth retardation in craniofacial and long bones[47]. Insulin-like growth factor-I is a protein that is another vital component of bone growth where it is synthesized in bone and cartilage and moderates the effects of growth hormone[48].

The periosteum participates in bone growth in this phase through the process of intramembranous ossification. As the cambium layer is in direct contact to bone, osteoblasts are stimulated to lay new bone tissue simultaneously as the osteoclasts in the endosteum resorps bone which will result in a harmonious growth of the diaphysis. This intramembranous process does not involve cartilage[49].

Periosteum During Adulthood:

In early adulthood, net bone formation is still positive but during the early 20s in females and later in males, bone mass reaches its highest levels[50]. As the aging process continues bone resorption becomes greater than bone formation and osseous tissue becomes increasingly mineralized, making them stiffer but more brittle[51].

In addition, periosteum undergoes changes during aging. The production of mainly collagen type III reduces significantly along with collagen types I and VI and production of collagenase increases, further degrading collagen found in bone, cartilage and periosteum[35]. Other studies have demonstrated that the periosteum calcifies with aging[52], the cambium layer

becomes thinner and a quantitative reduction of Sharpey's fibers, all contributing to weaker potential for bone and cartilage regeneration[35]. All of these factors in turn, alter the organic and inorganic qualities of bone and periosteum that also adds to the resorption processes taking place.

The rate of resorption is not identical in all aging individuals. There are other factors that may increase or decrease the rate of resorption including estrogen and exercise. Animal and immunohistochemical studies have shown that exercise increases the density and thickness of Sharpey's fibers in addition to collagen type III content and periosteal blood flow leading to an augmented osteogenic potential[35, 53, 54]. Postmenopausal women are at an increased risk of osteoporosis due to lack of estrogen. The major estrogen (E_2), has been noted to play a role in periosteal induced osteogenesis as osteoblasts are found to contain estrogen receptor alpha. These receptors are activated once estradiol binds to them triggering signaling pathways and cellular regeneration of osteoblasts[55, 56].

Role of Periosteum in Bone Repair and Regeneration:

Bone repair includes several composite processes working simultaneously to achieve bone regeneration in defects and fractures, where the mechanism involving periosteum plays a prominent role[49].

Endochondral bone repair as it is named, begins with hematoma formation and inflammatory phase after injury. The hematoma is contained by the elastic fibrous layer of the periosteum while osteoblasts proliferate and osteoprogenitor cells differentiate into osteoblasts in the cambium. Further away from the injury site where vascularity is intact, the cambium layer produces woven bone by membranous ossification. At the site of the injury, the periosteum stabilizes the area by producing cartilaginous internal and external calluses that undergoes

endochondral ossification, a process analogous to bone formation during the fetal stages of life[34, 49, 57].

Paired related homebox protein 1 (Prx-1), a transcription factor produced during embryogenesis[58], alpha smooth muscle actin (aSMA), and Sox9[59, 60] are some examples of cell surface antigens (markers) that have been identified to assist in labeling the periosteal progenitor cells. Studies in mice have shown that expression of Prx-1 was noted during fetal development[61] and that significant deformities in the extremities and craniofacial region resulted from the marker's absence (by experimentally inducing an inactivating mutation) that causes disruptions in the normal development of osteoprogenitor cells[62]. aSMA, a myofibroblast marker, is expressed in periosteal osteogenic and chondrogenic precursor cells, giving these cells the capability to differentiate as necessary during bone healing[59]. Similarly, Sox9 is involved in osteogenic differentiation and it is also expressed in osteogenic and chondrogenic precursor cells[60].

Insulin like growth factor-I (IGF-I) is important during bone development and fracture repair. IGF-I and II bind to IGF-I receptor initiating a series of processes activating several pathways and kinases[63, 64], which promote further differentiation of osteoblasts[65]. Several studies[66, 67] have also described a quantitative increase in IGF-I and IGF-I receptor in the periosteum further enhancing callus formation, as previous research has shown that a decrease or absence of this protein and its receptor negatively affects the quality of callus formation resulting in healing failure due to lower bone mass and mineralization[68-70].

There are a group of proteins involved in bone healing belonging to fibroblast growth factor (FGF) family that includes FGF 2, 9 and 18. One of the principal functions of FGF2 is activating periosteal cells resulting in increased osseous generation through endochondral ossification[71].

Previous studies have shown that FGF2 deletion resulted in a substantial reduction of bone formation and subsequently bone mass and that bone marrow stromal cells had markedly less osteoblast differentiation greatly due to reductions in glycogen synthase kinase 3 (GSK-3), a destruction complex involved in the Wnt pathway, and dickkopf-related protein-2 (DKK-2), a protein produced during embryogenesis [72, 73]. FGF9 is involved in bone repair and its absence has been linked to congenital deformities such as achondroplasia[72]. Therefore, it has been suggested that in the primordial cartilage, FGF9 boosts vascular formation and hypertrophy of chondrocytes that will undergo ossification. The chondrocytes produce matrix with abundant collagen and angiogenesis starts taking place driven by VEGF, eventually leading to the recruitment of osteogenic cells into the area.[74]. Additionally, osteoblast differentiation and proliferation are promoted by FGF18[72], albeit it is not clear whether it promotes or suppresses chondrogenesis[75, 76].

Another contributor in the process of bone repair is periostin, a protein that is produced in the extracellular matrix. It is involved in processes such as wound healing, organ development[77] and several pathologies including cancers by activating signaling pathways and triggering abnormal cellular growth and eventually invasion and metastasis[78, 79]. As perostin plays a role in wound healing, it also does with bone healing. Studies have demonstrated that osteoblasts in the periosteal cambium layer expressed this protein abundantly while its deficiency has been shown to affect periosteal osteogenic activities negatively thus resulting in impaired fracture healing and non-union[78, 80].

Periosteal Tissue Engineering:

Research in the field of tissue engineering is promising and continuously expanding to manufacture new biologically viable bone, albeit the numerous challenges[81, 82]. The pillars of tissue engineering include, scaffolds resembling the osseous extracellular matrix that also possesses the 3D form and mechanical characteristics of the original bone being substituted, osteoprogenitor cells capable of differentiation into different bone cells and repopulation of the defect site and proper blood supply necessary for cell and tissue survival[82, 83].

Periosteal tissue engineering is a niche that has not been extensively explored. But there have been few studies published recently reported using acellular dermal matrix as a scaffold for engineering periosteum[84, 85]. In addition, translation from in vitro to in vivo environments is a challenge that new emerging research have shown some solutions to overcome those difficulties to allow more appropriate evaluations of tissue engineered periosteum[81, 86, 87].

Huang et al[81] describe the use of rabbit bodies as bioreactors and triggering the body to regenerate a new bone, where they placed decellularized bone matrix scaffolds in a muscular pouch in the femoral muscle of one group and compared it with another where the scaffold was wrapped by a pedicled periosteal flap. They concluded that the periosteal flap group had more bone regenerated with a denser vascular network. This study followed from previous studies using the bioreactor approach where muscular pouches, arteriovenous loops and bundles were used[88-90]. Baldwin et al[86] used human tissue engineered periosteum constructs as xenografts and implanted them NOD scid gamma mice that are capable of receiving xenografts including humans[91].

Clinical Applications of Periosteum:

The osteogenic and chondrogenic potentials of periosteum has favored its use in a wide variety of surgical applications in the fields of plastic surgery, orthopedic surgery and dentistry. Over the years, oral and craniofacial surgeons have studied and utilized the bone generative capacity of periosteum in multiple surgical procedures then resulting to various degrees of success. Skoog[92] pioneered the use of periosteal flaps in maxillary clefts, however the results were not entirely satisfactory. Massei[93] modified Skoog's techniques by abandoning the use of the pedicle flap and replacing it with an island flap that has a richer vascular supply leading to enhanced osteogenic activities. Sierra et al[94] recently reported a case of mandibular reconstruction where a vascular fibular periosteal flap was used along with a bone graft which then demonstrated favorable results. Similar results were also attained with the use of vascularized flaps in comparison with the non-vascularized flaps for scaphoid non-union, demonstrating better osteogenesis with the vascularized flaps[95].

Maxillary sinus floor augmentation is one of the methods used in cases such as atrophic maxillae. One of the complications of this method is perforating the maxillary sinus, due to the clinical manifests of bleeding, the wound infections and the sinusitis in the post-operative period[96]. Several methods have been used to surgically correct these sinus defects, but a recent study involving 24 patients have shown the use of autogenic periosteum for an effective defect coverage, where the periosteal graft was incised from the retromolar area of the mandible. They reported that all 24 patients recovered and that the procedure was in fact effective[97].

A promising novel technique, periosteal distraction osteogenesis (PDO), has been shown with several animal studies to promote bone regeneration and eliminating some of the disadvantages of distraction osteogenesis (DO). PDO works by gradually elevating the periosteum

over a period of time by distraction devices and thereby creating a space between the cortex and the periosteum. In comparison to DO, PDO does not involve carrying out an osteotomy, thus it is less invasive with a shorter treatment period. The clinical use of PDO is still limited and has been applied to repairs of cleft defects and alveolar atrophy[98].

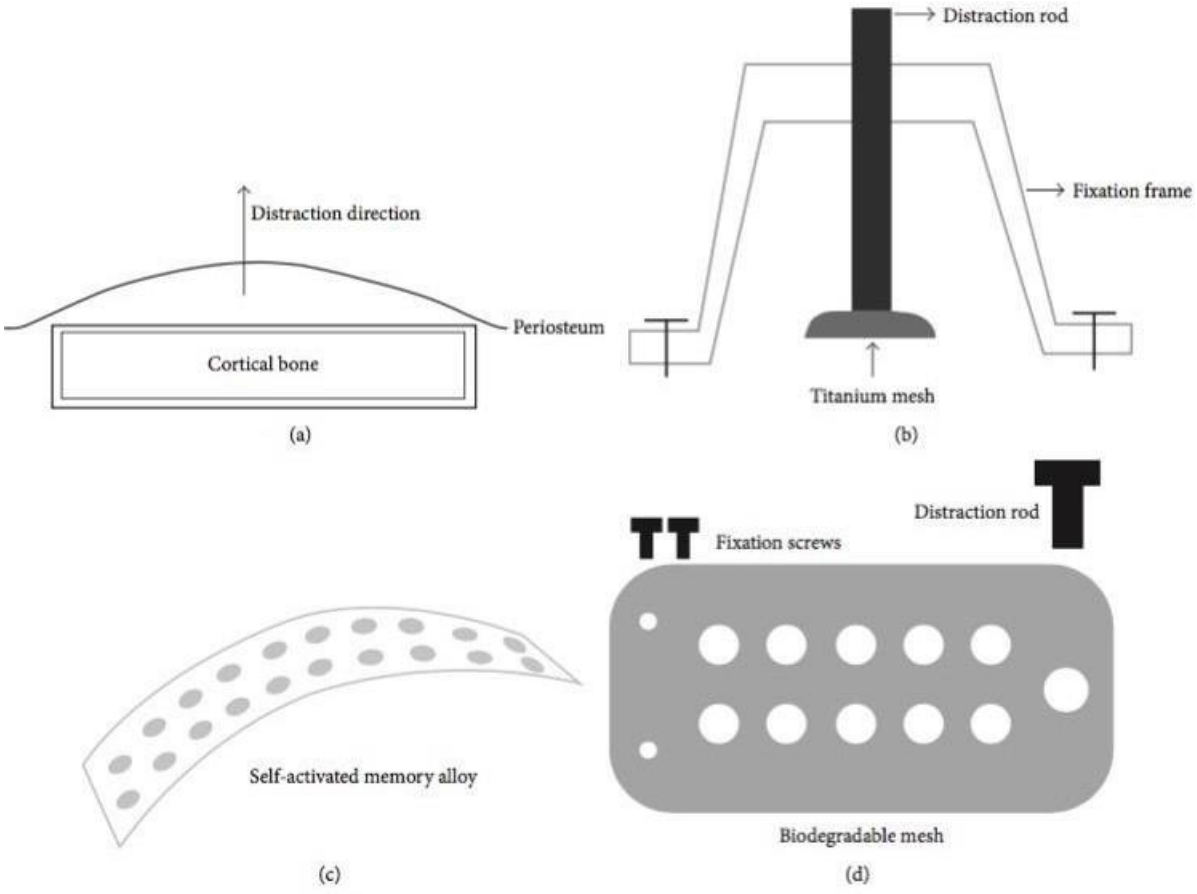

Figure 6. The mechanism (a) and devices (b, c, d) of PDO. The mechanism (a) and devices (b, c, d) of PDO. (a) PDO creates an artificial space between the bone surface and periosteum to generate new bone by expanding the periosteum, muscle, and skin at the same time. (b) U-shaped distractor composes of three different parts: fixation frame, distraction rod, and titanium mesh. Bilateral fixation legs can be fixed rigidly to the surface of cortical bone by titanium screws, and then through the rotation of middle distraction rod, the titanium mesh can be lifted off the ground of bone and distract the periosteum simultaneously. (c) SMA leaves out distraction screws. (d) Biodegradable PLLA/HA mesh instead of titanium mesh for distracting periosteum[98].

Yamauchi et al[99, 100], described using a technique that allows the expansion/elevation of the periosteum by installing a self-activated memory alloy (figure 5, c) that does not require placement of screws. It is a minimal invasive technique that allows the formation of a space that stimulates the periosteum to undergo osteogenesis and bone regeneration in that space. But they have reported that the amount of new bone that had formed was inadequate.

Periosteum and Masquelet's induced membrane:

The Masquelet Technique was the focus of this project and it is further discussed in the following chapters. It is important to note that one of the purposes of using the Masquelet Technique is to induce the formation of a periosteum-like fibrous membrane. By provoking a foreign body reaction, this fibrous membrane is created by placing a bone spacer in a bone defect which is left in place for several weeks[101].

The composition of this fibrous membrane has been shown to share many similarities with the periosteum. It is a highly vascularized and a slightly thicker two-layered membrane with an inner cellular epithelial layer and an outer less cellular layer with abundant collagen[102].

The fibrous membrane also possesses some functions like the periosteum, as it produces mesenchymal stem cells and a variety of molecules that are pro-angiogenic and pro-osteogenic, thus providing favorable conditions for bone regeneration and healing. In addition, the membrane serves as a protective barrier to the defect site, where it prevents fibrous tissue formation and the resorption of the bone grafts that are placed in[102].

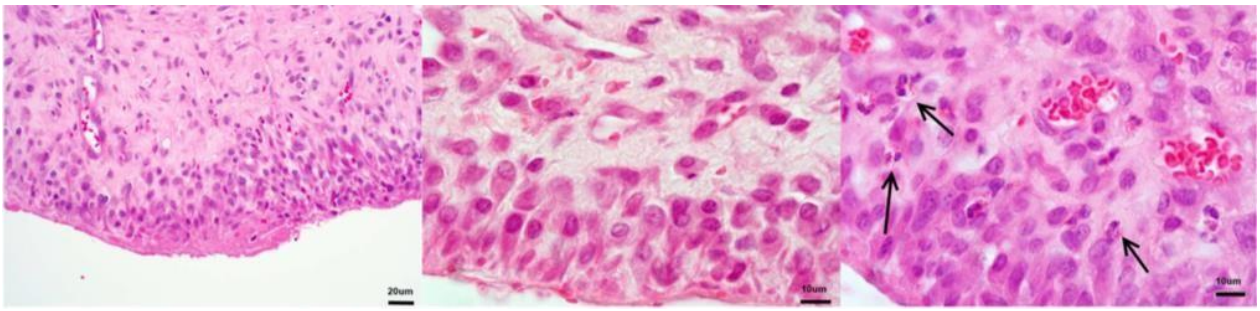

Figure 7. H & E sections of the capsule formed as a result of the PMMA spacer. A: Showing transition of cell orientation deeper into the tissue (400x). B: Showing no basement membrane (1000x). C: Presence of eosinophils (arrows) within the tissue (1000x).[103]

Chapter 3:

Current Approaches to Healing Large Segmental Bone Defects

Introduction:

Reconstructing critical sized bone defects (CSBD) is a common challenge facing orthopedic surgeons. There is no defined cut-off to include a defect in the "critical sized" category, although it is generally agreed that surgical stabilization of these defects is not sufficient and requires further surgical reconstruction due to the inability of CSBDs to heal and regenerate naturally[104]. It is important to note that there are several other factors that play a role in the ability of a CBSD to regenerate naturally. There are factors related to the defect site such, the shape and location of the bony defect and the state of the surrounding soft tissue, while other factors are related to the age and general conditions of the patient and presence/absence of comorbidities[104, 105]. It is noteworthy to indicate how these factors can affect the healing process, where some reports indicated that that the preservation of the soft tissues around the femur, large defects up to 15 cm have healed spontaneously[106, 107].

There are many types of grafts and materials used for reconstructing CSBDs, but autogenic grafts remain the gold standard[108]. Some studies[109-111] have argued that "massive defects" that measure >6 cm in long bones require reconstruction with vascularized grafts due to the reason that it reduces the risk of graft resorption and enhances the delivery of blood supply and nutritive substances to the grafts[112, 113]. But the systematic review published by Allsopp[112], have revealed no supporting evidence for the use of vascularized graft in massive defects. Some of the available treatment options for massive defects include, vascularized fibular grafts, Ilizarov technique and Masquelet technique.

Free Vascularized Fibular Grafts (FVFG):

The structural anatomy of the fibula and its main blood vessel (peroneal artery) allows it to be a good treatment choice for massive defects in long bones. The fibula can measure up to 40 cm of which up to 30 cm of it can be harvested, depending on the size of the primary defect[114], where it can be used to reconstruct long bones in the upper and lower limbs.

FVFGs have a variety of applications that includes reconstructing large osseous defects caused by trauma, as well as other circumstances such as infections and malignant tumors as they can cause substantial destruction[114]. Infection induced defects may be challenging as this requires initially treating the infection after debriding the effected zone, where reconstruction should only be performed after the infection has settled. Albeit FVFGs is the most appropriate method in these cases, these defects pose a challenge as the infections usually result in an unfavorable environment for vascular anastomosis[115]. Other applications of FVFGs where studies have shown its considerable efficiency include management of avascular necrosis[116] and joint arthrodesis[117].

FVFGs are not without complications at the surgical sites. At the early postoperative phase, as with any free flap procedures, considerable amount of blood leaking or thrombi formation may block the site of anastomosis, compromising the viability of the flap. Another acute complication is compartment syndrome due to an increased pressure at the donor site compromising blood flow and oxygen delivery into the limb. Infection can occur as an early or late complication, that can be caused by inadequate vascularity of the graft or the surrounding soft tissue and/or inadequate eradication of infected tissue at the defect site. On the longer term, grafts may not heal and or

fracture, which is especially true when reconstruction large bones such as the femur, as the graft is not capable of baring weight due to its thinner diameter in comparison with the femur[114].

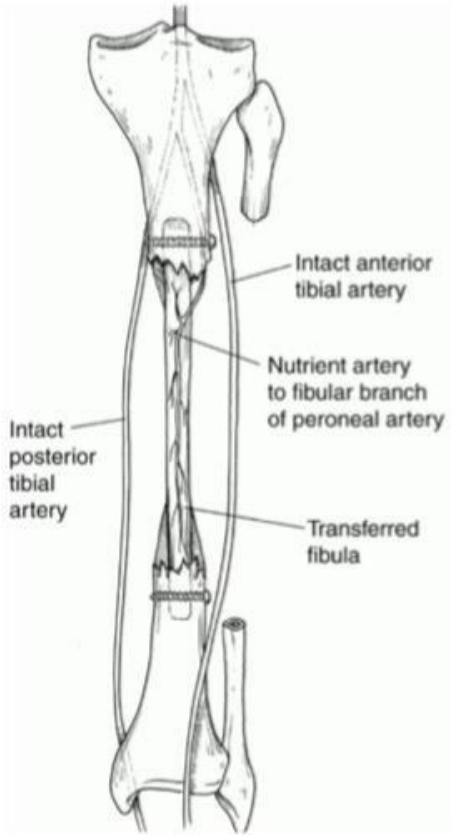

Figure 8. Vascularized Fibular Graft. Transfer of the adjacent fibula on its vascular pedicle with screw fixation[118].

Ilizarov Technique:

This technique is also known as distraction osteogenesis, and as the name suggests, the callus is distracted by using a device shown in figure 7, that triggers new bone formation due to the application of controlled mechanical stress. There are several phases involved where they typically start after creating an osteotomy followed by the week-long latency phase[119, 120] that triggers angiogenesis and callus formation[121]. Thereafter, bone formation runs in parallel with angiogenesis during the distraction phase where it is recommended that a 1 mm/day distraction rate is optimal[120, 122]. The process finalizes with the consolidation phase, where the defect is filled with the mineralized callus and new bone closes the gaps. The external fixator that is initially applied to provide mechanical stress to induce osteogenesis, is kept for some time (usually months) to allow enough time for the new bone to gain sufficient strength to endure weight baring[120].
It is still uncertain what type of ossification that occurs when Ilizarov's technique is performed. Studies conducted on sheeps[123, 124] tibiae and radial bones have reported that endochondral and intramembranous ossifications were observed when their samples were analyzed.

Distraction osteogenesis is an efficient method due to its osteogenic potentials and capabilities. It has been widely used since it was pioneered by Dr. Gavriil Ilizarov six decades ago[125]. This was followed by numerous studies to establish the mechanism and efficiency of the procedure. The technique's use is not only limited for the reconstruction of bone defects, but includes limb lengthening, malalignments and joint contractures[125]. A systematic and meta-analysis by Papakostidis[126] demonstrated the ability of Ilizarov Technique to reconstruct large bony defects, management of associated infections and minimizing the rates of amputations.

Significant complications have been reported. Notably, patient tolerance is a major issue as the treatment is prolonged (months to years), where many patients request getting an

amputation[120, 126-128]. In addition, extreme pain resulting from adjustments made to the external fixator that irritates the nerves and muscle[129] and pin tract infections[130].

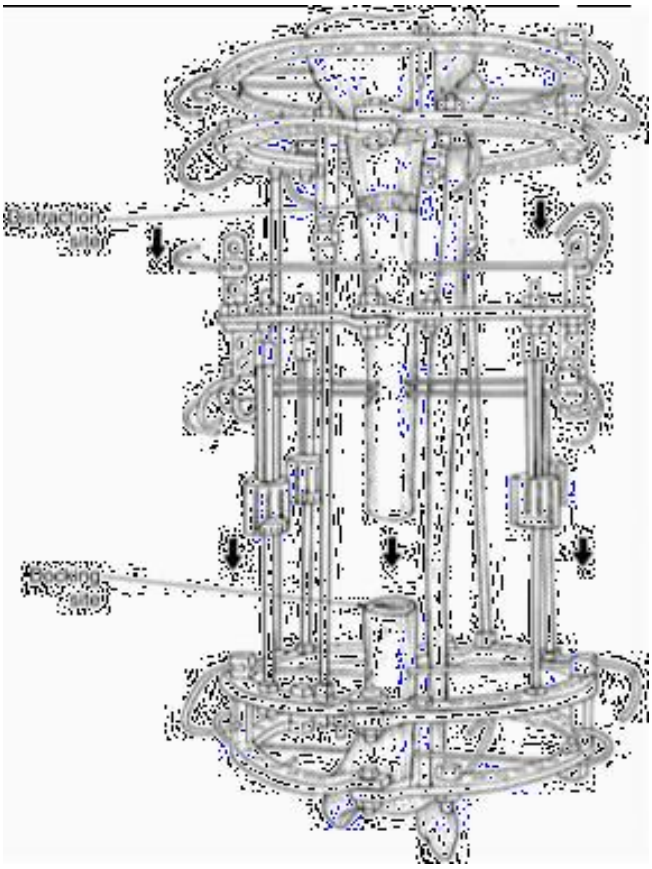

Figure 9. Bone Transport Distraction Osteogenesis. Bone transport distraction osteogenesis with a circular frame and proximal corticotomy. The middle segment is transported distally (arrows)[118].

Masquelet Technique:

This technique is also known as the induced membrane technique that was first developed by the French orthopedic surgeon Dr. Alain Masquelet for almost four decades. This technique can be used in various clinical situations to manage complex bone injuries and defects that can result due to trauma, tumors, infections, etc. It is comprised of two stages, where it starts the first procedure that involves the placement of a bone spacer in the bony defect, followed by a second

surgical procedure with the removal of the spacer and placement of bone grafts, 6-10 weeks later[101]. The most commonly used cement is Polymethylmethacrylate (PMMA) and Masquelet in his recent review[101] advised against the use of an antibiotic-loaded cement because it is ineffective in combating bacterial infections and provides an enhanced ability to monitor cases of re-infections. Nevertheless, the main purposes of bone cement placement are the development of the "induced membrane" around the cement due to foreign body reaction, providing stability to the defect and the surrounding soft tissue and inhibition of fibrous tissue and hematoma development[120, 131].

The fibrous "induced membrane" plays a crucial role in this technique and has been compared to synovial tissues that is 1-2mm thick[132], albeit its mechanism and functions remains not fully understood. It has been shown that this fibrous membrane acts in a similar fashion to the natural periosteum. It produces abundant amounts of molecules and growth factors that provoke angiogenesis followed by osteogenesis and bone formation. These include, BMPs, VEGF, vWF and various interleukins that are seen produced in the natural process of bone healing[102]. A study[133] published 2013, studied the composition of the induced membrane at bone defect sites in rats and compared it to induced membranes formed in subcutaneous pockets and periosteum. They reported thicker measurements of the membranes, close to 1mm that is almost 20 times thicker than the periosteum, due to the overall larger fiber and cellular populations. They also noted that the induced membrane surrounding bony defects had much higher rates of vascularization in the initial weeks post-operatively, which then plateaued a month later. In comparison, the periosteum has been noted to have a more modest rate of vascularization[133].

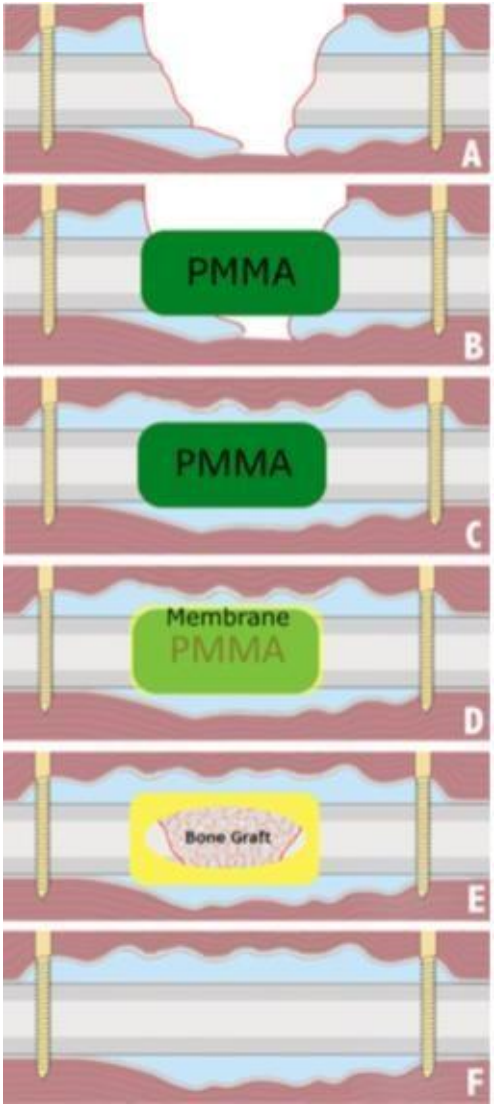

Figure 10. The Masquelet Technique. Images showcase the step-by-step the two-staged Masquelet Technique. Periosteum is represented by the blue area surrounding the bone. From top to bottom – A) First Surgery: Critical-sized bone defect irrigated and debrided at both bone ends. B) PMMA cement fills the defect, surrounding both proximal and distal bone ends. C) Surrounding soft tissue healing with PMMA in place. D) A membrane forms around the PMMA. E) Second Surgery: Induced membrane exposed, incised, and PMMA removed. Defect site filled with bone graft, membrane sutured closed. F) Defect site showcasing healed bone[134].

Mesenchymal stem cells (MSCs), that have the ability to differentiate into osteoblasts, were noted to be in negligible quantities at the initial two weeks post-operatively, that were located at the outer most regions of the membranes. But after a month post-operatively, MSCs were not detectable. In contrast, MSCs were found in abundance especially in the periosteum's cambium inner layer[133]. The presence of osteogenic markers, such as Ki67, Stro-1^{+} and ALP^{+}, were present in the induced membranes but in much lower quantities than that found in periosteum[133]. These results have shown to be in accordance with other studies that have described that vascularization and production of growth factors such as BMP-2 and TGF-β1, that seem to play crucial roles in generating new bone[135, 136].

Most clinical studies in the literature show encouraging results with the use of the Masquelet Technique, but many drawbacks and issues remain to be addressed. Masquelet recommended leaving the bone spacer for 6 to 10 weeks[101]. But some studies have shown that the induced membrane's peak in angiogenic and osteogenic growth factors production occurs at around 4 weeks. Another issue is that larger defects require larger graft quantities and harvesting autogenic grafts locally maybe difficult and worsens morbidity[120]. Synthetic bone grafts can be effective, but further research should show what material is optimal and if any growth factors should be added to supplement it. There are also clinical complications that have been reported with the use of this technique. Multiple surgical interventions were required to manage some complications that included infections, fracture recurrence, nonunion and amputations[120, 131].

Tarchala et al[134] described a simplified version of the Masquelet technique that could be a reasonable method to reduce the rate of complications. They described performing a single step technique where a PTFE membrane was placed at the defect site, overlapping with the distal and proximal ends of the defect, followed by placement of a bone graft in the same surgical procedure.

Their results have shown comparable rates of bone regeneration in comparison with the traditional technique. Although this version has the potential benefit of eliminating an additional surgical procedure, therefore reducing morbidity, further animal and clinical studies would provide clearer answers to these issues.

Chapter 4:

Effectiveness of Masquelet Technique with β-TCP Grafts in Critical Sized Bone Defects in Rabbit Models

Introduction:

Clinically, autogenic bone grafts are considered the gold standard in the management of large complex bone defects due to its favorable characteristics that include production of mesenchymal stem cells where growth factors initiate its differentiation into osteogenic cells and its osteoconductive properties that allow osteoblasts and blood vessels to grow within the graft[98, 120]. In complicated cases with large bone defects, harvesting autogenic grafts proves to be a challenging task and may increase patients' morbidity. Therefore, these issues should be addressed to find an optimal synthetic material that can act as a defect filler and that possesses similar favorable characteristics as the autogenic grafts.

One of the synthetic materials that has been studied is β-TCP, which is a calcium phosphate scaffold that have been shown to possess favorable qualities as a bone substitute. Its porosity and pore interconnectivity allow ingrowth of cells and blood vessels that will eventually lead to graft bioresorption and formation of stable and mature bone[137]. There remain some factors to be addressed pertaining to the graft's geometry that might enhance its performance which includes the amount of porosity and the sizes of pore interconnections and whether supplementing it with growth factors would be beneficial.

In this study, β-TCP was used to evaluate its effectiveness in comparison with autogenic bone grafts through the utilization of the Masquelet technique.

Materials and Methods:

Animals:

Surgical procedures were carried out on New Zealand rabbits aged six months weighing around 3.5 kg (Charles River Laboratories). Each rabbit was caged separately at our large animal facility in controlled conditions with adequate temperatures, 12-hour light/dark cycles and provided with food and water. All animals were acclimatized for 7 days prior to the surgical procedures. Animal experiments and surgical procedures were approved by the animal compliance committee at our institution.

Surgical Methods:

In total, our experiments included fourteen New Zealand rabbits. Surgeries were carried out on both forelimbs of each rabbit (n=28). Figures 9 and 10 show the different study groups. Starting with a pilot experiment involving eight ulnar defects (n=8) measuring 2cm, where six defects where left empty for the entirety of the experiment and the other two defects were filled with autogenic bone grafts, 4 weeks after the creation of the defect. Then, the experimental group involved ulnar defects measuring 3.5cm, where four ulnar defects (n=4)(negative controls) were left empty for the entirety of the experiment, in addition to seven defects (n=7)(positive controls) filled with autogenic bone grafts and six more (n=9) with β-TCP grafts (experimental controls).

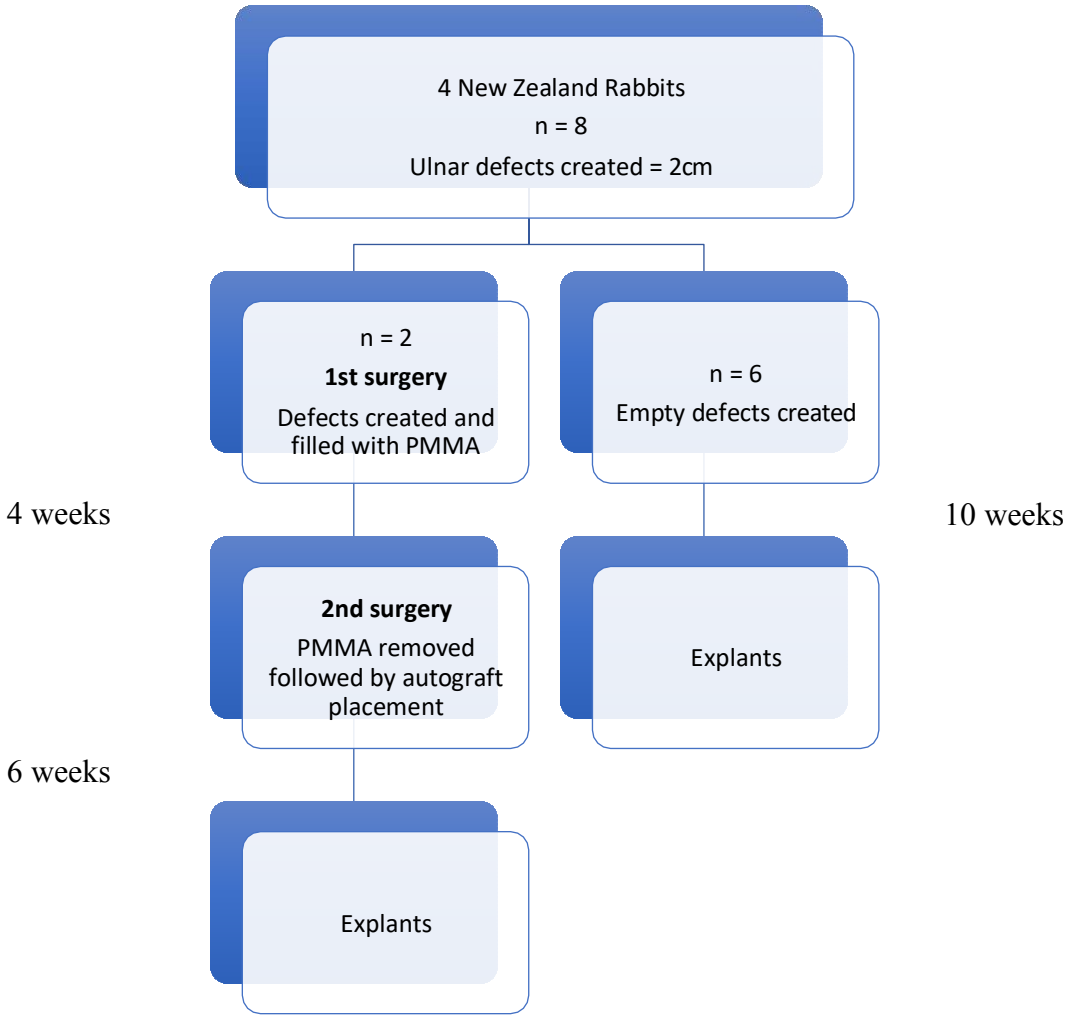

Figure 11. Experimental design of the pilot study. Four New Zealand rabbits, for a total of eight forearms, were divided into two groups. The right arm consisted of six ulnar measuring 2 cm were created and left empty for 10 weeks, after which the rabbits were euthanized and samples explanted. The left arm consisted of two samples where the Masquelet Technique was performed. After the creation of the 2-cm defect, a bone spacer (PMMA) was placed at the defect site which was left in for four weeks. The second procedure involved removing the spacer and placing autogenic bone grafts that were left in for six weeks, after which the rabbits were euthanized and samples explanted.

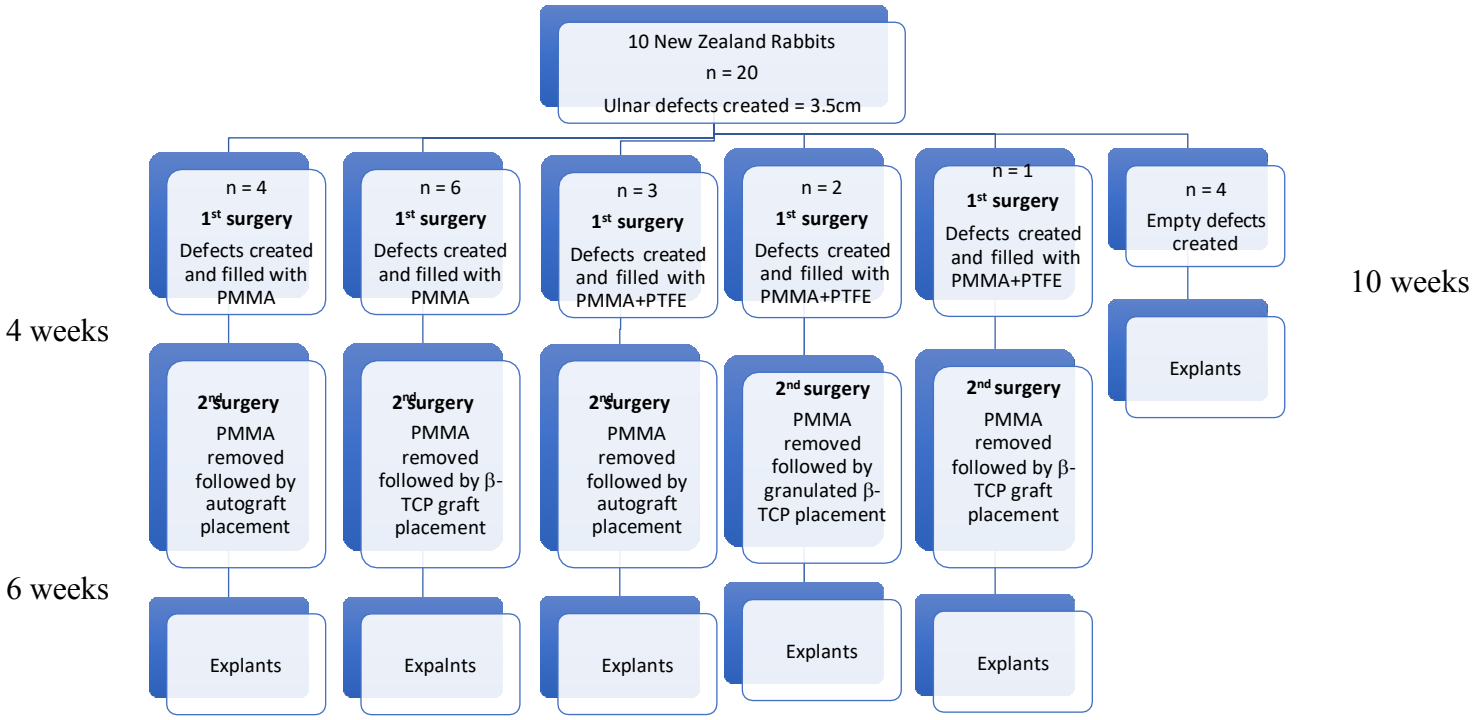

Figure 12. Experimental design. Ten New Zealand rabbits, for a total of twenty forearms, were divided into six groups. Four ulnar defects measuring 3.5 cm were created and left empty for 10 weeks, after which the rabbits were euthanized and samples explanted. The remaining samples had the Masquelet Technique performed on them. After the creation of the 3.5-cm defect, a bone spacer (PMMA) was placed at the defect site which was left in for four weeks. The second procedure involved removing the spacer and placing autogenic bone grafts (n=4), β-TCP grafts (n=6), autogenic grafts+PTFE, granulated β-TCP+PTFE (n=2) and β-TCP block + PTFE (n=1) that were left in for six weeks, after which the rabbits were euthanized and samples explanted.

Anesthesia was carried out by a veterinary technician starting by sedating the animal with xylene/acepromazine and ketamine intramuscularly. Rabbits were intubated and isoflurane was used for the induction and maintenance of anesthesia. Surgical procedures for both forelimbs were performed back to back, and were shaved, prepped and draped in a sterile manner prior to the commencement of the surgery.

All rabbits included in this study received the traditional Masquelet Technique on all forelimbs bilaterally. Starting with the 2cm defect experiments (n=8), the surgery started by skin incision and muscle dissection to reach the ulna. A defect measuring 2 cm was created in the ulna. Polymethylmethacrylate (PMMA, Palacos) was prepared and used to fill the defect and ensuring to cover bone ends on both sides of the defect. Once PMMA solidified, the surrounding muscles were sutured using vicryl followed by suturing the skin with monocryl. The bone that was removed after creating the defect was rinsed in a saline/gentamicin solution then kept in a -80°C freezer and kept until the second stage of the Masquelet Technique. As mentioned previously, the critical sized defect was created similarly in the left forelimb and was kept empty for the entirety of the experiment until euthanasia of the animal (10 weeks). PMMA was left for 4 weeks and no constraints were placed on rabbits' mobility and were allowed to bear weight.

The second stage of the Masquelet Technique was performed four weeks after the first surgery and the rabbits were prepared and anesthetized as described previously for the first stage of the surgery. After incising the skin and dissecting the muscles, the induced membrane created by PMMA was visualized. After incising the membrane, PMMA was extracted. Bone graft was thawed in warm saline then crushed into small pieces and filling it in the defect site. The induced membrane was firmly sutured with vicryl, then muscles and skin were sutured as described previously. Similarly, no constraints were placed on the rabbits' mobility and were free to bear

weight. Six weeks after the second stage of the Masquelet Technique, rabbits were euthanized and samples were extracted.

The rabbits that were subjected to 3.5cm defect (n=20) were subjected to the same surgical procedures described previously where four of the defects were left empty, seven were filled using autogenic bone grafts and nine were filled with synthetic β-TCP grafts for the second stage of the surgery (at week 4).

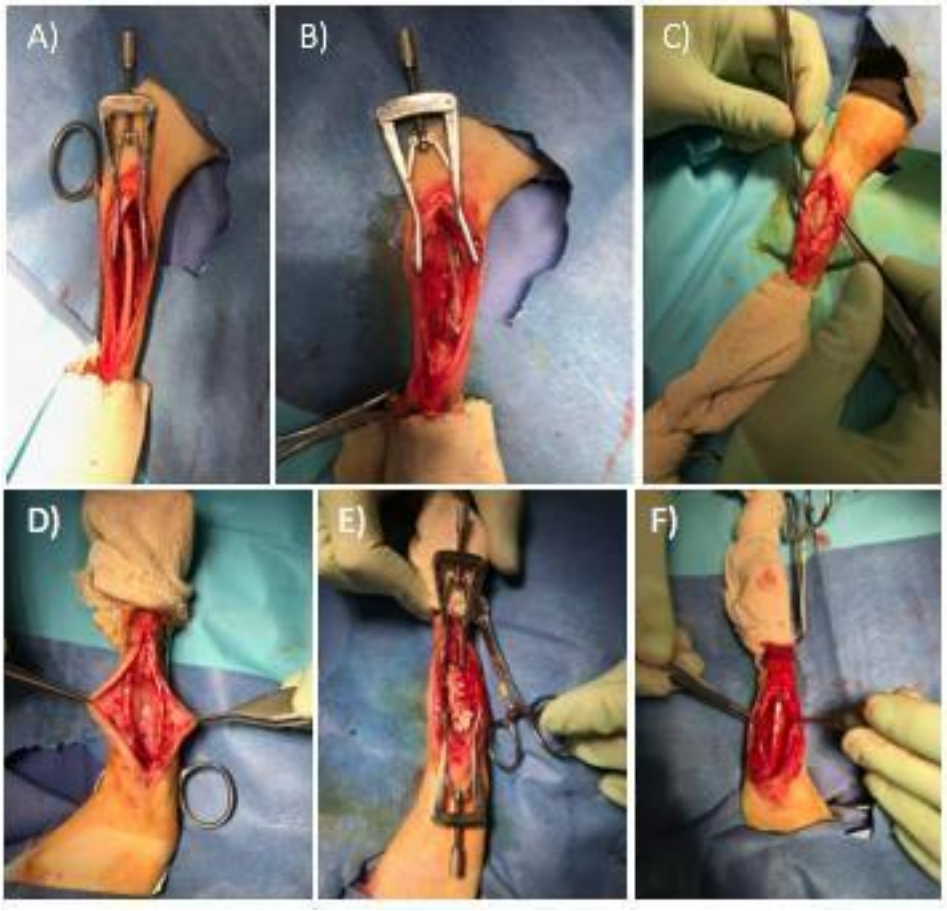

Figure 13. The Masquelet Technique. A) Rabbit ulnar bone exposed during the first surgery of the of the two-step technique. B) A massive defect is created, where PMMA spacer is inserted. C) Four weeks later during the second stage, PMMA spacer is exposed. D) Induced membrane visible after spacer removal. E&F) Defect site was filled with either autogenic bone grafts or β-TCP, then induced membrane was sutured tightly around the grafts.

β-TCP block measurements:

In this study, in order to accurately fit in the β-TCP grafts in our ulnar defects, several measurements have been taken, as shown in figures 12 and 13. The total length of a 6-month-old New Zealand rabbit measured around 9cm. The 3.5cm ulnar defect was recreated at middle section of the bone. Both cross sectional ends of the defects were measured as follows, at the proximal end the length and width measured 6mm and 4mm respectively, while the length and width of the distal end measured 6mm and 3mm respectively.

To ensure adequate coverage of both ends of the defect with β-TCP grafts, it was decided that the most appropriate shape would be a right-angled triangle measuring 5mm on both perpendicular legs and a hypotenuse of 7.07mm, since most of the ulnar defect bone highly resembles a right-angled triangle.

Figure 14. A) Total length of a rabbit ulna measuring 9cm. B) 3.5cm defect taken from the middle section of the ulna.

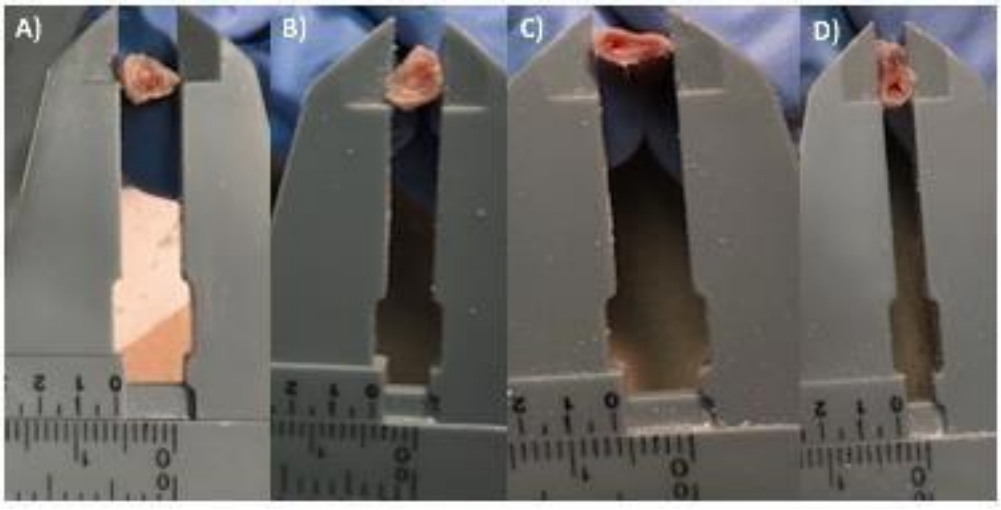

Figure 15. Cross-sectional dimensions of the two sides of defect ends. A) Proximal end, length measured 6mm. B) Proximal end, width measured 4mm. C) Distal end, length measured 6mm. D) Distal end width measured 3mm.

Follow up:

Live x-ray images of the rabbits' forelimbs were taken after the first and second surgeries and finally one week before euthanasia. The purpose of this is to make sure that no other structures (i.e. radius) were damaged during the surgical procedures and to monitor if there were any indications of bone regeneration in the limbs containing the critical sized empty defects.

At the time of euthanasia, samples were explanted and placed in 4% paraformaldehyde for 72 hours, then micro CT analysis was carried out to compare the bone volume (BV) between the three models. After that, samples were dehydrated by increasing gradients of ethanol (70%-100%) for around a week. Finally, samples were embedded in polymethylmethacrylate (PMMA) and sections were made and stained with tartrate-resistant acid phosphatase (TRAP) and methylene blue/basic fuchsine (MB/BF) to identify the regions of bone regeneration.

Results:

Micro-CT analysis:

Micro-CT analysis of the 2cm ulnar defects showed that the two defects containing autogenic bone grafts (n = 2) had a mean bone volume of 185.30mm^3 (SD 17.82) and the six empty defects (n = 6) had a mean bone volume of 38.48mm^3 (SD 55.72) (P = 0.0128). Whereas the 3.5cm ulnar defects showed that the defects (n = 4) containing autogenic bone grafts had a mean bone volume of 593.36mm^3 (SD 147.35), the samples containing β-TCP grafts (n = 6) had a mean bone volume of 616.93mm^3 (SD 86.04) and the four empty defects (n = 4) had a mean bone volume of 104.11mm^3 (SD 62.98). It is noted that in regards to the three arms of the larger defect sizes (3.5cm), the results show statistical significance when comparing the empty defects with the autograft and β-TCP samples, P = 0.0009 and P < 0.0001, respectively. Whereas, there was no statistical significance when the autograft and β-TCP arms were compared, P = 0.7549.

The remaining samples had PTFE membranes added to the defect site. Of these, three (n=3) had autografts with a mean bone volume of 372.26mm^3 (SD 18.3). One sample (n=1) had a β-TCP block with a bone volume of 835.29mm^3. And two more samples (n=2) had granulated β-TCP particles with a mean bone volume of 278.72mm^3 (SD 75.41).

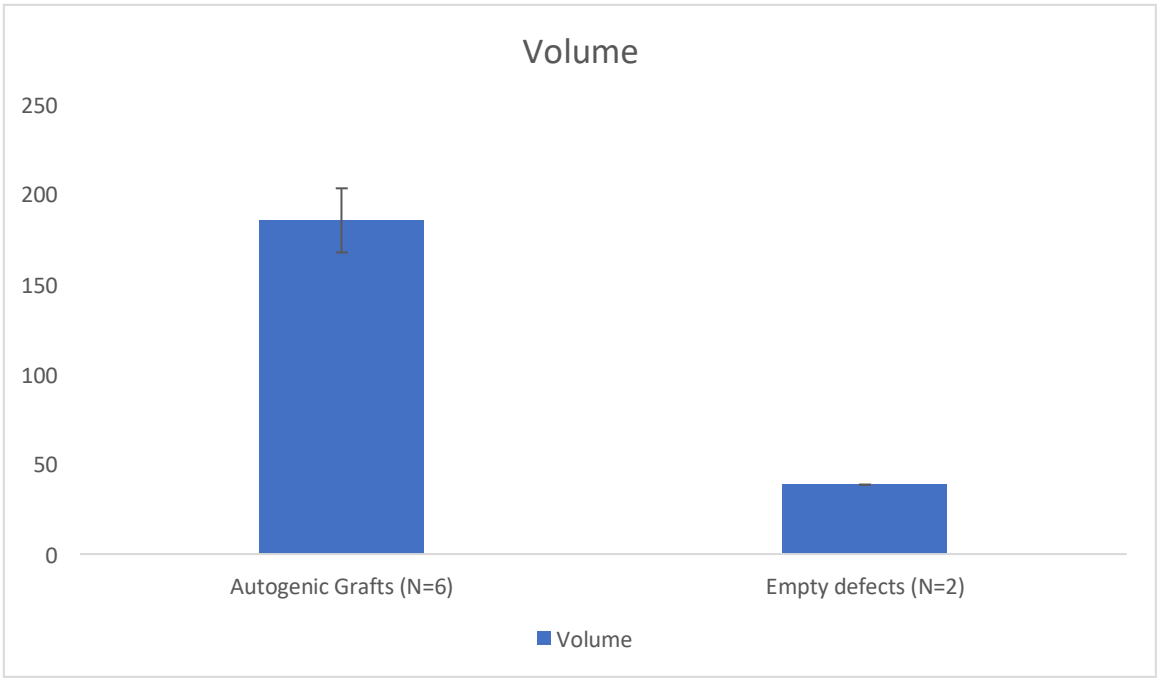

Figure 16. This graph compares the mean bone and graft volumes between the two experimental groups with 2cm defects. Positive control group (autogenic grafts) had a mean BV 185.3mm^3 (SD 17.8) and the negative control group (empty defects) had a mean BV 38.5mm^3.

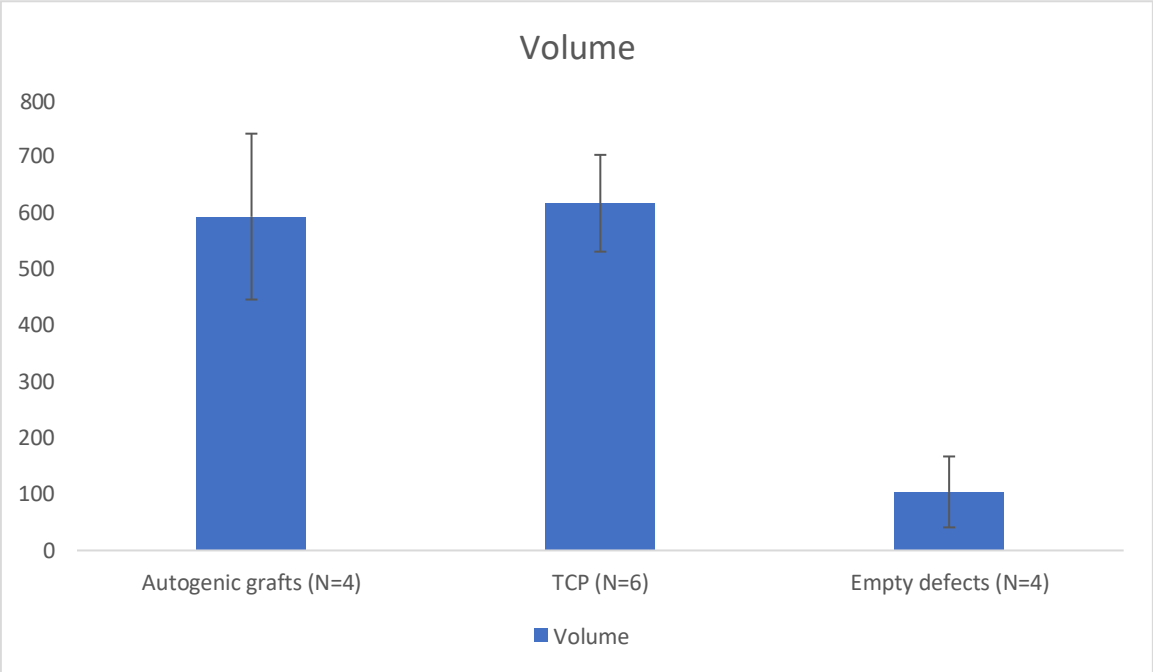

Figure 17. This graph compares the mean bone and graft volumes between the three experimental groups with 3.5cm defects (without PTFE). Positive control group (autogenic grafts) had a mean BV 593.36mm^3 (SD 147.35), experimental control group (β-TCP) had a mean BV 616.93mm^3 (SD 86.04) and the negative control group (empty defect) had a mean BV 104.11mm^3 (SD 62.98).

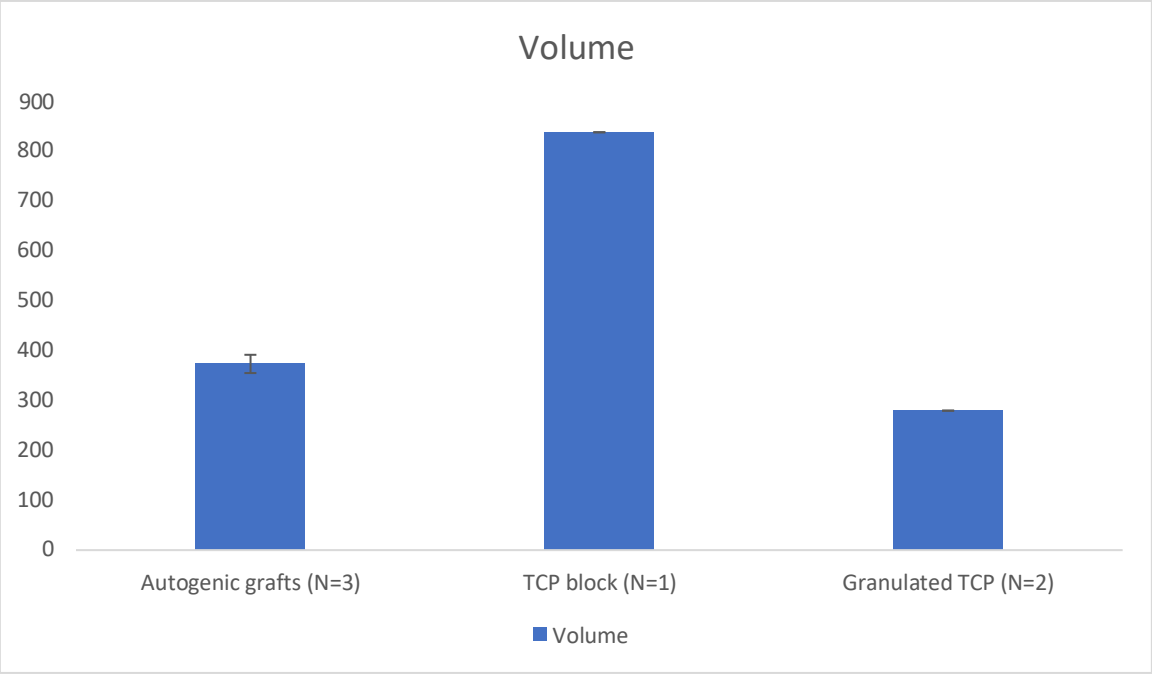

Figure 18. This graph compares the mean bone and graft volumes between the experimental groups with 3.5cm defects (PTFE). Positive control group (autogenic grafts) had a mean BV 372.26mm^3 (SD 18.3), experimental control group (β-TCP) had a mean BV 835.29mm^3 and the experimental control group (granulated) had a mean BV 278.72mm^3.

In addition, all the autograft and β-TCP samples exhibited complete bridging running the entirety of the defect sites from one edge to the other, where bone volumes of the bridges were measured and showed a mean of 292.79mm^3 and 255.31mm^3, respectively. No statistical significance was shown when the bridge volumes were compared between the two groups, P = 0.2406. It is worthy to note that the empty defect arm has shown minimal and incomplete bridges that were generated from the edges of the defect. The mean length of the outgrowths was 6.89mm with a mean bone volume of 50.51mm^3. When this was compared with the autograft and β-TCP arms there was statistical significance at P = 0.0003 and P = 0.0002, respectively.

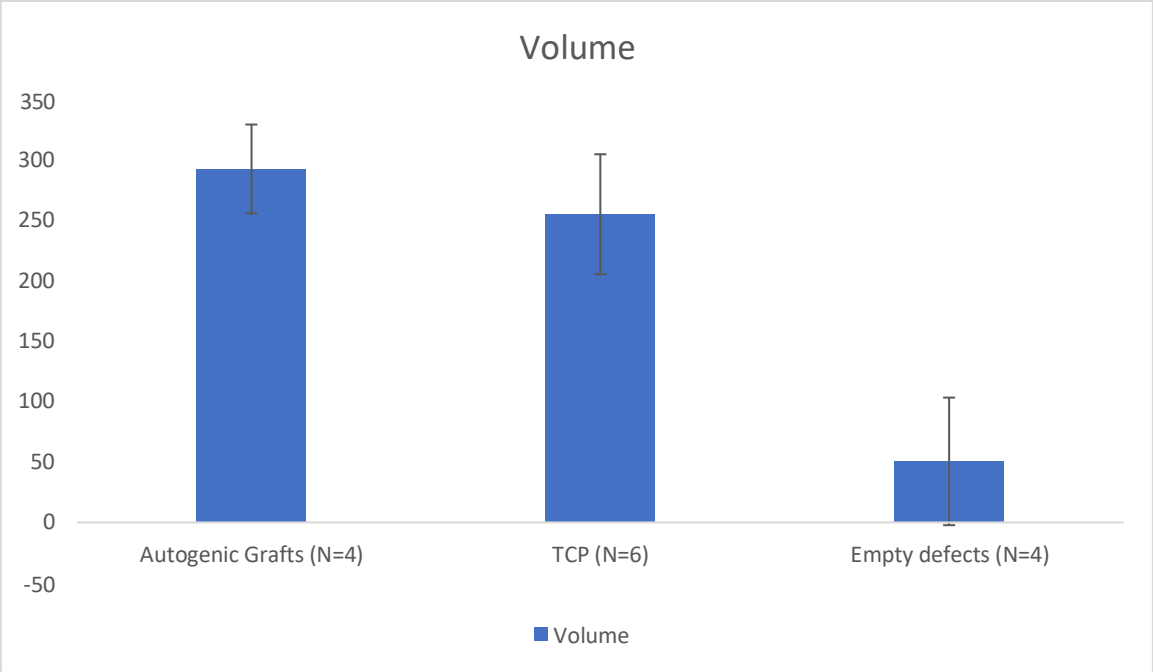

Figure 19. This graph compares the mean bone and graft volumes of the bony bridges between the experimental groups with 3.5cm defects (without PTFE). Positive control group (autogenic grafts) had a mean BV 292.79mm^3 (SD 36.8), the experimental control group (β-TCP) had a mean BV 255.31mm^3 (SD 49.63) and the negative control group (empty defect) had a mean BV 50.51mm^3 (SD 52.84).

Similarly, the samples containing PTFE, had developed bone bridges between the defect edges, although the total bone volumes were less compared to the samples that did not contain PTFE membranes. Autograft samples (n=3) had a bridge bone volume of 188.76mm^3 (SD 25.24). One sample containing β-TCP block (n=1) had a mean bone volume of 332.81mm^3. While the two samples containing granulated β-TCP (n=2) had a mean bone volume of 143.16mm^3 (SD 24.44).

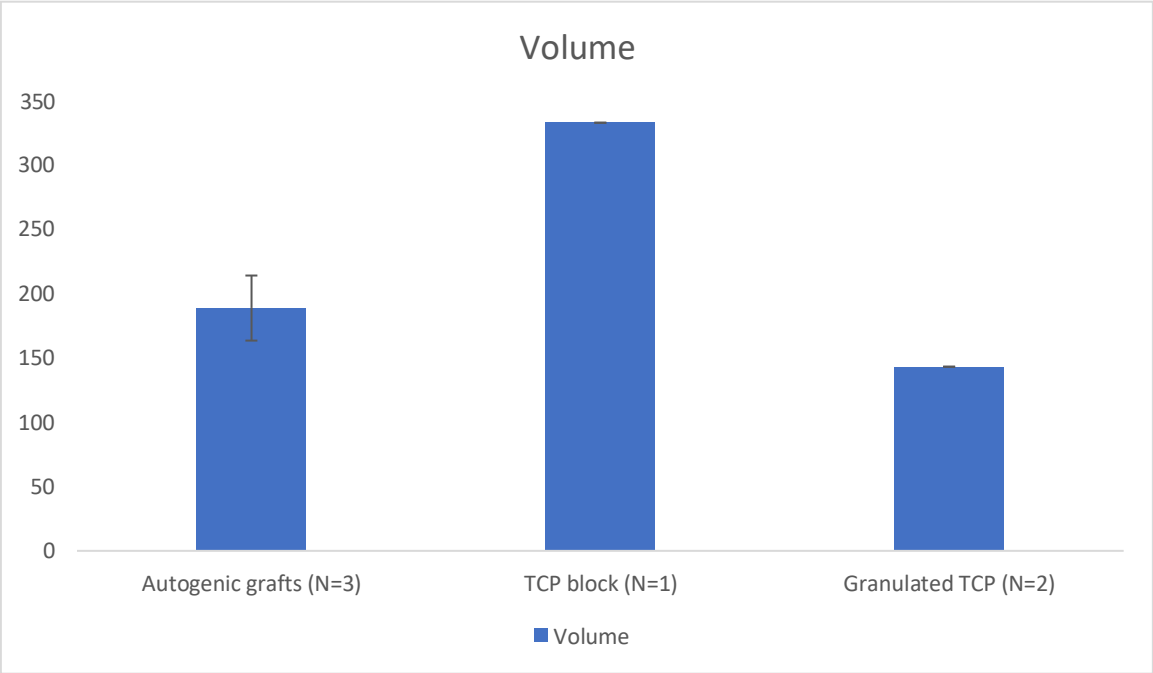

Figure 20. This graph compares the mean bone and graft volumes between the experimental groups with 3.5cm defects (PTFE). Positive control group (autogenic grafts) had a mean BV 188.76mm^3 (SD 25.24), experimental control group (β-TCP) had a mean BV 332.81mm^3 and the experimental control group (granulated) had a mean 143.16mm^3.

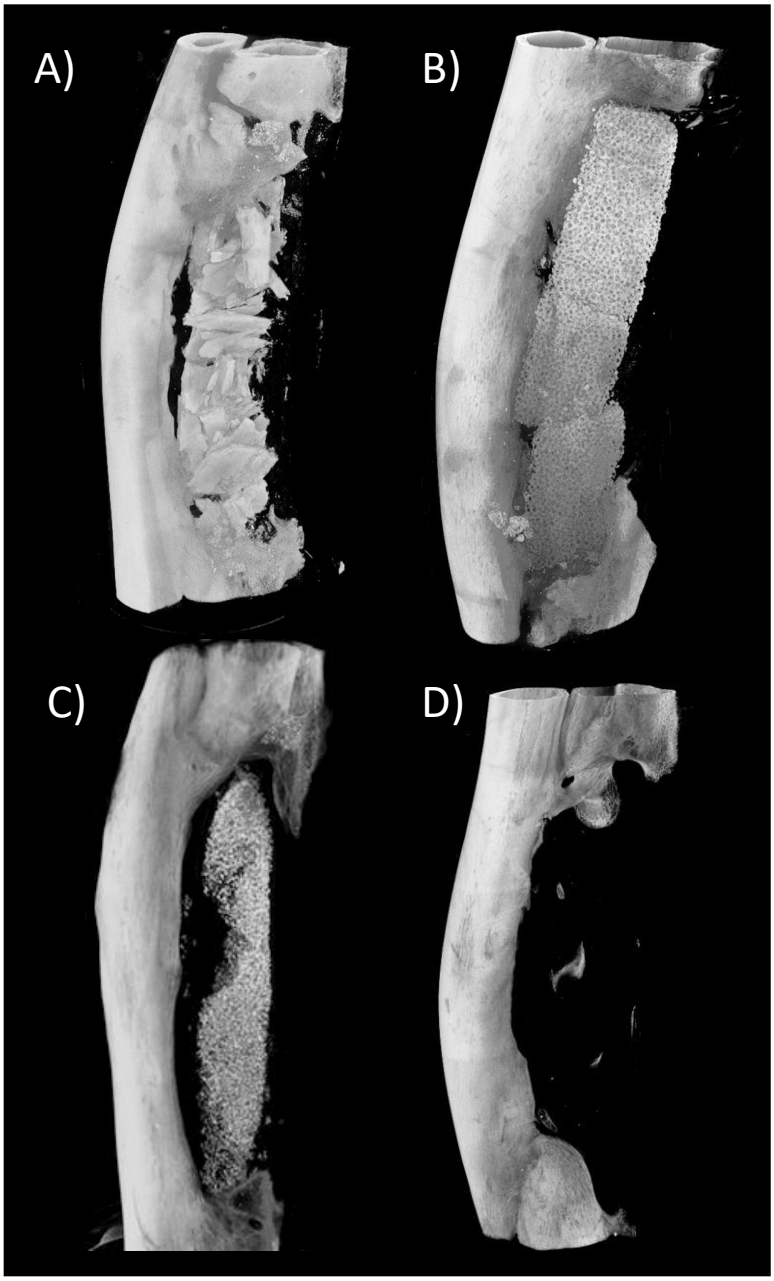

Figure 21. 3D reconstruction images obtained from micro-CT analysis. A) Autogenic bone graft. B) β-TCP graft. C) Granulated β-TCP graft. D) Empty defect showing minimal bone regeneration.

Histological Analysis:

All samples were embedded in methylmethacrylate (MMA). Histological sections were stained with tartrate-resistant acid phosphatase (TRAP) that identifies osteoclast activity and methylene blue/basic fuchsine (MB/BF) that stains newly formed bone in red and fibrous tissue in blue.

Quantitative analysis of the TRAP staining shows an increase in TRAP activities in the autograft and β-TCP groups while showing less activities in the empty defect group, with a mean surface ratio of 0.68 (SD 0.09), 0.76 (SD 0.15) and 0.46 (SD 0.11), respectively. The autograft and β-TCP groups were compared with the empty defect group to show statistical significance at ($P = 0.0169$) and ($P = 0.0079$), respectively. It is worthy to note that the β-TCP has shown slightly elevated TRAP activity compared to the autograft group, but there was no difference statistically, ($P = 0.3682$).

Futhermore, the MB/BF stains have shown statistically significant higher levels of newly formed bone tissues in the autograft and β-TCP groups in comparison with the empty defect group, with a mean surface ratio 0.35 (SD 0.16), 0.21 (SD 0.05) and 0.13 (SD 0.026), respectively. The autograft and β-TCP groups were compared with the empty defect group to show statistical significance at ($P = 0.037$) and ($P = 0.0185$), respectively. While the autograft group have shown a slightly higher amount of newly formed bone, they were not statistically different, $P=0.0728$.

The samples that contained PTFE membranes underwent similar staining techniques and analysis. Quantitative analysis of the TRAP staining showed comparative results between the autograft and β-TCP group with a mean surface ratio of 0.52 (SD 0.16) and 0.42 (SD 0.07), respectively ($P=0.3774$). Similarly, (MB/BF) staining showed comparative results between the autograft and

β-TCP group with a mean surface ratio of 0.35 (SD 0.03) and 0.34 (SD 0.09), respectively (P =0.864).

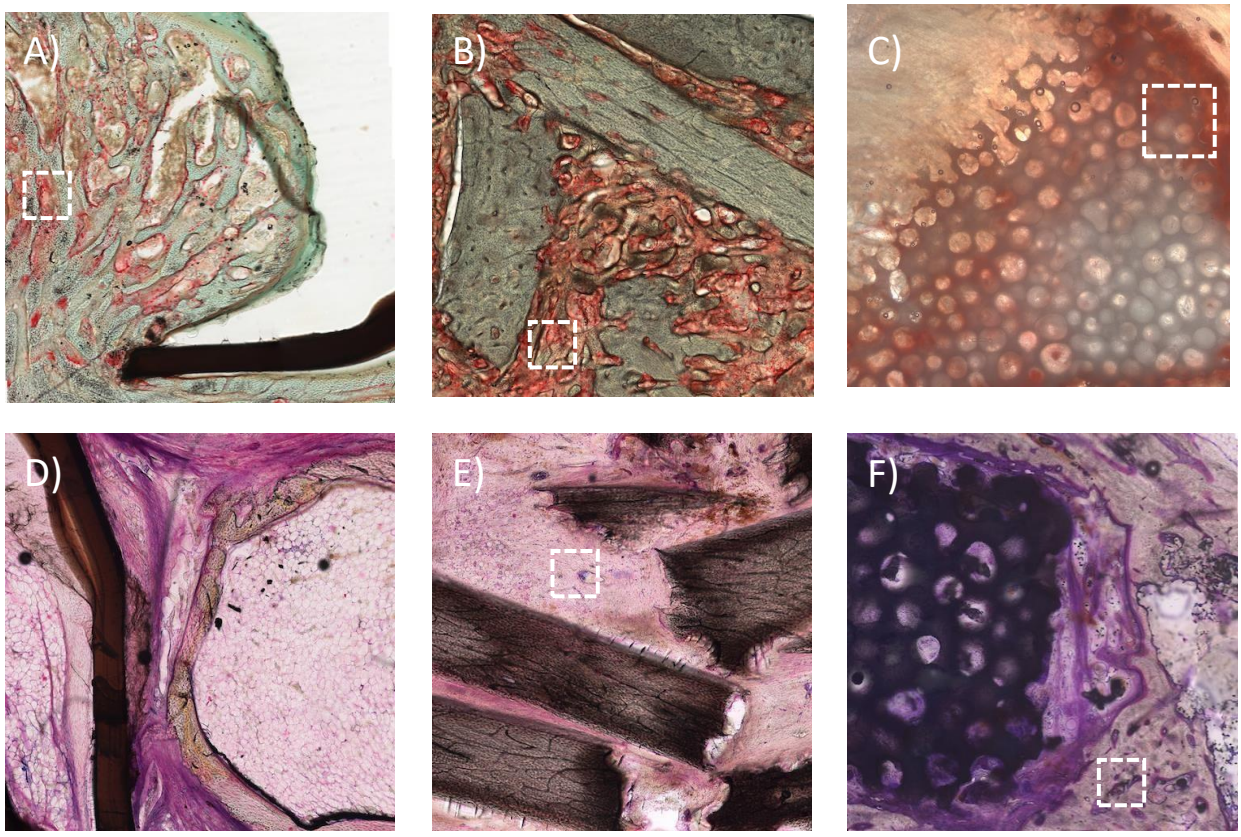

Figure 22. The annotative boxes in the top row showcases the level of TRAP activity and the lower row showcases the amount of osteoid formed stained in blue with methylene blue/basic fuchsine (MB/BF). A) Edge of an empty defect sample showcasing lower TRAP activity. B) Autogenic graft sample showcasing higher TRAP activity, compared to the empty sample. C) β-TCP graft sample showcasing high TRAP activity surrounding the graft as well as the pores. D) MB/BF stain of an empty defect. E) MB/BF stain of an autogenic graft. F) MB/BF stain of a β-TCP graft.

Discussion:

In bone regeneration research, rabbit models are one of the most commonly used[138]. But one of the drawbacks of rabbit models is their rapid rate of regenerating bone and turnover[139], which poses some challenges in defining "massive bone defects" in rabbits, where bones are not able to regenerate without any intervention. In this study, it was noted that in the 2cm group, two defects showed significant bone regeneration while all the defects in the 3.5cm showed minimal bone regeneration in relation to their sizes, indicating to the inability of the native bone to heal spontaneously.

The Masquelet Technique is ideal in managing these massive defects as it addresses several issues. First, the technique provides mechanical stability to the defect site that is essential to the healing process, by PMMA placement and applying rigid fixation[101, 140], as unstable fixation have been shown to result in non-union[141]. As noted previously in the methods section, no fixation devices were used during the surgical procedures in this project. Stability was provided mainly through the PMMA spacer during the first stage of the technique and the adjacent radius bone throughout the duration of the experiment. Due to the tight tissue existing between the ulna and radius in the rabbits' forelimb anatomy, creating a defect on the ulnar site and relying on the radius as the main bone is a standard concept in bone regeneration studies[142].

In addition, PMMA spacer results in the formation of the "periosteum-like" surrounding fibrous membrane, due to foreign body reaction[142]. This induced membrane seems to possess osteogenic and healing properties where it contributes to the formation of extracellular matrix and production of growth factors, important for the healing process[143]. PMMA also prevents the formation of fibrous tissues in the defect site, which is a major cause for non-union[120]. In this project, the induced membrane was not evaluated histologically. Future studies may benefit from comparisons with them membrane and the native periosteum. As previously discussed in

Chapter 2, the possibility that the membrane acts as a pro-regenerative periosteum like membrane is one of the proposed mechanisms of action of the Masquelet technique.

Another important factor is vascular supply. Both surrounding soft tissue[120] and induced membrane vasculature contribute to the healing process by delivering oxygen and nutrients, removing waste[144] and transporting various cytokines and growth factors[145]. Some of the factors that is transported to healing site includes, VEGF and vWF[143]. VEGF is known to initiate and promote angiogenesis and its functional absence negatively affects bone formation due to absence of formation of new blood vessels[143]. Studies are suggesting that VEGF has an osteogenic role in bone healing by stimulating osteoblast differentiation and recruitment of osteoclasts[143, 144]. While vWF ensures that the new blood vessels are formed normally[146].

The edges of bony defects are important sources for generating new bone. The induced membrane and the other factors merely facilitate bone formation, whereas the starting point of new bone and the main source of osteoprogenitor cells and therefore its successor, the osteoblasts, originate at the defect edges[147]. All this has been observed in this study, where in the larger positive and experimental groups have exhibited a bony bridge connecting one side of the defect to the other. This seemingly had started from the defects edges and had the necessary support from the factors delivered through the Masquelet Technique (bony edges, induced membrane, vascularization, surrounding soft tissue, materials, etc.). On the other hand, the larger empty defect samples have shown minimal bone regeneration mostly at the edges of the defects, indicating that initially, there was an attempt by the edges, represented by osteoprogenitor cells, to initiate bone healing but had failed due to the absence of the previously mentioned facilitators.

β-TCP materials have been used previously in animal and human studies[148]. These studies have observed various timings at which the materials are resorbed, of course depending on

the animal model used and defect sizes, but can take up to 26 weeks[149]. In addition, β-TCPs possess osteoconductive properties, meaning that they facilitate the expansion of a variety of tissues, cells and vasculature[150]. This is the first study to use β-TCP in a rabbit model after creating a massive defect, that is almost 40% of total length of the ulnar bone.

β-TCPs are very useful in grave clinical situations. This material is gaining popularity with surgeons due to several factors, such as in cases of insufficient sources of autogenic or allogenic grafts, relatively inexpensive and no risk of infection transmission[148, 150]. Studies have claimed that the strength and structure of β-TCPs are comparable to cancellous bone[150, 151].

Although we have not observed total resorption and degradation of our graft materials, due to relatively short period (6 weeks), multiple animal studies have reported complete resorption after at least four to six months[149, 152, 153]. But in this study, we have several indications about the progress of osteo-integration. Micro-CT analysis revealed quantities of bone volumes comparable to autograft samples, studied for the same period. Although new bone was not observed at the centre of the defects, bone bridges were observed spanning the entire length of the defect, similarly to the ones observed with the positive controls (autografts).

The reasoning behind using PTFE membranes in other samples, in both positive and experimental controls, was to attempt to allow bone regeneration at the edges of the defect and limit bone regenerating from the adjacent bone (radius). These samples have shown in general less bone volume compared to the samples that did not have PTFE membranes due to the fact that decreased bone generated from the adjacent radial bone. Increasing the implantation time would possibly allow sufficient time for β-TCP blocks to integrate and resorb in massive defects of 3.5cm in rabbit models.

Another interesting aspect to consider, which was evaluated previously at our laboratory by Tarchala et al[134], is the modified single step Masquelet Technique. This included only one surgical procedure that eliminated the use of bone spacers without formation of any membrane. In that work authors used both non-porous and porous PTFE membranes that were placed in the defect and directly filled with bone allograft samples. They compared their results with the traditional Masquelet Technique and found no difference in bone formation. As one of the future directives, it would be compelling to study the applicability of the single step technique with β-TCP, using PTFE or other materials such as silicone for guided bone regeneration. Additionally, comparisons between direct placement of bone grafts and their substitutes with the induced membrane technique may help us better understand how to better optimise the approach.

Chapter 5:

Conclusion and Future Directions

The burden of massive/critical sized bone defects on the healthcare system is immense. Whether they are caused by polytrauma, bone tumors or infections, increasing costs and patient morbidity are challenging tasks. Unfortunately, there is no single best method for the management of these large defects as every modality has its own advantages and disadvantages. The Masquelet Technique is one of the promising methods that has gained popularity in the past two decades but questions remain. The role of the fibrous "induced" membrane is not fully known, although it has been shown to resemble the periosteum structurally and functionally[102].

Another issue is the optimal type of bone graft to use in combination with the Masquelet Technique to fill the defect site. Autogenic bone grafts are effective but practically difficult especially in large defects. Synthetic β-TCP bone substitutes have shown effectiveness but their use raises further questions. There is no consensus on the optimal geometry of the material. In this study, β-TCP grafts were highly porous (75%) and had an average pore size of 510μm, and has been shown to allow enhanced cell and blood vessel ingrowth within the scaffold[137].

Although our results have shown some bone forming activities mainly around the edges of the defect, the graft itself has not shown evidence of complete resorption and osteointegration. This might be explained by the relatively short implantation time in this study (6 weeks) in comparison with other animal studies[154] that have reported promising results with the use β-TCP grafts for longer periods, although without the utilization of the Masquelet Technique. It would be interesting to study Masquelet's fibrous membrane and its effect on the β-TCP scaffold in longer implantation periods (e.g. 12 weeks).

Furthermore, studying the effects of supplementing β-TCP grafts with growth factors using the Masquelet Technique would be interesting, as previous studies showed that several molecules and growth factors (BMPs, TGFs, etc.) are effective in augmenting bone regeneration and formation[155, 156].

Chapter 6:

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