STUDIES ON THE PHYSICOCHEMICAL, BIOMECHANICAL AND BIOLOGICAL PROPERTIES OF NOVEL DECELLULARIZED BOVINE SCAFFOLD FOR BONE REGENERATION

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UNIVERSITI SAINS MALAYSIA

2024

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

SEPTEMBER 2024

ACKNOWLEDGEMENT

In the Name of Allah, the Most Gracious, the Most Merciful

First and foremost, I shall thank Allah the Great who provided me with life and patience. I shall thank my family, my wife, my parents, and my kids for their patience during my period of study. I thank my supervisor, Prof. Dr. Azlina Ahmad for her guidance, patience and understanding in making my PhD a feasible and enjoyable study, without her support, this work would not have been possible. I deeply appreciate my field supervisor, Prof. Dato' Ab. Rani Samsudin for his enthusiastic guidance, persistent motivation, support, and advice throughout my PhD project who taught me how to love science and how to become a passionate researcher and clinician, it would have been impossible for me to complete this thesis without his supervision. My sincere gratitude also goes to those who encouraged me to take up this challenging project. My heartfelt gratitude goes to Prof. Sausan Al Kawas Vice Dean at the University of Sharjah for her continuous follow-up and support during my study, where she assists at any time needed. I deeply appreciate my co-supervisor Prof. Dr. Suzina Sheikh Ab Hamid for her step-by-step support, motivation and advice and her continuous technical support and supply of materials from the Tissue Bank during the period of my study. I would also like to thank my co-supervisor Dr. Johari Abdullah from the Craniofacial Imaging Laboratory for his expertise, knowledge, and guidance and all whole staff of the Craniofacial laboratory for their support and guidance. I want to extend my special thanks to Dr. Aghila Rani Nair from the Research Institute for Medical and Health Science for her close and deep work in making this study valuable and real, and for spending time and effort during my study journey in a teamwork manner. Finally, I would like to thank all staff of the Animal Facility and Advanced Materials Research Laboratory, University of Sharjah and Universiti Sains Malaysia for their assistance and support.

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LIST OF SYMBOLS

α	Alpha
bp	Base pair
^{0}C	Degree celsius
d	Day
Ø	Diameter
et al	Et alia
HU	Hounsfield Unit
µL	Micro liter

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ARS	Alizarin Red S
ASTM	American Society for Testing and Materials
ARASC	Animal Research and Service Centre
ATR	Attenuated total reflection
ANOVA	Analysis of variance
BCP	Biphasic calcium phosphate
BTE	Bone tissue engineering
BMP-2	Bone morphogenetic proteins 2
BSE	Bovine spongiform encephalopathy
BV	Blood vessels
BBM	Bovine bone mineral
BSE	Bovine spongiform encephalitis
CSBD	Critical-sized bone defect
CTRL	Control
Ct	Cycle threshold
CDA	Calcium-deficient apatite
CDHA	Calcium-deficient hydroxyapatite
CBA	Cytometric Bead Array
CD8+	Cytotoxic T lymphocytes
DBM	Demineralized bone matrix
DCC	Decellularized bovine bone
DICOM	Digital Imaging and Communication in Medicine
DPSCs	Dental pulp stem cells
DSCs	Dental stem cells
DMSO	Dimethyl sulphoxide
DM	Defect margin
DTA	Differential thermal analysis
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
DMB	Demineralized bovine bone
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EDS	Energy-dispersive X-ray
EDTA	Ethylene diamine tetra acetic acid
EO	Endochondral ossification
FDA	Food and drug administration
FDBA	Freeze-dried bone allograft
FS	Fibrin sealant
FT	Fibrous tissue
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GT	Granulation tissue
GFs	Growth factors
GPa	Gigapascals
H & E	Hematoxylin and eosin

H	Hours/Hour
HCL	Hydrochloric Acid
HBOT	Hyperbaric oxygen therapy
HAP	Hydroxyapatite
HU	Hounsfield unit
IGF-I	Insulin-like growth factor I
ICC	Intraclass correlation coefficients
IACUC	Institutional Animal Care and Use Committee USM
Mm	Millimeter
kVp	Kilo volt peak
kDa	Kilodalton
kGy	Kilogrey
LAL	Limulus Amebocyte Lysate
Mg	Milligram
Ml	Milliliter
Micro-CT	Micro-computed tomography
MSCs	Mesenchymal stem cells
NB	New bone formation
ng	Nanogram
nm	Nanometer
nHAP	Nano-hydroxyapatite
OC	Osteocalcin
OPN	Osteopontin
ON	Osteonectin
OBs	Osteoblasts
PO_4^{3-}	Phosphate
PLG	Poly(lactide-co-glycolide)
PLGA	Poly (lactide-co-glycolic acid)
RT-PCR	Reverse transcription polymerase chain reaction
Runx2	Runt-related transcription factor 2
RANK	Receptor activator of NF-kB
RC	Residual composite
ROI	Regions of interest
RPM	Revolutions per minute
SIMR	Sharjah Institute for medical research
SPSS	Statistical Package for the Social Sciences
SO	Sham Operated
SD	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
TCP	Tricalcium phosphate
TE	Tissue engineering
TGF	Transforming growth factor
TGF-β1	Transforming growth factor beta-1
CD4+	T helper cells
3-D	Three dimensional
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

LIST OF APPENDICES

KAJIAN SIFAT FIZIKOKIMIA, BIOMEKANIK DAN BIOLOGI KE ATAS KERANGKA BOVIN NYAHSELULAR BAHARU UNTUK REGENERASI TULANG

ABSTRAK

Pemindahan tulang, amalan pemindahan kedua paling biasa selepas pemindahan darah, melibatkan cabaran besar dalam memindahkan sel tulang penderma xenogenik kepada penerima disebabkan oleh potensi tindak balas imunologi. Kajian ini bertujuan untuk meneroka keberkesanan penghasilan perancah tulang spongiosa lembu dengan mengekalkan matriks ekstraselular (ECM) sambil menghapuskan sel tulang asli, serta membandingkan sifat fiziko-kimia, mekanikal, dan biologi mereka dengan perancah tulang spongiosa yang dinyahmineral. Kajian ini dijalankan dalam tiga fasa. Dalam Fasa I, blok tulang spongiosa yang diambil dari kepala femoral lembu telah dibersihkan secara fizikal, dinyahlemak secara kimia, dan diproses menjadi dua jenis perancah: perancah tulang spongiosa lembu yang dinyahmineral (DMB) dan perancah tulang spongiosa lembu yang dibuang selnya (DCC). Kedua-dua perancah ini kemudian dibekukan kering dan disinarkan gamma. Pelbagai analisis termasuk histologi, mikroskopi elektron imbasan, spektroskopi sinar-X penyebaran tenaga, dan spektroskopi inframerah transformasi Fourier telah dijalankan untuk menilai perancah-perancah tersebut. Kajian pengisian semula sel menggunakan sel osteoblas manusia menunjukkan bahawa perancah DCC menghasilkan ECM tanpa sel yang lengkap dengan liang yang lebih luas, mengekalkan fibril kolagen, dan mempamerkan pelekatan sel, percambahan, dan mineralisasi yang lebih baik berbanding DMB. Dalam Fasa II, keserasian imun perancah DMB dan DCC diuji pada model tikus Balb/c jantan selepas implantasi peritoneal. Keputusan

menunjukkan bahawa perancah DCC menimbulkan kiraan sel darah putih yang lebih rendah dan keradangan sistemik yang lebih sedikit berbanding dengan DMB dan tulang asli yang tidak dirawat. Analisis imunotoksik menunjukkan bahawa kumpulan DMB mempunyai kiraan CD4+ yang lebih tinggi dan peningkatan ekspresi sitokin pro-radang, manakala kumpulan DCC menunjukkan tindak balas imun yang lebih baik, dengan lebih banyak sel T CD8+ dan morfologi organ yang normal, menunjukkan keserasian imun yang lebih baik. Fasa III memberi tumpuan kepada keupayaan regenerasi tulang perancah DMB dan DCC dalam kecacatan saiz kritikal kalvaria tikus jantan Sprague-Dawley. Kajian ini mendapati bahawa perancah DCC secara signifikan menggalakkan pembentukan tulang baru, dengan penutupan kecacatan yang lebih baik dan ketumpatan tulang yang lebih tinggi yang diperhatikan dalam analisis micro-CT berbanding DMB. Tapak DCC juga menunjukkan peningkatan tahap mRNA penanda osteogenik seperti osteonektin, osteopontin, dan osteokalsin. Spektroskopi RAMAN menunjukkan peningkatan kehadiran kolagen dan mineral tulang, terutamanya ion fosfat (PO_4^{3-}) dalam perancah DCC. Kesimpulannya, teknik penyahselan berjaya menghasilkan perancah DCC tanpa sel dengan kerosakan ECM yang minimum dan potensi osteogenik yang unggul dalam model in vitro dan in vivo. Perancah DCC menunjukkan keserasian imun yang lebih baik dan potensi regenerasi tulang yang lebih tinggi berbanding DMB, menjadikannya pilihan yang menjanjikan untuk aplikasi pemindahan tulang.

STUDIES ON THE PHYSICOCHEMICAL, BIOMECHANICAL AND BIOLOGICAL PROPERTIES OF NOVEL DECELLULARIZED BOVINE SCAFFOLD FOR BONE REGENERATION

ABSTRACT

Bone grafting, the second most common transplant practice after blood transfusion, involves significant challenges in transferring xenogeneic donor bone cells to recipients due to potential immunological responses. This study aimed to explore the efficacy of producing bovine cancellous bone scaffolds by preserving the extracellular matrix (ECM) while eliminating native bone cells, comparing their physicochemical, mechanical, and biological properties with demineralized cancellous bone scaffolds. The study was conducted in three phases. In Phase I, cancellous bone blocks harvested from the bovine femoral head were physically cleansed, chemically defatted, and processed into two types of scaffolds: demineralized bovine cancellous bone (DMB) and decellularized bovine cancellous bone (DCC). Both scaffolds were freeze-dried and gamma-radiated. Various analyses, including histology, scanning electron microscopy, energy-dispersive X-ray spectroscopy, and Fourier-transform infrared spectroscopy, were performed to evaluate the scaffolds. Recellularization studies was done using human osteoblast cells showed that DCC scaffolds produced a complete acellular ECM with wider pores, retained collagen fibrils, and exhibited better cell attachment, proliferation, and mineralization compared to DMB. In Phase II, the immuno-compatibility of DMB and DCC scaffolds was tested in male Balb/c mice models following peritoneal implantation. The results revealed that DCC

scaffolds elicited significantly lower white blood cell counts and systemic inflammation compared to DMB and untreated native bone. Immunotoxicity analyses showed that the DMB group had higher CD4+ counts and increased pro-inflammatory cytokine expression, while the DCC group exhibited a more favourable immune response, with more CD8+ T cells and normal organ morphology, indicating better immuno-compatibility. Phase III focused on the bone regeneration capabilities of DMB and DCC scaffolds in male Sprague-Dawley rat calvarial critical-size defects. The study found that DCC scaffolds significantly promoted new bone formation, with enhanced defect closure and higher bone density observed in micro-CT analyses compared to DMB. DCC sites also demonstrated elevated mRNA levels of osteogenic markers such as osteonectin, osteopontin, and osteocalcin. RAMAN spectroscopy showed an increased abundance of collagen and bone minerals, particularly phosphate ions (PO_4^{3-}), in DCC scaffolds. In conclusion, the decellularization technique effectively produced an acellular DCC scaffold with minimal ECM damage and superior osteogenic potential in both *in vitro* and *in vivo* models. DCC scaffolds demonstrated better immuno-compatibility and greater bone regeneration potential than DMB, making them a promising option for bone grafting applications.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Repairing residual bone defects can be challenging, especially after bone disease, cancer surgery, accidents, degenerative conditions, or congenital abnormalities. These conditions often lead to defects that require repair or regeneration. In dentistry, bony defects, including those in the alveolar bone, can result in resorption and eventual atrophy of the basal bone in edentulous sites or ridges (Wang & Lang, 2012). The loss of bone tissue can cause soft tissue collapse, resulting in functional loss and decreased quality of life. Bone substitutes play a crucial role in promoting optimal healing, particularly in critical-size bone defects (Walsh et al., 2017). The conventional autografts, acknowledged as the universally accepted standard for bone graft procedures, are devoid of immune rejection. However, they are constrained by limited donor site bone availability, necessitating additional surgery with associated surgical risks and healthcare costs (Schmidt, 2021). A successful bone grafting procedure typically involves a traditional tissue engineering triad comprising cells, bone scaffold, molecules, and environment (Mhanna & Hasan, 2016). The elements of this triad are cells, bone scaffold, molecules, and environment (Fig 1.1).

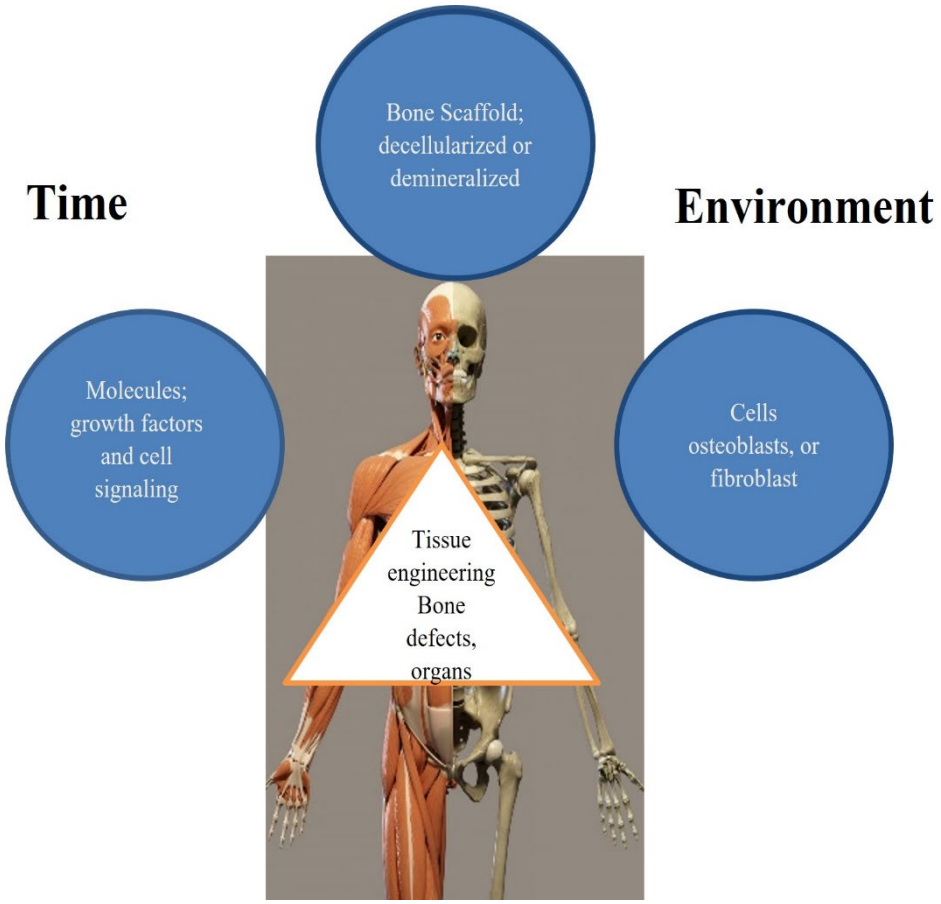

Figure 1.1 Fundamentals of tissue engineering triad adapted from Mhanna & Hasan, (2016).

Bone allografts are esteemed, and their obtainability is diminished because of concerns regarding infection transmission and significant acquisition costs. Consequently, alloplasts synthetic bone, particularly hydroxyapatite substitutes, have largely met these demands (Donnaloja et al., 2020).

Despite advancements like 3D-tissue printing and nanotechnology, significant challenges persist in replicating the intricate natural bone structure essential for effective interaction with the cellular microenvironment using synthetic alloplastic scaffolds (Alaribe et al., 2016). Unfortunately, there is currently no suitable synthetic or biological bone graft substitute in surgical practice capable of fully replacing lost bone. The search for an ideal bone graft material continues to expand, with ongoing

limitations. However, the selection criteria for bone grafts must prioritize the replacement of bony defects while preserving osteogenic properties, containing both osteoinductive and osteoconductive capacities (Georgeanu et al., 2023).

Due to the limited availability of allogenic bone grafts, there is an urgent need for bone substitutes that are compatible with the body, leading to exploration into xenogenic tissues sources. For more than five decades, processed bovine bone graft scaffolds have been utilized extensively, showing different levels of effectiveness and ongoing favor because of their plentiful availability (Alaribe, 2016; da Silva et al., 2020; Donnaloja, 2020). However, xenotransplantation poses inherent risks, and concerns are growing regarding the potential enduring consequences of introducing genetic material from other species into the host which can cause immunological reactions and then rejection of the transplanted organ or graft (Keane et al., 2012).

Bone regeneration plays a fundamental role in clinical practice, targeting defects caused by injuries, congenital abnormalities, and the removal of tumors. While existing clinical approaches can manage many of these defects, non-union defects, characterized by incomplete closure, remain challenging. In our experience, an ideal animal experimental model should exhibit high reproducibility, suitability for evaluating various materials or strategies, relevance to relevant clinical scenarios, compatibility with multiple analysis methods, and minimal morbidity and mortality until the intended experimental endpoint (Spicer et al., 2013). Additional considerations in assessing animal models involve the duration required to collect statistically significant data, related costs, and the expertise needed to proficiently conduct experimental procedures. Orthotopic animal models provide the most clinically relevant evaluation for bone regeneration strategies, particularly in non-

union cases. However, alternative non-weight-bearing sites, like calvarial defects, may be used for functional testing. In the context of bone tissue engineering, orthotopic animals refer to animal models in which engineered bone tissue, grafts, or scaffolds are implanted into the natural anatomical site of the bone where the tissue is intended to grow or regenerate. This means the engineered construct is placed at the specific bone location (such as the femur, tibia, or skull) that mimics the environment in which the bone would naturally develop, repair, or remodel (Amini et al., 2012). In orthotopic models, engineered bone tissue or scaffolds are implanted in the original bone site, allowing researchers to assess tissue integration, healing responses, and biomaterial performance in a realistic environment, making these models more predictive of human outcomes compared to heterotopic models (Spicer et al., 2012). While assessing bone regeneration functionally *in vivo* may not be feasible within calvarial defects, alternative models allow for such assessments. Hence, based on the aim of the bone regeneration approach or biomaterial, the rat calvarial defect may offer a swift, high-capacity method for assessing bone regeneration *in vivo* (Calasans-Maia et al., 2009).

Evaluation of bone grafts in animal models is essential to assess the immunogenicity of decellularized bone and to ensure they are devoid of prions, preventing disease transmission and immunological reactions (You et al., 2018). An essential factor of any bone xenograft is to be free from any prions which allows disease transmission in the future to the recipient body and prevents any immunoreactions (Bracey et al., 2018; Singh et al., 2016).

Contemporary understanding of transplant and regenerative sciences have endorsed the use of xenogeneic bone grafts which has promoted their utilization as

reliable alternatives to aid in the recovery of critical-size bone defects. However, xenotransplantation poses inherent risks, and there is increasing recognition of the potential long-term consequences associated with transferring xenogeneic genetic material into the host (Abdullah et al., 1999; Al Qabbani et al., 2018; Keane et al., 2012).

Recently, the utilization of decellularized bone matrix from xenograft sources has gained prominence due to its ability to provide a natural environment biochemically and in biocompatible grade, and promotion of effective bone formation with minimal immunogenicity in host tissue. While contemporary allo- and xeno-tissue grafts can alleviate acute inflammatory host responses and modulate innate immunity reactions, their impact on cell-mediated immunity remains inadequately explored (Fishman et al., 2013).

Unfortunately, many xenogeneic tissue grafts, including xenogeneic bone, have elicited prolonged cell-mediated immunity after initially overcoming essential immunity. This phenomenon may manifest as chronic inflammation at the xenografted site, characterized by persistent clinical symptoms such as pain, swelling, and tenderness long after graft surgery, often resulting in failure and rejection due to the presence of residual native cells, nucleic acids, and alpha-gal epitopes despite rigorous processing efforts to eliminate them (Kasravi et al., 2023). The consideration of cell-mediated immunity against implanted xenogeneic bone grafts extends beyond traditional scope of 'biocompatibility' emphasized by tissue engineering scientists. Consequently, various novel processing strategies, including effective tissue decellularization processes, are being proposed for the development of future immuno-compatible xenogeneic tissues.

1.2 Research Background

Decellularization, employed in biomedical engineering, the process entails removing the resident cells from a tissue's extracellular matrix (ECM), resulting in an ECM scaffold that mimics the original tissue. This technique finds applications in artificial organs and tissue regeneration. Stephen F. Badylak led the pioneering efforts in decellularization in 2006, at the McGowan science institute for regenerative medicine, University of Pittsburgh (Gilbert et al., 2006). This technique created natural biomaterial to act as a scaffold for cell growth, differentiation and tissue development. According to Chen and Lv (2018), the use of decellularized tissues in tissue engineering applications has gained significant popularity, particularly concerning decellularized bone matrix. The delicate focus on this matter arises from the complex composition of both the internal and external structure of bone (Chen & Lv, 2018). Decellularization emerges as a potential method for creating natural scaffolds for tissue regeneration endeavors. While effective decellularization has been documented across diverse tissues like skin, tendons, organs, and cartilage, there remains a gap in research concerning hard tissue decellularization, notably in the context of bone (Lee et al., 2016). Decellularized bone tissue is now preferred and favored over demineralized bone because it effectively preserves the ECM (Gilbert et al., 2006). This preservation enables appropriate cellular attachment which maintains the structural integrity and mechanical of the bone, comparable to its natural state. Additionally, the application of a decellularized bone matrix reduces the likelihood of immunogenic reactions occurring in the host tissue, as highlighted by the studies conducted by (Lee et al., 2016; Tapias & Ott, 2014). Biological active substances like cytokines and growth factors are maintained within the decellularized bone matrix which allows the osteoinductive ability of the scaffold (Song & Ott, 2011).

Preclinical *in vivo* trials (animal models) are essential for testing any processed grafting material to ensure the biocompatibility and efficacy of this material in bone formation. This can be achieved by inducing a critical-sized defect in rodents for testing new bone substitutes (Brennan et al., 2014; Spicer et al., 2012).

Bovine derived bone matrix which are demineralized has gained widespread recognition in dentistry throughout the last fifty years (Gruskin et al., 2012). It serves as a commercially available biomaterial with osteoconductive and osteoinductive properties, accepted as a medical device for addressing bone defects. While it has a substantial history of both clinical successes and failures, its use has been somewhat limited due to its low mechanical strength, mainly utilized to fill minor bone defects in periodontal conditions and cystic spaces. Nevertheless, in more intricate skeletal reconstruction procedures, it has been combined with platelet-rich blood end products and growth factors to augment its regenerative potential (Bezerra et al., 2019). The primary method for preparing demineralized bovine bone matrix involves extraction of acids of the inorganic matrix while maintaining a significant portion of the bone's proteinaceous components. This process also preserves minor quantities of inorganic phosphates and calcium-based solids while retaining native cellular remnants. These remnants may present an enduring, yet unidentified, risk to the recipient in the long term. Harvesting of xenogeneic bone from carefully selected healthy bovine donors and subjecting these cortico-cancellous materials through physical washing, application of chemicals and detergents and enzymatic treatment may guarantee patients with a supply of safe and effective xenografts for bone regeneration. The challenges in meeting the biocompatibility requirements of bone substitutes have been dealt with, with considerable success through the understanding of cell-material interaction at the clinical, cellular and molecular levels (Williams, 2008).

One of the most common approaches utilized to remove any remaining cell debris in the tissue grafts is via the process of decellularization. In this method, ECM will be devoid of its original cells and genetic material, forming a natural scaffold that poses no risk of genetic material transfer (Wong & Griffiths, 2014). While the decellularization technique has found favor in the field of soft tissue biomaterials, there remains a lack of comprehensive understanding regarding its application in the development of natural bone graft substitutes (Baldwin et al., 2019; Gruskin et al., 2012; Keane et al., 2012).

The primary benefit of the decellularization approach is its ability to thoroughly remove all components from the cellular tissue while maintaining the structural integrity of the ECM. The complete and accurate operation of this process is very complicated but can be accomplished to some extent. As a result, efficient decellularization should prioritize the removal of cellular components and genetic material, while minimizing disruption to the ECM, thereby preserving its three-dimensional ultrastructure, biological activity and distinctive biomechanical properties (Amirazad et al., 2022; Keane et al., 2015).

The processing techniques used in decellularization development of bone scaffolds typically involve a series of physical methods, biological and chemical reagents along with enzymatic treatment to perform cell lysis, followed by thorough rinsing to eliminate cell debris, DNA, and damage-associated molecular pattern (DAMP) molecules which are released by stressed, dying or damaged cells. However, the potential effects of these processing stages on the host immune response have not been thoroughly investigated in an attempt to validate it scientifically. Incomplete decellularization of bone tissue graft can trigger immune responses in the host elicited

by remnant cellular components, fatty acids, DNA or Gal epitope retained on scaffolds (Ling et al., 2021). This would often lead to robust immune rejection and heighten patient discomfort, which may lead to graft failure and endanger patients' lives.

Preclinical immunological investigations *in vitro* or using animal models such as rodents *in vivo* may help characterize both the native and acquired immune system response to biomaterials. The adaptive nature of the immune system enables it to employ alternative mechanisms to address functional deficiencies. This rationale underpins the suggestion that utilizing *in vivo* animal model assessments, rather than *in vitro* tests, may offer a more precise assessment of immune competency and immunotoxic potential. While the innate immune response has been well understood and documented, the cell-mediated host-tissue responses to ECM products could be characterized by analyzing the mononuclear cell infiltrates and T-cell responses, evidence of systemic inflammation and immunological injury in immune-related organs including the spleen, liver, and kidney. Immunocompatibility and immuno-toxic status of xenogenic bone graft can be investigated by performing material implantation of lyophilized decellularized (DCC) and lyophilized demineralized (DMB) bovine cancellous bone graft into the peritoneal cavity of BALB/c mice over a 21-day observation period. Hematological and immunological data shall be screened together with histological examination of the liver and kidney to ascertain evidence of overcoming host defense and immunological organ injury.

In clinical practice, the persistence of native cells within bovine bone graft scaffolds can incite immune reactions, potentially contributing to the enduring the chronic inflammatory reaction that remains incompletely understood at the xenogeneic graft site. These clinical manifestations manifest long after the healing of the surgical

wound at the bone grafting operation site. While there is an urgent demand for a cell-devoid bone scaffold to secure implantation, the objective to create an ECM scaffold without cellular components demands meticulous physical and chemical treatments. Nonetheless, these processes might compromise the microstructure, chemical makeup, and mechanical integrity of the original natural material, all of which are vital for supporting cell growth. Various methods for tissue decellularization have been explored, yet consensus on the optimal bone decellularization approach remains elusive. Ideally, this technique should yield an ECM suitable for applications in osseous regenerative medicine (Wong & Griffiths, 2014; You et al., 2018). This research endeavors to assess the effectiveness of an innovative decellularization method in generating bovine cancellous bone scaffolds. It aims to compare the physicochemical, mechanical, and biological attributes of these scaffolds with demineralized scaffolds through *in vitro* and *in vivo* investigations. Although *in vitro* studies allow a wide range of physicochemical and biological tests to be performed on biomaterials, *in vivo* studies involving animal models will certainly provide high evidence of efficacy for future clinical studies.

Before advancing to larger animal models for possible use in the craniofacial region of the human body, researchers can utilize the rat calvarial defect model to assess bone healing and evaluate tissue engineering on different biomaterials or biostructural framework. For rat calvarial defects, the generally accepted critical size is 5 mm. However, smaller defects have been explored in models featuring two defects per animal. This approach allows for the utilization of fewer animals in each study. Nonetheless, researchers must carefully consider this advantage considering the study's objectives, as subcritical size defects may heal naturally without intervention. Furthermore, potential interactions between adjacent defects should be considered.

The calvarial defect in rat model serves as a platform for assessing bone regeneration and evaluating various tissue engineering or biomaterials structural framework before advancing to larger animal models for potential application for use in human craniofacial contexts. This study outlines the preparation, surgical technique, and potential analysis of bone regeneration in calvarial defects in rats, a model that has been employed in our laboratory for over the past decade. Critical-sized defects may vary in definition according to the literature, some researchers defined the defects according to the animal model, location of the defect and size of the circumferential bone (Schemitsch, 2017; Sun et al., 2014).

A numerous range of bone graft materials is readily accessible for utilization in medical and dental procedures. These materials originate from various sources and undergo distinct processing methodologies (Blaudez et al., 2020; da Silva et al., 2020; Rasch et al., 2019). Nonetheless, finding an optimal bone graft material remains a challenge, as it is difficult to surpass the expectations set by autografts, which are harvested from the patient during surgery and considered the "gold standard" against which the success of other grafting methods is measured (Schmidt, 2021). A various selection of deproteinized and demineralized bone has been used in oral and craniofacial defects for decades (Grgurevic et al., 2017; Laurencin & Jiang, 2014), nevertheless because cellular by-products are still present, these materials are prone to causing chronic inflammatory reactions (Rodriguez & Nowzari, 2019). Inappropriate and early resorption is yet another concern, which leaves the surgical site in need of a second access to receive another bone graft material (Keane et al., 2012).

The advancement of innovative technologies in bone tissue processing has continuously progressed, resulting in the successful development of biomaterials

based on decellularized bone matrix for repairing, replacing, and regenerating bone defects (Tran et al., 2020). The decellularization process eliminates immunogenic and cellular contents from tissues while sustaining the mechanical properties and natural constituents of the ECM. These features are crucial for facilitating the supply of nutrients and oxygen to the organ (Marquez et al., 2013). The decellularized bone matrix further revealed a significant advantage in providing the mechanical, physical and biological environment that cells require to live and grow (Yuan et al., 2016). According to their structural similarity with the native tissue, these grafts imitate the local microenvironment required for promoting cellular growth, adhesion, and activation of bone-forming signals (Amirazad et al., 2022).

Utilizing bovine cancellous bone in xenotransplantation poses inherent risks, and there is a growing recognition of the potential long-term consequences associated with introducing xenogeneic genetic material into the host (Keane et al., 2012). Concerns have been raised regarding the risk of transmitting bovine spongiform encephalopathy (BSE), a destructive degenerative disease which affects the nerves in cattle caused by abnormal prion proteins. BSE has been correlated to variant Creutzfeldt-Jakob disease (vCJD) in humans who have consumed infected meat products or been exposed to contaminated materials. However, decellularized bone grafts eliminate the risk of BSE transmission as the decellularization process removes all cellular components, including potential prions. Itoh and his group demonstrated the safety and effectiveness of decellularized bone grafts in various orthopedic procedures such as spinal fusion, arthroplasty, and bone defect repair (Itoh et al., 2023). Their study observed no adverse reactions or disease transmission associated with the use of decellularized bone grafts. As a result, decellularized bone grafts do not pose a risk of transmitting BSE or other prion diseases. The utilization of bovine-

derived materials in medical applications undergoes rigorous monitoring and regulation to safeguard patient well-being (Qabbani et al., 2017). Over the last three decades, thorough examinations and risk evaluations have been undertaken to assess the potential transmission of BSE through bovine bone-derived bone graft substitutes employed in dental procedures. These assessments consistently demonstrate that the risk of disease transmission is minimal when strict protocols are adhered to during the procurement and processing of raw bovine bone for commercial purposes (Sogal & Tofe, 1999). It is important to maintain an effective risk management program during operations involving xenogenic tissue processing for human use. As a result, clinicians have become less apprehensive about choosing bovine-origin bone substitutes when they are indicated for their patients.

The main classifications of bone grafts of natural source employed in dental practice incorporate demineralized, deproteinized and decellularized bone graft materials. Over the last half-century, demineralized bone grafts have been subject to inspection, exhibiting diverse outcomes and occasional delayed failures. In contrast, decellularized bone grafts show promise in mitigating immunogenicity in long-term and delayed chronic inflammatory reactions. There is a significant increase in the investigation of advanced decellularized bovine bone grafts, which are posited to provide improved biocompatibility and heightened osteogenic characteristics, suggesting potential clinical applicability (Ling et al., 2021; Solarte David et al., 2022).

1.3 Justification of the study

Clinical experience demonstrated chronic inflammation at sites of bovine substitute implantation without evidence of infection or trauma. The delayed inflammatory conditions do not resolve with antibiotic therapy. The study introduces

and evaluates a three-phase approach to produce bovine cancellous bone scaffolds through decellularization, aiming to preserve the ECM while removing native bone cells. This research study includes both *in vitro* and *in vivo* evaluations comprehensively by examining the physicochemical, mechanical, and biological characteristics of the produced scaffolds. This comprehensive assessment provides a valuable understanding of the efficacy and potential applications of the decellularized bone scaffolds. There is a need to produce bovine grafts that are biocompatible and immunocompatible with no trace of native bovine DNA. Current understanding points towards the presence of residual native cells, nucleic acids DNA that stimulates host cell-mediated immune response in the demineralized bone substitute. The decellularization technique is a preferable method in processing a biocompatible bone substitute and preserving the biological component of the ECM which can allow cell proliferation and attachment in addition to enhancing the osteogenic potential of bone defects with a desirable mechanical strength that can bear the loading forces. This method could offer a promising alternative to traditional bone grafting techniques. This study demonstrates the effectiveness of the decellularization method in generating acellular scaffolds with minimal ECM damage is confirmed, sustaining the success of the decellularization process by comparing the decellularized scaffolds with demineralized bone and native bone. Moreover, the scaffolds exhibit osteogenic potential both *in vitro* and *in vivo*, suggesting their suitability for bone regeneration applications.

There is a critical need to design a new processing technique that can eliminate the residual native cells, nucleic acids, and DNA in bone substitutes and develop a scientific method to validate its effectiveness in protecting from cell-mediated immunity. The findings have potential clinical implications, indicating that

decellularized bone scaffolds offer superior immuno-compatibility and osteogenic properties compared to demineralized bone scaffolds. This could explore the way for the development of improved bone grafting procedures with enhanced outcomes for patients. The study contributes to advancing knowledge in the field of bone regenerative medicine and tissue engineering, offering insights into the development of safer and more effective bone grafting techniques.

1.4 Null Hypothesis

Decellularized bovine bone scaffolds do not support regeneration of bone in healing sites of critical size defects. There is no significant difference between DCC and DMB in terms of biocompatibility and immunocompatibility.

1.5 Aim of the Study

1.5.1 General Objectives

The aim of this study was to develop a decellularized bovine cancellous bone and evaluate its biocompatibility and immunocompatibility in supporting the healing of critical-sized defects *in vitro* and *in vivo*.

1.5.2 Specific Objectives

1- To develop a method of decellularized bone graft substitute of bovine origin.

2- To investigate the physicochemical properties of the decellularized bovine bone graft substitute.

3- To determine the biomechanical properties of the decellularized bovine bone graft substitute.

4- To investigate the biocompatibility of the decellularized bovine bone *in vitro* and *in vivo*.

5- To investigate the immunocompatibility of the decellularized bovine bone *in vivo.*

6- To investigate the regenerative osteogenic potential of the decellularized bovine bone substitutes and the healing capacity of critical-size bone defects by histological and histomorphometric analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Bone biology

Bone is a complex material made up of various components arranged in a heterogeneous manner. These components include a mineral phase called hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ in the highest proportion, followed by an organic phase composed mostly of type I collagen (~90%), noncollagenous proteins (NCPs) (~5%), and lipids (~2%) by weight. The relative amounts of these constituents in bone can vary depending on factors such as age, site within the body, gender, ethnicity, and health status. Consequently, the properties of a particular bone are defined by the quantity, characteristics and proper arrangement of each of these components both in terms of quantity and quality (Boskey, 1989, 2013; Young, 2003).

Bone, a connective tissue rich in minerals, consists of four specific cell varieties: osteoblasts, osteocytes, osteoclasts and bone lining cells (Figures 2.1 & 2.2). It serves essential roles in the body, including facilitating movement, providing structural support and safeguarding soft tissues, storing phosphate and calcium, and housing bone marrow (Florencio-Silva et al., 2015).

Proteins within the ECM of bone can be divided into two groups: structural proteins, like collagen and fibronectin, and proteins with unique functions. These specialized proteins perform various tasks, such as controlling collagen fibril size, acting as signalling agents, functioning as growth factors, acting as enzymes, and executing other specific functions.

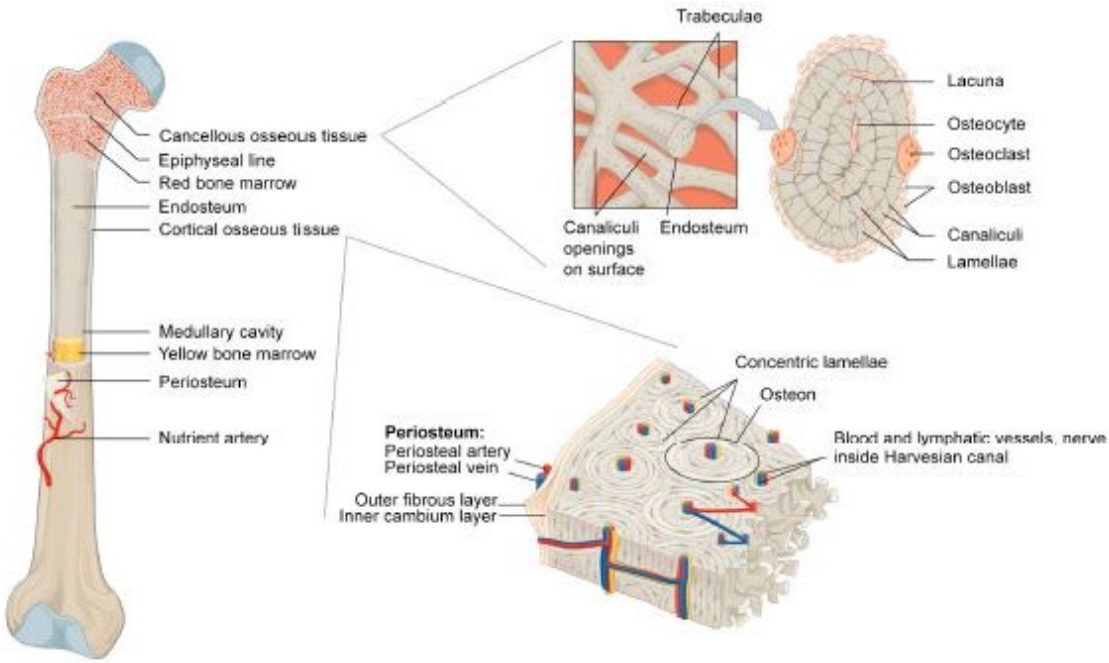

Figure 2.1 Bone anatomy adapted from Le et al., (2017).

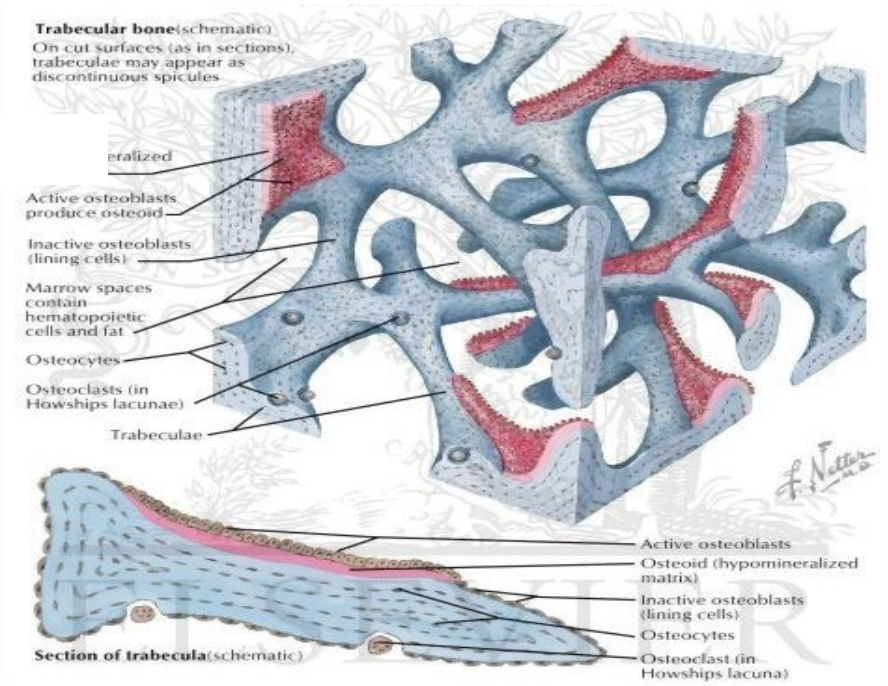

Figure 2.2 Structure of cancellous bone trabecula and involved cells adapted from Netter, (2014).

2.1.1 Osteoblasts

Osteoblasts derived from mesenchymal stem cells (MSCs), the commitment of osteoblasts to the osteoprogenitor lineage necessitates the timely activation of certain genes, particularly those involved in bone morphogenetic protein (BMP) synthesis. Crucial for osteoblast differentiation are the expressions of transcription factors like Distal-less homeobox 5 (Dlx5), Runt-related transcription factor 2 (Runx2), and osterix (*Osx*) (Capulli et al., 2014; Ducy et al., 1997). Of particular importance, Runx2 acts as a master regulator of osteoblast differentiation, as evidenced by the absence of osteoblasts in *Runx2*-null mice (Ducy et al., 1997; Komori, 2019). Runx2 orchestrates the upregulation of key osteoblast-related genes, including *ColIA1*, *ALP*, *OCN, BSP*, and *BGLAP* (Fakhry, 2013). Following the establishment of a pool of osteoblast progenitors expressing *ColIA1* and *Runx2*, a proliferation phase ensues, characterized by alkaline phosphatase (*ALP*) activity in preosteoblasts (Capulli et al., 2014). The transition to mature osteoblasts is marked by increased *Osx* expression and the secretion of bone matrix proteins like bone sialoprotein (*BSP*) I/II, osteocalcin (*OCN*), and collagen type I. Additionally, morphological changes occur in osteoblasts, leading to their enlargement and acquisition of a cuboidal shape.

2.1.2 Bone lining cells

Cells of the bone lining, characterized as resting, flat-shaped osteoblasts, serve to overlay bone surfaces where neither bone formation nor bone resorption occurs. These cells exhibit a thin, flat nuclear shape, with their cytoplasm stretching along the bone's surface and containing limited cytoplasmic structures like Golgi apparatus and rough endoplasmic reticulum profiles. Certain bone lining cells show extensions into canaliculi, with visible gap junctions connecting osteocytes and neighbouring bone lining cells (Miller et al., 1989). The secretory behavior of bone lining cells is contingent

upon the physiological state of the bone, with the potential for these cells to resume their secretory function, leading to an increase in size and a transition to a more cuboidal morphology (Donahue et al., 1995). While the precise functions of bone lining cells remain incompletely understood, evidence suggests their role in preventing direct interactions between bone matrix and osteoclasts when bone resorption is not warranted. Additionally, bone lining cells contribute to osteoclast differentiation by producing receptor activator of nuclear factor kappa-B ligand (*RANKL*) and osteoprotegerin (*OPG*) (Andersen et al., 2009). Moreover, bone lining cells, along with other bone cell types, form an essential part of the basic multicellular unit (BMU), a structural element crucial to the bone remodelling process (Everts et al., 2002).

2.1.3 Osteocytes

Osteocytes account for 90–95% of all bone cells, making them the most abundant and long-lasting cell type, with a lifespan extending up to 25 years (Franz-Odendaal et al., 2006). Unlike osteoclasts and osteoblasts, which are defined by their specific functions in bone formation and resorption, osteocytes were initially identified based on their shape and distribution within bone tissue. MSCs are derived from the lineage through osteoblast differentiation, osteocytes undergo a multi-stage process involving the young osteocyte, osteoid-osteocyte, preosteocyte, and mature osteocyte phases (Franz-Odendaal et al., 2006). After a bone formation cycle concludes, some osteoblasts transform into osteocytes, becoming part of the bone matrix. This change involves significant morphological and ultrastructural changes, such as a decrease in the rounded shape of osteoblasts.

While the osteocyte cell body resides within a lacuna, its cytoplasmic extensions, which can number up to 50 per cell, extend through small channels originating from the lacuna, termed canaliculi, thereby forming the osteocyte lacuna-

canalicular system (Manolagas, 2006). These cellular projections form links with neighbouring osteocyte processes through gap junctions, as well as with the extensions of bone lining cells and osteoblasts on the bone surface, aiding in the transmission of small signaling molecules like prostaglandins and nitric oxide (Florencio-Silva et al., 2015). Furthermore, the intricate network of lacunae and canaliculi within osteocytes is closely linked with the vascular system, facilitating the supply of oxygen and nutrients to osteocytes (Dallas et al., 2013).

2.1.4 Osteoclasts

Osteoclasts, multinucleated cells with specialized functions, undergo terminal differentiation from mononuclear cells within the hematopoietic stem cell lineage, a process regulated by various factors. Among these factors, macrophage colony-stimulating factor (M-CSF) plays a crucial role, produced by osteoprogenitor mesenchymal cells and osteoblasts, alongside RANK ligand secreted by osteoblasts, osteocytes, and stromal cells (Yavropoulou & Yovos, 2008). These factors collectively stimulate the activation of transcription factors and gene expression in osteoclasts.

Excessive osteoclast formation and intensified activity contribute to certain bone disorders like osteoporosis. In this condition, resorption surpasses formation, resulting in reduced bone density and elevated incidence of bone fractures (Kim et al., 2008). In certain pathological circumstances, in conditions like metastases of bone and inflammatory arthritis, aberrant activation of osteoclasts results in painful osteolytic lesions and periarticular erosions, respectively (Feng & McDonald, 2011).

2.1.5 Extracellular Bone Matrix

Bone consists of both mineral inorganic salts and an organic matrix (Boskey et al., 2002). The primary constituents of the organic matrix comprise collagenous proteins, primarily type I collagen, making up 90% of the matrix, along with

noncollagenous proteins such as *osteonectin*, *osteocalcin, fibronectin, osteopontin, and bone sialoprotein II*, *BMPs*, and growth factors. Whereas the inorganic portion of bone predominantly consists of calcium ions and phosphate. However, notable amounts of potassium, sodium, bicarbonate citrate, magnesium, fluorite, strontium, carbonate, zinc, and barium, are also present. Hydroxyapatite crystals are formed through the combination of calcium and phosphate ions, which, together with noncollagenous matrix proteins, establish a framework for hydroxyapatite deposition.

The interaction between these components plays a significant role in determining the unique strength and flexibility of bone tissue (Datta et al., 2008). The ECM is a three-dimensional framework secreted by cells into their surrounding space, consisting of specific polysaccharides and proteins. Each bone tissue's ECM has a unique composition and structure throughout its development (Frantz et al., 2010). Responsible for providing tissues with integrity and flexibility, the ECM undergoes continuous remodelling influenced by variations in receptor levels, growth factors, and local pH levels. These factors collectively regulate tissue and organ development, function, and maintenance (Bonnans et al., 2014). Within the scope of bone tissue engineering, the ECM is recognized as a pivotal component, representing the fourth element. Comprising 40% organic and 60% inorganic compounds, the bone matrix exhibits variability in composition based on factors such as sex, age, and health status. Comprising primarily of trace elements and calcium-deficient apatite, the inorganic constituents stand in contrast to the intricate nature of the organic ECM, which is predominantly constituted of collagen type I (90%) and noncollagenous proteins (10%). These organic components are primarily synthesized by osteoblasts before the mineralization phase (Mansour et al., 2017).

The organic ECM contains noncollagenous proteins, which can be classified into four types: g-carboxyglutamate-containing proteins, glycoproteins, proteoglycans, and small integrin-binding ligands N-linked glycoproteins (SIBLINs). These proteins interact dynamically with osteoblast-lineage cells and osteoclasts, playing a crucial role in coordinating the formation of new bone during the regeneration process (Paiva & Granjeiro, 2017).

2.2 Bone trauma and wounds

Bone wounds can be broadly classified into two main types; open (compound) bone wounds and closed (simple) bone wounds. Open bone wounds are wounds where the skin is broken, exposing the bone to the external environment. They can result from trauma, fractures, or surgical procedures. Open bone wounds are at higher risk of infection due to the direct exposure of the bone to pathogens. Closed (simple) bone wounds: These are bone wounds where the skin remains intact, covering the injured bone (Fig 2.3). Closed bone wounds commonly occur in less severe fractures or injuries where the bone does not break through the skin (Kumar & Narayan, 2014). While they may not be as prone to infection as open wounds, they still require appropriate medical attention and treatment (Harper et al., 2014).

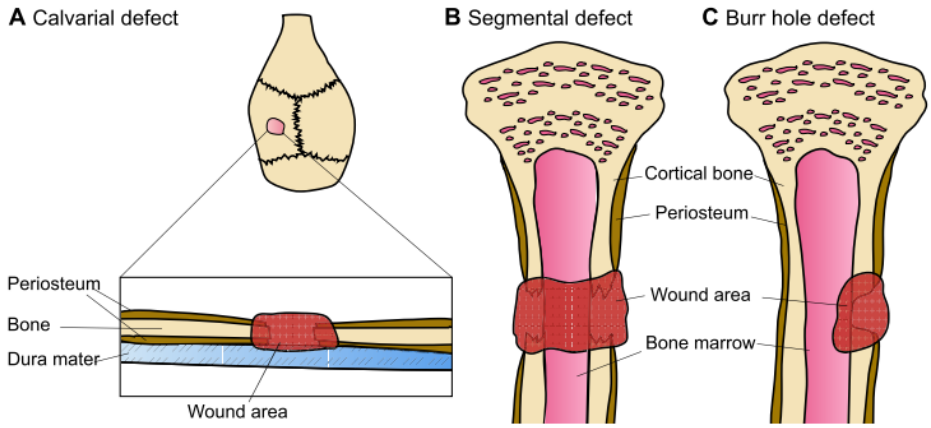

Figure 2.3 Bone defect models. Bone defect models utilized in research to simulate specific types of bone injuries. These models include: (A) Calvarial defects: created by drilling a circular burr hole and removing the resulting bone disk without harming the underlying dura mater. (B) Segmental bone defect model: Involves surgically removing a portion of bone, resulting in a gap between bone edges. This gap is stabilized using a fixation device and/or filled with a tissue-engineered bone substitute to investigate bone healing and formation. (C) Burr hole or partial defect model: Involves drilling an incomplete hole into the bone's side, often penetrating the cortical bone and possibly reaching the underlying cancellous bone or bone marrow cavity. This model typically induces injury on one side of the bone. Adapted from McGovern et al., (2018).

While bone possesses notable regenerative abilities depending on the nature of the bone injury, its capacity for healing may be restricted or inadequate in certain scenarios, such as critical-sized defects following trauma, revision surgeries, or tumor resection (Poser et al., 2014). Restoration of aesthetics and functional abilities in the maxillofacial and musculoskeletal regions is based upon restoring adequate volume of bone tissue whether it is a compound or simple bone wound (Liu et al., 2010). The repair rate of a bone defect is directly proportional to the size of the bone defect (Schmitz & Hollinger, 1986).

A bone wound indicates any harm or impairment to bone tissue, which can arise from diverse factors including trauma, fractures, surgeries, infections, or underlying medical issues. The severity of bone wounds can vary significantly, ranging from minor fractures to substantial breaks or loss of bone tissue (Gerstenfeld et al., 2003). The