

MASTER'S
ORAL REHABILITATION

INFLUENCE OF BONE GRAFTS SUBSTITUTES IN THE BIOLOGICAL RESPONSE TO ZIRCONIA IMPLANTS: *AN IN VITRO* STUDY

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"Segue o teu destino

Rega as tuas plantas

Ama as tuas rosas

O resto é a sombra,

de arvores alheias"

-Ricardo Reis

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ABSTRACT

Introduction: Bone is a complex dynamic tissue that undergoes constant remodelling. Formation as well as repair of bone are nuanced complex procedures that are dependent not only the host factors, but also the local cellular factors. A cascade of events starting from the proliferation of mesenchymal and hematopoietic stem cells into osteoblasts, osteoclasts etc., to the formation of well a structured connective tissue, bone serves an important organ that provides structural as well as mechanical support to the body. Although bone with small defects is capable of healing itself, larger defects often require grafting procedures to restore its volume and quality. In order to regenerate bone, bone graft substitutes of different origins are employed, depending on the site and the nature of the defect. In the field of dental implantology specifically, bone regeneration is often required with concomitant implant placement to ensure optimal osteointegration of the implant. Implantology encompasses several aspects, such as biological, mechanical, morphological, and physicochemical considerations that serve as variables for predictable treatment outcomes. Within the realms of biological considerations, two important aspects of implantology include choice of materials of the implant and adequate bone volume for implant loading. Although titanium has been a long- standing gold standard as a choice of materials, limitations such as corrosion, metal allergies, poor aesthetics have led to the favour of zirconia implants. Since the interaction of implant with bone is crucial in the success of implant therapy, zirconia's osteoconductive potential must be harnessed to have predictable results. In order to maximize its potential, one such approach is to observe and assess the biological response of osteoblast-like cells, towards various bone grafts, cultured over zirconia. To further explore this, the present study aims to understand whether its presence alters the influence of various origin bone substitutes on osteoblast-like cells through an *in vitro* study model.

Objectives: To evaluate the cellular metabolic activity and ALP expression of osteoblast-like cells in two ways: When indirectly exposed to various bone-graft substitutes (bovine origin, synthetic biphasic calcium phosphate and bioglass); when grown over zirconia substrates and placed in close proximity to the said bone graft substitutes and observe the differences.

Materials and Methods: The presented study used a xenograft (Cerabone™, Botiss, Germany), a synthetic biphasic calcium phosphate (Maxresorb™, Botiss, Germany), and a synthetic bioactive glass (Novabone Dental Putty™, Osteogenics, USA), along with custom-made zirconia discs (Patent™, Zircon Medical Management AG, Germany). These biomaterials underwent surface characterization using SEM and EDS. Cytocompatibility assays were performed with osteoblast-like cells, evaluating their metabolic activity and

alkaline phosphatase expression at defined time points. Two approaches were employed: first, cells were indirectly exposed to bone graft substitutes; and second, cells were cultured on zirconia and placed in close proximity to the bone graft substitutes. The results were analysed with one-way ANOVA, followed by the Tukey's post hoc ($\alpha=0.05$).

Results and Discussion: With the indirect exposure to the bone graft substitutes, the metabolic assay of the cells revealed significantly lower values in the bioglass group on days 3 and 7, in comparison to control. The alkaline phosphatase activity revealed significantly lower values in xenograft and bioglass groups, on day 7, in comparison to control. Cells cultured over zirconia, when placed in close proximity to the bone grafts substitutes, revealed significantly lower metabolic activity on day 11 in all experimental groups, in comparison to control. The alkaline phosphatase activity revealed significantly higher values in bioglass group in comparison to control. The inverse results underscore how different bone graft substitutes can exert a varied influence on the cellular response and the presence of zirconia of can significantly alter the cellular response by modulating the cellular environment owing to its osteoconductive nature.

Conclusions: Through this *in vitro* study, it can be concluded that bone grafts can have varying degrees of influence on cell proliferation and differentiation in the presence of zirconia materials. The results underscore interesting insights that could additionally implicate that the composition of bone graft substitutes plays a vital role in influencing cellular response in the presence of zirconia.

Keywords: Zirconia, Bone regeneration, Bone graft substitutes, Osteoconductive potential.

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LIST OF ABBREVIATIONS

3Y-TZP: 3-Yttrium stabilized tetragonal zirconia polycrystals

Al: Aluminium

ALP: Alkaline phosphatase

BCP: Biphasic calcium phosphate

BG: Bioglass

BMPs: Bone morphogenic proteins

C: Cubic

Ca: Calcium

Ca-P: Calcium phosphate

CO₂: Carbon dioxide

CpTi: Commercially pure titanium

ECM: Extracellular matrix

EDS: Energy dispersive spectroscopy

HA: Hydroxyapatite

IL-1: Interleukin-1

IL-6: Interleukin-6

M: Monoclinic

M-CSF: Macrophage colony-stimulating factor

MSC: Mesenchymal stem cells

OPG: Osteoprotegerin

P: Phosphorus

RANKL: Receptor activator of nuclear factor- κ B ligand

RUNX2: Runt-related transcription factor 2

SEM: Scanning electron microscopy

Si: Silicone

T: Tetragonal

TCP: Tricalcium phosphate

Ti: Titanium

Tissue CP: Tissue culture plate

TNF- α : Tumour necrosis factor- α

V: Vanadium

XENO: Xenograft

Zr: Zirconia

PREFACE

The pursuit of optimal health is an infallible force that drives the art of medicine. The humble yet clever beginnings of medicine involving plants and herbs in pre-historic times eventually evolved into the sophisticated multitude of sciences we know today.

Over the course of evolution, as man progressed from a primarily raw diet to using stone tools and fire for cooking, the need for a strong masticatory system reduced. This, in turn, led to changes in the orofacial structures, such as narrower jaw bones and smaller teeth, and even modulation of bone metabolism.¹ Even though modern foods contribute to the increased prevalence of dental diseases and subsequent tooth loss, these issues are not exclusive to modern times.

Throughout history, various civilizations have documented dental intervention and techniques to treat oral health diseases. The earliest attempt dates back to 2000-3000 BC when the ancient Egyptians and Chinese used silk and gold ligatures to hold lost teeth in place.² By the 5th century BC, Hippocrates and Aristotle documented several interventions, including treating decayed teeth, gum disease, tooth extractions, and the use of gold ligatures to reinforce loosening teeth.³ Remarkably, in the 1970s, revolutionary discoveries showed the works of Mayans in 600 AD, where they used sea shells to replace missing lower incisors, marking the first successful attempt at osseointegration.^{2, 4}

Nevertheless, as the years went on, the Middle and Modern Ages witnessed more refined treatment modalities in both surgical and non-surgical interventions in Dentistry. Groundbreaking works of Ambroise Paré, the father of modern surgery, pioneered wound management, adoption of ligatures for haemostasis, and interventions of delayed tooth eruption in children, amongst other innovations, as described in his book, *“Les oeuvres d'Ambroise Paré”*.⁵ In 1728, Pierre Fauchard, known today as the father of modern Dentistry, published the first edition of *“Le Chirurgical Dentiste ou Traité des Dents”* which was an extensive compilation of his observations and treatment strategies, in which he not only coined the term “dental caries” but also revolutionized the field of dentistry by offering an unprecedented, detailed compilation of dental diseases, treatment strategies, along with his professional observations.^{6, 7}

The publication of extensive dental literature in the 18th century marks the beginning of dental sciences into specialized fields. The first notable work dedicated extensively to prosthetic dentistry is attributed to Claude Mouton who released works dedicated specifically to dental prosthetics in 1746. Following the years, Jourdain in 1778, released a two-part book dedicated to oral surgery.⁸

As years went by, prosthetic dentistry witnessed a colossal development in 1952. The term *osseointegration* was coined by Prof. Per Ingvar Branemark, who elucidated the biology behind the fusion of titanium and bone and defined it as “a continuing structural and functional coexistence, possibly in a symbiotic manner, between differentiated, adequately remodelled, biologic tissues and strictly defined and controlled synthetic components, providing lasting, specific clinical functions without initiating rejection mechanisms.”⁹⁻¹²

Upholding this definition as the gold standard, the field of implantology has grown exponentially ever since, with numerous improvements and innovations not only in surgical techniques but also in implant design along with its prosthetic components.¹³ As far as implants are concerned, they have undergone several improvements in terms of their material composition, macrostructure, and prosthetic connections. When focusing on the choice of implant biomaterial, continual research and developments have led to the development and use of various titanium alloy formulations with different surface modifications, to optimize biocompatibility and biomechanics, followed by exploration and subsequent adoption of ceramics for medical and dental applications. Although titanium was discovered in 1798 by Reverend William Gregory, its adoption wasn't well established until the 1900s owing to its exorbitant manufacturing costs. What began as the choice of materials for military and aircraft applications, titanium and its alloys, particularly Ti-6Al-4V eventually paved its way into medical applications owing to its favourable mechanical and biological properties. Although it has set the precedence for gold standards in implantology, titanium poses its own challenges that has furthered research into alternative materials, as ceramics.

The leap to ceramics started with the first documented report of zirconia's biocompatibility which can be attributed to Helmer and Driskell, who inserted a 6% yttria-stable pellet into a monkey femur, reporting the lack of adverse reactions and noticeable bone growth. After its use in orthopaedics for over 30 years, zirconia made its way into dental applications that started as a choice of materials for dental prosthetics, such as tooth and implant-supported crowns and bridges; implant components, such as abutments; and eventually, as a choice of implant material especially where metal-free options are indicated.

14-17

In addition to the implant material, a crucial aspect of implant dentistry is its dependence on adequate bone volume and quality. Patients with insufficient bone often require regenerative procedures, making bone substitutes a crucial area of research and development.¹⁸ Several bone substitutes exist today that can be broadly classified based on their origin: autografts, allografts, xenografts, and synthetic grafts; or their regenerative capacities: osteogenic, osteoinductive, or

osteoconductive.^{19, 20} Each type of bone substitute serves different purposes depending on the clinical indication, ranging from sinus floor elevation to alveolar ridge reconstructions.^{21, 22} These regenerative procedures are often challenging, requiring not only clinical expertise but also a deep understanding of the biological and physicochemical properties of each material.

Maximizing the regenerative potential of biomaterials, including both implants and bone substitutes, requires a thorough understanding of each material's contribution to osteogenesis to enhance predictability and longevity.

Accordingly, this study aims to explore the cell response to zirconia substrates in conjunction with various bone graft substitutes of different origins. The focus is set on analysing the potential synergic effects on osteoblastic proliferation and differentiation.

I. LITERATURE OVERVIEW

1. Bone tissue

Bone is a specialized, dynamic connective tissue that serves the dual role of both an organ and a tissue, contributing not only to protection and locomotion but also aiding in mineral homeostasis through metabolic pathways. As an organ, it comprises marrow, calcified cartilage, cartilaginous joints, and cortical and cancellous mineralized structures. As a tissue, bone consists of both organic and inorganic components within the cortical and cancellous regions.²³

By proportion, bone forms the largest constituent of the connective tissue system in the body and continuously responds to ever-changing biomechanical demands placed upon it. This phenomenon of continual remodelling was first comprehensively described by Wolff in 1892 as *“Every change in the form and function of bone or their function alone is followed by certain definite changes in their internal architecture, and equally definite alteration in their external conformation, in accordance with the mathematical laws”*.²⁴

1.1. Classification and macro features of bone

Although bone can be classified in several ways that may overlap, from a macroscopic point of view, it can be classified based on either morphology or structure.²⁵

The structure of bone can be further classified based on its hierarchical organization,²⁶ extending from its macroscopic features, down to the sub-nanoscope level, which will be elaborated on in later sections.

1.1.1. Morphological classification

Based on its **morphology**, bone can be broadly classified into 5 types (Figure 1):

- **Long bones:** Cylindrical shaped with a greater length-width ratio, primarily found in the limbs. Typical examples include the femur, radius, and ulna.
- **Short bones:** Nearly equal in width and length, providing stability. Prime examples include tarsal and carpal bones.
- **Flat bones:** Thin and flat, serving as attachment points for muscles and protection for organs. Examples include the skull, scapula, pelvis, and sternum.
- **Irregular bones:** Lacking specific dimensions and serving as protective bones for organs such as the vertebrae.
- **Sesamoid bones:** Shaped like sesamoids, these bones cushion the tendons from compressive forces. A classic example of a sesamoid bone is the patella.²⁷

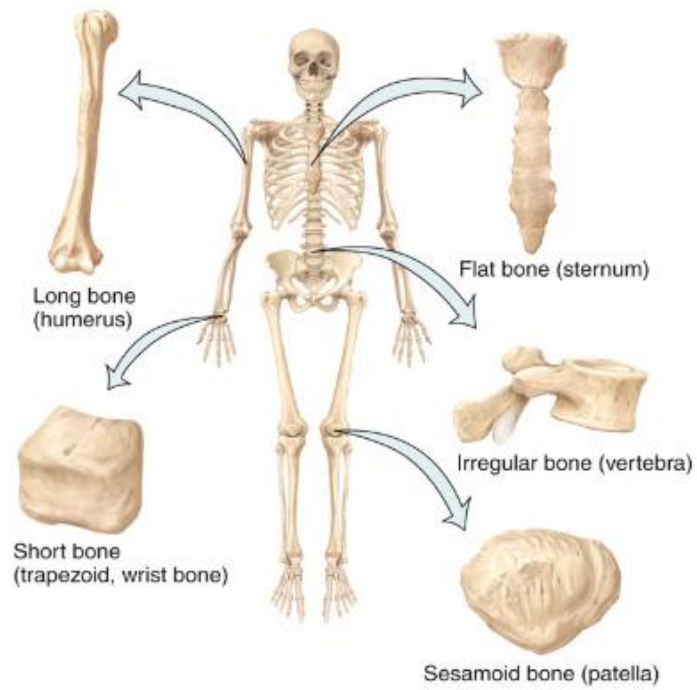


Figure 1: Classification of bones based on morphology.

Source: Adapted from Biology forum gallery²⁸

1.1.2. Structural classification

Based on its **structure**, bone can be classified into two types: cortical and cancellous bone.

- Cortical bone:** Forming about 80% of the skeleton, cortical bone is dense, hard, and calcified, with a slow turnover rate. It is surrounded by periosteum externally and endosteum internally (Figure 2). The periosteum consists of two layers: the outer layer is fibrous and highly vascularized, and the inner layer is rich in a cellular network consisting of Mesenchymal stem cells (MSCs). The internal surface of bone is lined by the endosteum, that is rich in a cellular network and lacks fibrous tissue. The basic building blocks of cortical bone are *osteons* or *Haversian systems*. An osteon consists of several tightly packed layers of lamellar rings surrounding the central Haversian canal, which contains bone's vascular, lymphatic, and nerve supplies, as well as progenitor cells located in the loose connective tissue. Between the lamellae, empty spaces called the *lacunae* are present, housing rings of osteocytes. The osteocytes are interconnected by *canaliculi* that run perpendicularly to the long axis of the bone and serve as a transport system for oxygen and nutrients to the osteocytes, while also removing waste. It is noteworthy that the alignment of osteons is dictated by the forces applied during bone formation. They are parallelly arranged along the long axis of the bone and separated by cement lines, where neither collagen fibres nor *canaliculi* pass through.²⁹
- Cancellous bone:** Consisting of intersecting bony latticework arranged in a rod and plate fashion, called *trabeculae*, cancellous bone is not as dense as the cortical bone and only accounts for 20% of the total skeletal mass. Unlike the *lamellae* found in the osteons of the cortical bone, the *lamellae* in cancellous bone are arranged in a parallel sheet-like arrangement that collectively forms the *trabeculae* (Figure 2). The *trabeculae* are widespread, and their arrangement is in response to the mechanical forces applied on bone during formation. The large spaces between the *trabeculae* are filled with marrow, which provides a scaffold for vascular structures – a distinguishing feature from the Haversian canal of cortical bone. This feature also results in a bone turnover rate that is 8

times higher than that of cortical bone. Cancellous bone also contains an endosteum that is connected to the marrow.²⁹

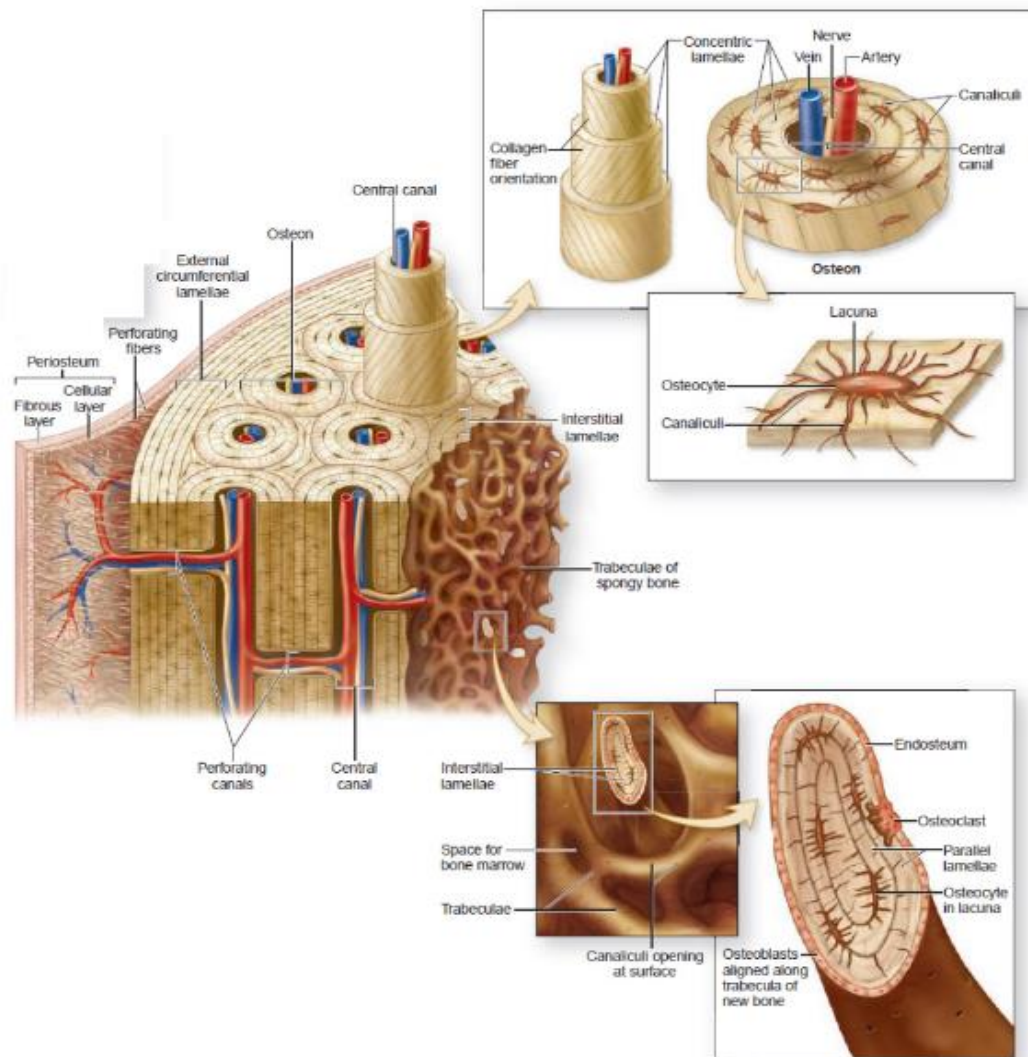


Figure 2: Diagram representative of cortical and cancellous bone and its various components.

Source: Adapted from John E. Davies³⁰

1.2. Microscopic features of bone

From a microscopic point of view, bone can be classified hierarchically depending on the size of the component being observed, ranging from the lamellar structure down to its chemical composition (Figure 3).²⁵

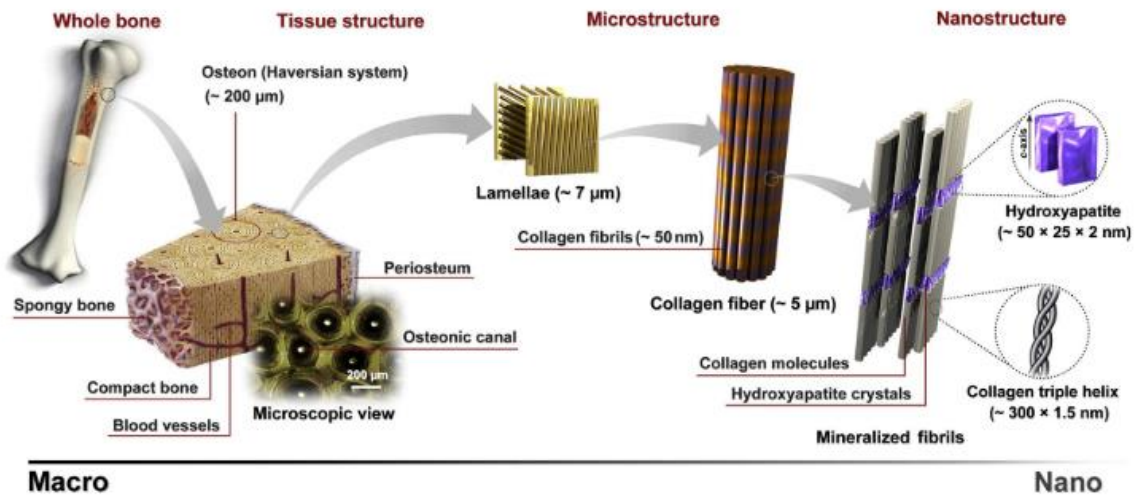


Figure 3: Hierarchal classification of bone from macro to nano structures

Source: Adapted from Sadat-Shojai *et al.*³¹

Both cortical and cancellous bone can consist of either woven bone or lamellar bone, depending on their maturation stage and physiological status.

- Woven bone:** Woven bone is found in areas of rapid growth, such as underneath the fibrous periosteum during the formation of primary bone, as in a developing foetus; at certain metaphyseal ends of bones in a growing skeleton; during remodelling phases of adulthood; and in alveolar bone in adults. It is usually a transient phase of bone formation, eventually being replaced by mature lamellar bone. Its continued presence otherwise may indicate pathologies, such as Paget's disease, hyperparathyroidism, fibrous dysplasia, osteogenesis imperfecta, renal osteodystrophy and several osteosarcomas. It is characterised by a disorganized arrangement of collagen as well as a high cellularity, where the crystals of the mineral components are smaller and weaker in comparison to those found in lamellar bone (Figure 4).^{30, 32, 33}
- Lamellar bone:** Lamellar bone is mature bone tissue, formed in a rather slow deliberate fashion, replacing the primary bone tissue (i.e., woven bone). It forms a consolidated network and an unyielding foundation that provides substantial strength and resistance to compressive forces. Depending on the location, lamellar bone can either be inner circumferential (near the endosteum) or outer circumferential (next to the periosteum). Lamellar bone is characterized by its

highly organized collagen arrangement, aligned in response to mechanical stresses, which contributes to its significant strength (Figure 4).^{34, 35}

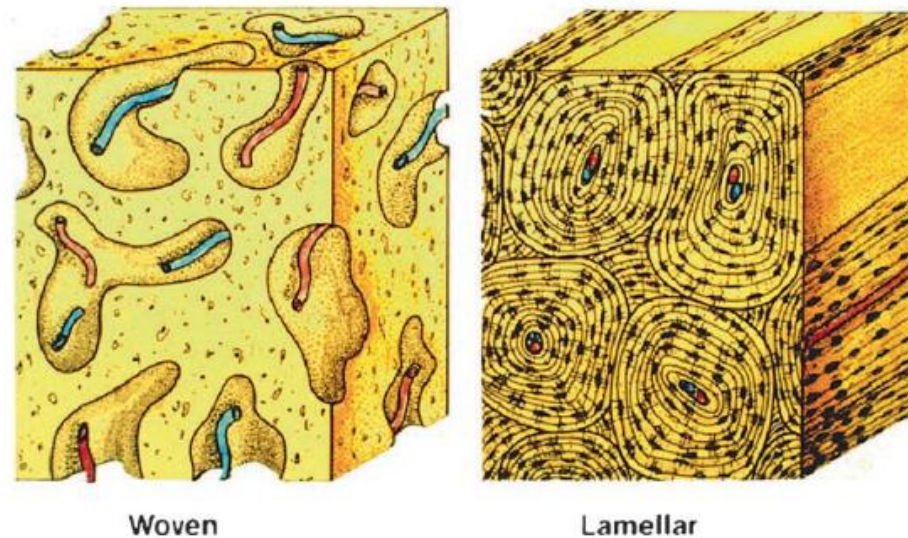


Figure 4: Diagram representative of architecture of woven bone and lamellar bone

Source: adapted from Safadi *et al.*²⁹

1.3. Composition of bone

Bone is a composite structure that consists of an organic phase (20%-40%), an inorganic phase (50%-70%), lipids (<3%), and water (5%-10%) by weight.³²

1.3.1. Organic phase of bone

The organic phase plays a vital role in maintaining the structural integrity and biochemical activity of bone, and provides flexibility and elasticity to the tissue. It primarily consists of type I collagen, which constitutes around 90% of the matrix, followed by non-collagenous bone proteins that form the remaining 10%, along with growth factors, cytokines and bone morphogenic proteins (BMPs), which are also present in small quantities.³⁶

- **Collagenous protein:** Around 90% of the protein structure of the matrix is contributed by type I collagen, followed by traces of types II, III, V, and XI. Type I collagen is composed of a triple helix chain, (two $\alpha 1$ chains and one $\alpha 2$ chain) and serves as the fundamental structural element of the matrix.³²
- **Non-collagenous protein:** Several non-collagenous proteins are present in the extracellular matrix (ECM), among which osteocalcin accounts for 10%-20%

of the total non-collagenous proteins and is the most widely researched, albeit its role remains eluded. Its apparent functions involve the regulation of bone mineralization as well as attracting osteoclasts to the bone surface for resorption. Other significant non-collagenous proteins include osteonectin, osteopontin, bone sialoprotein, fibronectin, bone acidic glycoprotein and bone-specific proteoglycans (biglycan and decorin). An important glycosylated protein, alkaline phosphatase, is also present in the ECM. These phosphate-binding proteins regulate the amount and size of hydroxyapatite crystal formation through complex enzymatic mechanisms with subsequent mineral deposition and serve as an important biomarker during early osteoblast differentiation.³⁷

1.3.2. Inorganic phase of bone

As for the inorganic content of the bone, its chemical composition consists of a hydroxyapatite complex composed of calcium hydroxide and tricalcium phosphate (chemical formula: $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$), along with traces of other ions such as magnesium, sodium, fluorine, carbonate and citrate, all arranged in a uniform microcrystalline structure.²³ This provides mechanical stability and rigidity to bone and acts as a reservoir for 99% of the body's calcium, and 85% of its phosphorus.²⁵

1.4. Cellular components of bone

At the cellular level, the key cellular components of bone include mesenchymal-derived cells, as osteoblasts and osteocytes; and haemopoietic-derived cells, as osteoclasts. Together, these collectively form the Basic Multicellular Unit (BMU) of bone.³² Formation of bone cells is a complex cascading process as described through the schematic representation in Figure 5 and Figure 6.

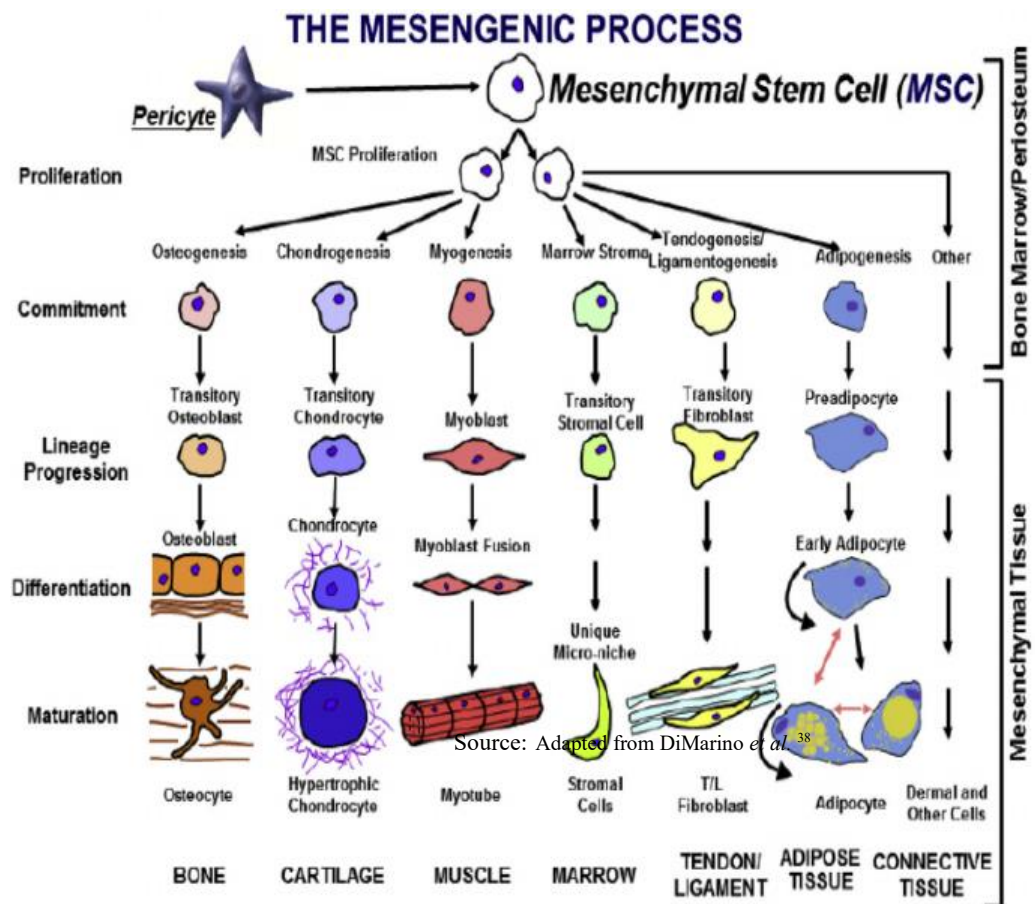


Figure 5: Schematic representation of mesenchymal stem cell differentiation

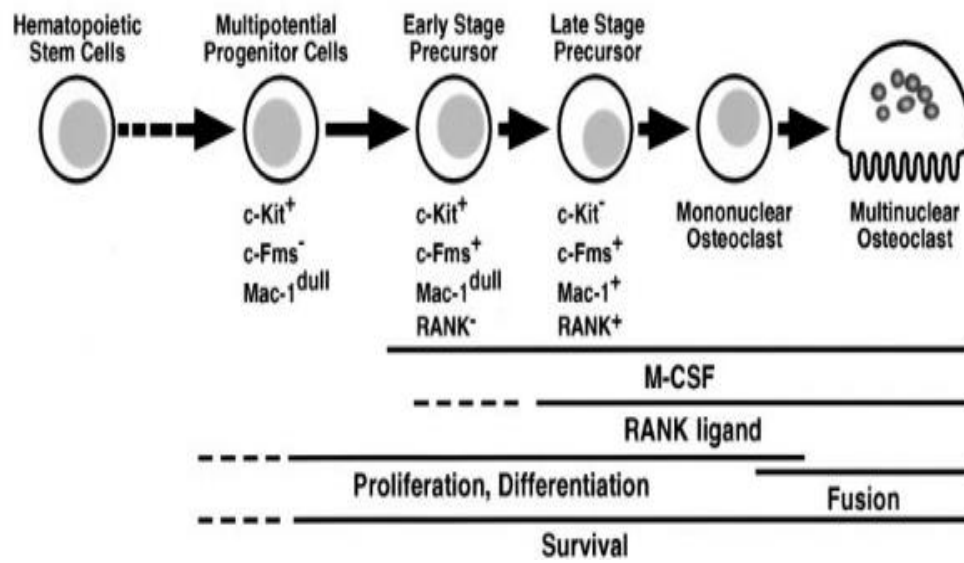


Figure 6: Schematic diagram representative of initiation and formation of osteoclasts

Source: Adapted from Arai *et al.*³⁹

1.4.1. Osteoblasts

Derived from MSCs, osteoblasts are the primary bone-forming cells responsible for secreting both collagenous and non-collagenous proteins, as well as facilitating bone mineralization (Figure 7E). The formation of osteoblasts starts with the activation of key transcription factors, most importantly *RUNX2* and *SP7*, imperative in the establishment of the osteoblastic phenotype. Depending on the stage of maturation and cellular activity, osteoblasts either differentiate into osteocytes or become bone-lining cells.⁴⁰

The cascading event of osteoblast differentiation goes as follows:

Mesenchymal stem cells → osteoblast precursors → preosteoblasts → osteoblasts

1.4.2. Osteocytes

Osteocytes possess variable morphology and chemical composition depending on their stage of maturity (Figure 7A,7B). Early-stage osteocytes found in the *trabeculae* of woven bone are densely, albeit irregularly packed in the matrix and possess a cytoplasm rich in alkaline phosphate. Their cellular processes inter-tangle easily and aren't as long as those of osteoblasts. Late-stage/mature osteocytes, with long branching processes, are found in a dense hard matrix, encompassed by either *canaliculi* (fine canals) or *lacunae* (cavities).⁴¹

1.4.3. Osteoclasts

Serving a significant role in bone remodelling, osteoclasts are of hematopoietic origin, unlike the other cells of the BMU (Figure 5). These cells are multinucleated (Figure 7C, 7F) and are formed by the fusion of precursor cells from the monocyte/macrophage cell lineage. Osteoclasts are found on the surface of bone undergoing active resorption, or in spaces known as “Howship’s *lacunae*”, which are empty cavities with ruffled borders, formed as a result of bone resorption.⁴²

1.4.4. Bone lining cells

Following the synthesis of bone matrix, some osteoblasts convert into bone lining cells of an inactive bone surface, wherein the flat morphology marks their reduced bone-synthesis abilities. Although the role of bone lining cells remains unclear, speculated corollaries suggest them to be a source for osteogenic precursors. Other speculated functions of these cells include

exchanging minerals and metabolites between other bone surfaces and bone marrow, maintaining fluid exchange, as well as circulation.⁴⁰

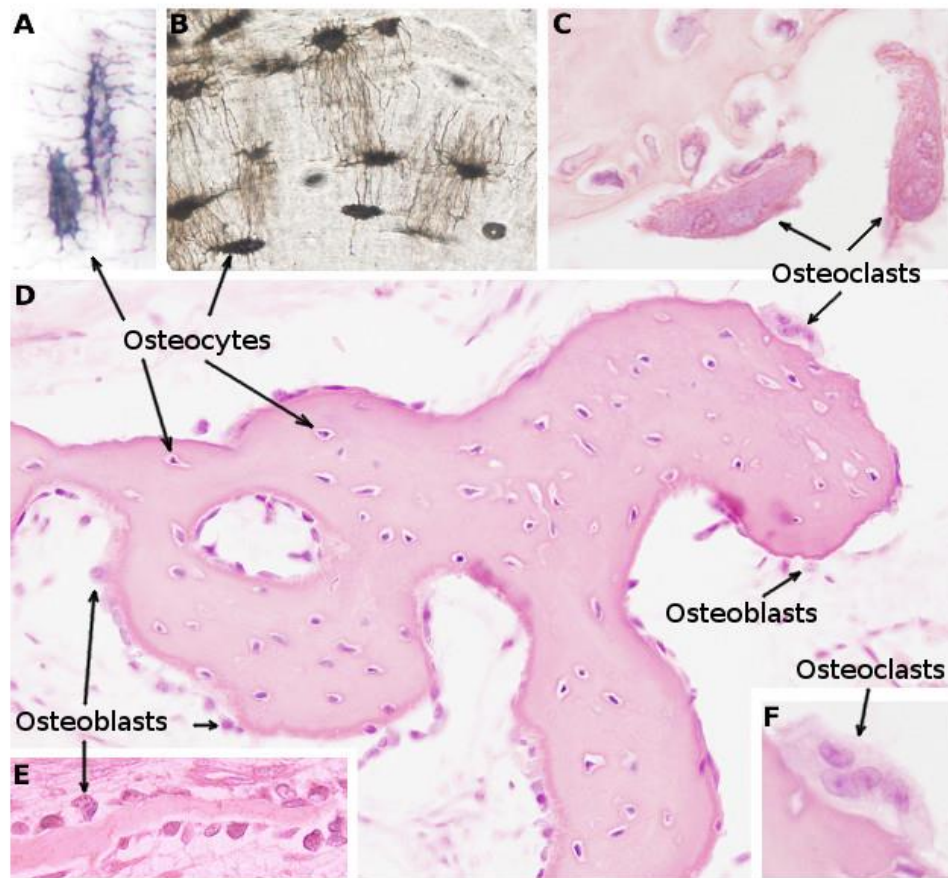


Figure 7: Histological section of compact bone(A-B) and growing bone (C-F) of several animal species, identifying various bone cells.

Source: Adapted from Megías *et al.*⁴³

1.5. Bone remodelling

The dynamic nature of bone is the result of the continual remodelling of the tissue, attributed to the combined synergistic processes of the BMU. The ongoing activity is a biological response to the mechanical and metabolic influences acting on the body, that result in a cascade of remodelling events. Various factors within the local cellular environment can modulate osteoblastic and osteoclastic signalling.⁴⁴ This cascade is illustrated through a schematic layout in Figure 8.

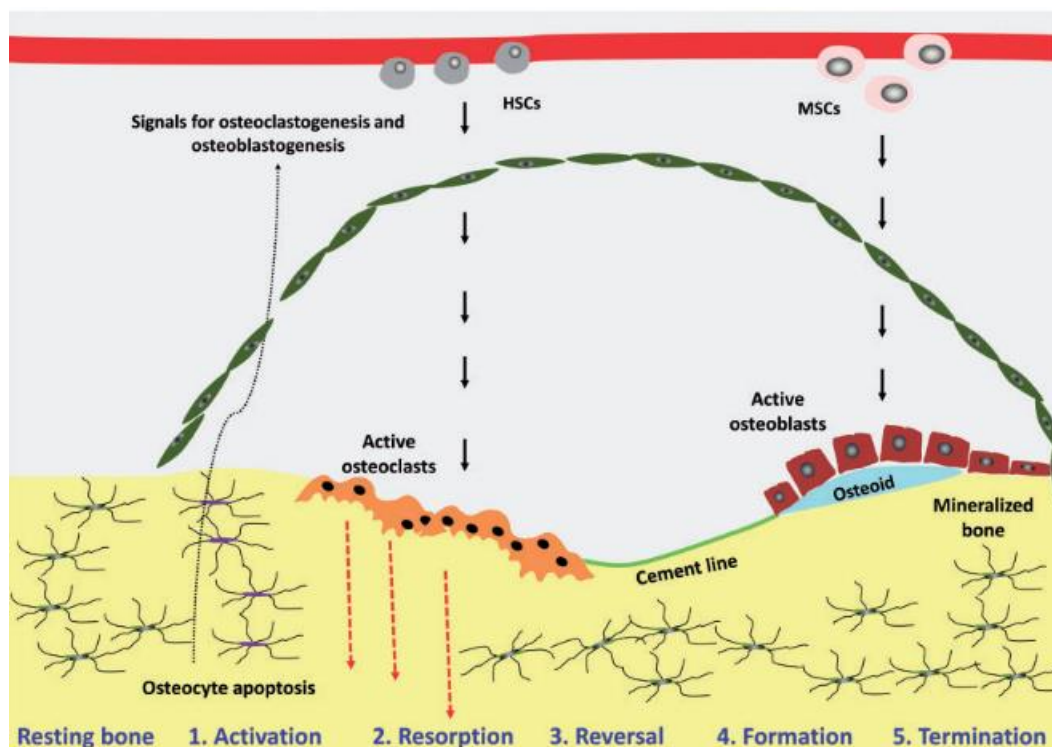


Figure 8: Schematic figure representative of the key stages of bone remodelling

Source: Adapted from Bassett *et al.*⁴²

Initiation of remodelling starts with local microdamage and ischaemic activity in the bone that triggers osteocyte disruption. Osteocytes, in-turn, cause an upregulation of macrophage colony-stimulating factor (M-CSF) and RANKL (receptor activator of nuclear factor- κ B ligand), both of which are key for osteoclastogenesis. OPG (osteoprotegerin), a decoy for RANKL, is also released by osteoblasts and osteocytes that regulate osteoclastogenesis by inhibiting excessive osteoclast activity.^{41, 45}

After the maturation of osteoclasts, the resorption phase initiates where the key process involves the recognition and subsequent adhesion of osteoclasts to the bone surface targeted for resorption. This recognition is mediated by both collagenous and non-collagenous bone proteins, as well as α v β 3 and α 2 β 1 integrins. These integrins also facilitate adhesion, a mandatory step in bone resorption. Various adhesion proteins are recruited to form podosomes, which delineate the bone surface undergoing resorption from the extracellular matrix. This creates the typical lacuna with a “ruffled border” that is an observable area for bone resorption. The lacuna has an acidic microenvironment which is imperative for the dissolution of the inorganic contents of the bone, followed by proteolysis of the organic content.^{42, 45}

Once the resorption phase concludes, with osteoclasts undergoing apoptosis, the reversal phase begins. This phase is marked by the appearance of macrophage-like mononuclear cells, which are thought to help in debris removal and further collagen degradation, subsequently preparing the surface for the new bone formation.⁴²

The final stage of bone remodelling involves the formation of new bone. It starts with the activity of large osteoblasts, which secrete collagenous fibre bundles that are cemented together to form an uncalcified matrix known as the osteoid. The osteoid is subsequently mineralized by deposition of miniscule calcium phosphate (Ca-P) complexes. Since the proliferation rate of osteoblasts is high, not all cells are able to exit the matrix, and cells that get entrapped in the matrix differentiate into osteocytes.³⁰

1.6. Bone healing

Bone healing is a complex process that can be divided into four key stages, that are modulated by both local and systemic factors (Figure 9):

Haematoma → Formation of granulation tissue → Formation of soft callus → bone remodelling.

Following the infliction of injury at the bone site, the formation of haematoma occurs. This is an acute inflammatory response that is guided by prostaglandins and lasting for approximately 7 days under physiological conditions. The site is infiltrated by fibroblasts and inflammatory cells such as macrophages, lymphocytes, and monocytes that release factors such as tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1) and interleukin-6 (IL-6) that promote the formation of a rich vascular network within the granulation tissue. It is noteworthy that TNF- α , IL-1 and IL-6 are crucial for the recruitment and osteoblastic differentiation of MSCs, promoting angiogenesis, and initiating callous formation.⁴⁶

During the early healing stage, the blood supply of the granulation tissue is mainly sourced from the exposed bone and muscle. However, following the formation of granulation tissue, a fibrin-rich network is laid by fibroblasts that form a stromal tissue capable of supporting the development of a new vascular network.⁴⁷

Once sufficient angiogenesis occurs, a collagen-rich matrix is deposited, leading to the formation of a soft callus. This structure, which is devoid of significant strength in the first 4-6 weeks, requires fixation and stability. The soft callus is eventually resorbed and replaced by a hard, mineralized callus. This process is similar to embryogenic bone formation, marked by

the significance of Wnt family of proteins that play a crucial role in embryogenic development and bone healing.⁴⁸

The hard callus starts with the mineralization of cartilage formed by chondrocytes. Once chondrocytes reach maturation and subsequent mineralization, the formation of woven bone is initiated. As a result, the hard callus achieves more stability and strength, albeit it doesn't achieve enough strength for loading. The woven bone is finally remodelled into lamellar bone with a central medullary cavity where, once again, IL-1 and TNF- α play a significant role.³⁷

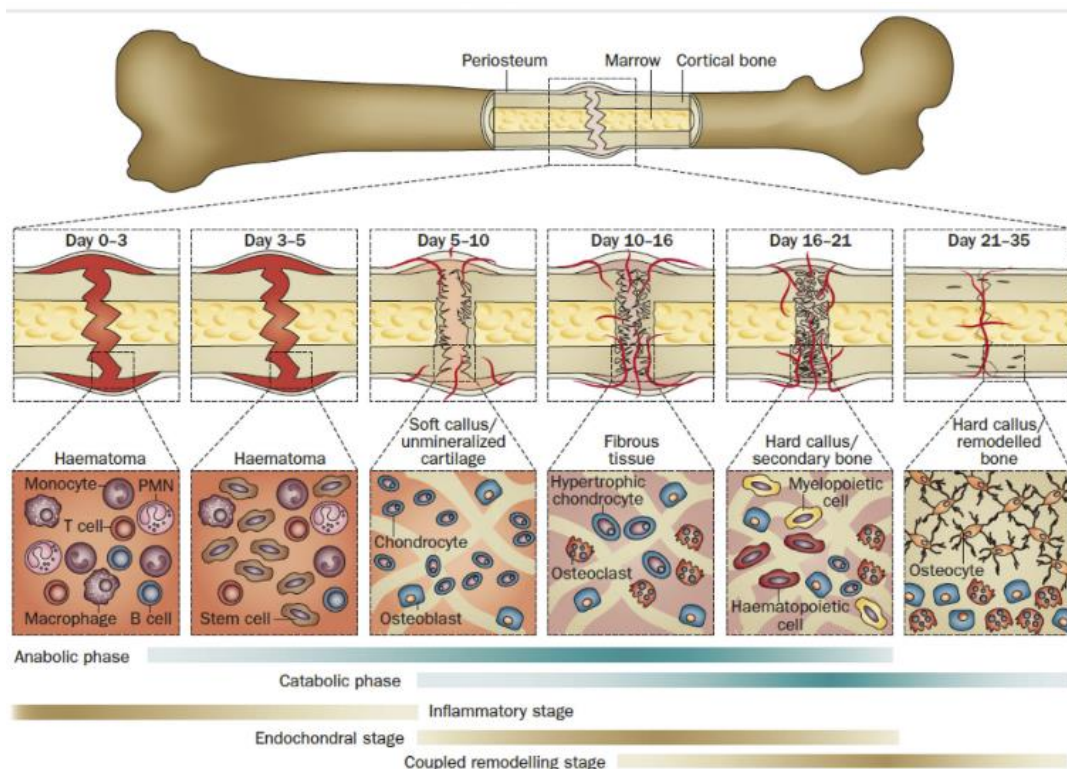


Figure 9: Schematic representation of the events in bone healing.

Source: Wang *et al.*⁴⁹

2. Implantology and implant biomaterials

2.1. Dental implants and their role in oral rehabilitation

Although an array of fixed and removable treatment modalities exists within the field of oral rehabilitation to replace missing teeth - broadly categorized into short and long-span bridges, partial and complete dentures - dental implants have become a routine treatment modality for edentulous patients. Treatments now being rendered with dental implants range from single-tooth rehabilitation and partially edentulous spaces to full-mouth rehabilitation.⁵⁰⁻⁵²

Implant-supported rehabilitation offers several advantages over tissue-supported alternatives that justify its growing popularity and preference. Key benefits include high predictability in terms of treatment protocols,⁵³ a high survival rate,⁵⁴⁻⁵⁶ preservation of adjacent teeth, soft tissues, and marginal bone,^{50, 57} along with excellent aesthetics⁵⁸ and high patient compliance and satisfaction.⁵⁹ These attributes make implants a suitable and effective form of treatment.

Globally, the dental implant and prosthetic market was valued at \$4.43 billion in 2023, and is projected to grow up to \$8.06 billion by 2032.⁶⁰ These projections highlight the increasing adoption of implants on a global scale, which in turn fuels further research to optimize materials, surfaces, as well as treatment protocols.

Although implant therapy offers unmatched advantages, it does come with challenges. The success and longevity of osseointegration are governed by multiple factors, and non-compliance with these factors may result in the failure of the implant.⁶¹

In 1998, Esposito *et al.*⁶² classified these failures into:

- Biological: early or delayed failure of osseointegration;
- Mechanical: fracture of implant, connecting screws, bridge framework;
- Iatrogenic: nerve damage or incorrect implant placement; and
- Inadequate patient adaptation: issues such as phonetic problems, aesthetic, or psychological problems.

A retrospective study conducted by Raikar *et al.*⁶³ concluded that biological factors, such as age, bone quality, the region of the mouth, and mechanical factors (e.g., implant length and diameter) were key determinants of survival rates. The results of this study were further corroborated by Yang *et al.*⁶⁴ enunciating that the most frequent risk factors affecting the survival of implants were age, poor bone quality, and poor implant design.

Biological and mechanical aspects are often closely interrelated, resulting in a need for incessant modifications and innovations to achieve more predictable outcomes and minimize the failure rate owed to these aspects. Some ways to improve biological outcomes include improved diagnostic tools such as routine adoption of cone beam tomography; resonance frequency analysis to measure implant stability; improvements in surgical techniques with soft and hard tissue augmentation; less traumatic protocols to improve healing; immediate implant placement (post extraction); guided bone regeneration to ensure more predictable regenerative outcomes; more focus on aesthetic outcomes; and adoption of digital tools for diagnosis and treatment.^{65, 66}

To improve mechanical outcomes, the primary focus has been on optimizing the choice of materials and refining manufacturing protocols for implants and their components, including considerations such as implant length, diameter, thread design, and abutment connections, in order to reduce mechanical complications.⁶⁷⁻⁶⁹

2.2. Implant biomaterials: Evolution and current trends

A vital criterion in choosing a biomaterial for medical applications involves its biocompatibility. Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any inflammatory, allergic, immune, toxic, mutagen, or carcinogenic effects in the recipient. Instead, the biomaterial should elicit the most appropriate beneficial cellular or tissue response for that specific situation, thereby optimizing the clinically relevant performance of the therapy.⁷⁰

From its onset in the 50s, implant biomaterials have evolved rapidly, focusing not only on enhancing the biocompatibility of the materials but also on improving mechanical stability. Today, implant biomaterials can be broadly classified, in accordance to their chemical composition, into metals, ceramics, and polymers, as described in Figure 10. Although several materials ranging from aluminium oxide to PEEK have been tested, the two major market holders as of today are titanium and zirconia implants, since these are the two materials with proven pre-clinical and clinical success.⁷¹

Implant Material	Common Name or Abbreviation
I. Metals	
Titanium	CpTi
Titanium Alloys	Ti-6Al-4V extra low interstitial (ELI)
	Ti-6Al-4V
	Ti-6Al-7Nb
	Ti-5Al-2.5Fe
	Ti-15 Zr-4Nb-2Ta-0.2Pd
	Ti-29Nb-13Ta-4.6Zr
	Roxolid (83%–87%Ti-13%–17%Zr)
Stainless Steel	SS, 316 LSS
Cobalt Chromium Alloy	Vitallium, Co-Cr-Mo
Gold Alloys	Au Alloys
Tantalum	Ta
II. Ceramics	
Alumina	Al ₂ O ₃ , polycrystalline alumina or single-crystal sapphire
Hydroxyapatite	HA, Ca ₁₀ (PO ₄) ₁₀ (OH) ₂
Beta-Tricalcium phosphate	β-TCP, Ca ₃ (PO ₄) ₂
Carbon	C
	vitreous,
	low-temperature isotropic (LTI),
	ultra-low-temperature isotropic (ULTI)
Carbon-Silicon	C-Si
Bioglass	SiO ₂ /CaO/Na ₂ O/P ₂ O ₅
Zirconia	ZrO ₂
Zirconia-toughened alumina	ZTA
III. Polymers	
Polymethylmethacrylate	PMMA
Polytetrafluoroethylene	PTFE
Polyethylene	PE
Polysulfone	PSF
Polyurethane	PU
Polyether ether ketone	PEEK

Figure 10: Broad classification of biomaterials used for dental implant fabrication.

Source: Adapted from Osman *et al.*⁷¹

2.3. Titanium

2.3.1. Background and properties

Titanium is a lustrous transition element with high ductility and low density, making it a versatile material with a wide range of applications. In medical applications, its biocompatibility and favourable mechanical properties justify the use of titanium in routine medical practice.

Two forms of Ti are commercially available that include:

- Commercially pure titanium (CpTi), an oxide of titanium, available in four grades, and
- Titanium alloys, which contain various elements in varying proportions.⁷²

Titanium exists in two temperature-dependent phases. In its α -phase, titanium has a hexagonal structure up to 882.5 °C, after which it transforms into β -phase, with a cubic structure. Several elements are used to stabilise these phases such as oxygen, nickel, aluminium, and vanadium. Out of all the alloy compositions, **Ti-6Al-4V** is the most widely used composition due to its excellent mechanical properties, including high tensile strength ($\approx$ 950 Mpa), high yield strength ($\approx$ 880 Mpa), low density ($\approx$ 4.43 g/cm³), and a high modulus of elasticity ($\approx$ 113.8 Gpa).⁷³

2.3.2 Major Advantages

Broadly encompassing its advantages, from a biological perspective, the foremost preference lay in titanium's ability to form a close physical bond with bone without eliciting an adverse tissue response.⁷⁴ Secondly, titanium's ability to form a protective oxide layer prevents it from surface degradation, thereby reducing its corrosiveness. Although titanium particles have been detected in the serum and blood after implantation, they typically do not provoke an immunogenic response, although recent studies have suggested otherwise.^{75, 76}

From the perspective of biomechanics, titanium offers superior advantages in terms of force dissipation at the bone-implant interface and the ability to adequately sustain occlusal loads. This is due to its modulus of elasticity being closest to that of bone.⁶⁸

2.3.2 Limitations

Since titanium is known to release metal particles in its microenvironment, studies cite it as a potential concern with the probable association to peri-implantitis. Titanium ion

dissolution has a potential effect on the oral microbiome that can disrupt the functional balance of the surrounding tissues and compromise its integrity.^{77, 78}

Surface degradation also emerged as a potential drawback that can accelerate the ionic leeching of particles. Factors such as occlusal loading, bacterial biofilm formation, and even chemical-induced degradation due to surface doping, have been associated with surface degradation.^{68, 75}

Clinical reports also indicate possible metal hypersensitivity in patients, which can potentially lead to peri-implantitis and external tooth resorption, according to some authors.⁷⁹

Low patient satisfaction has emerged as another concern since titanium imparts the “black line effect” on soft tissues, which can result in an inferior aesthetic outcome. Recession of the soft tissue around the implant components, exposing the metal, also adds to its unesthetic appeal.⁸⁰

2.4. Zirconia

2.4.1. Background and properties

Zirconia is the crystalline dioxide form of zirconium and exists in three distinct allotropic forms: monoclinic (m), tetragonal (t), and cubic (c). These phases are martensitic in nature and undergo transformation with changes in temperature and external force. At room temperature, zirconia exists in its monoclinic phase and transforms into various allotropes as temperature changes, as described in Figure 11.

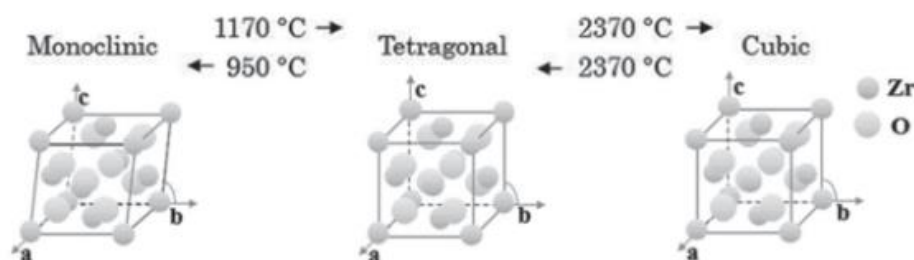


Figure 11: Schematic diagram representative of temperature-dictated phase transformations of zirconia.

Source:

Adapted

from

Hanawa.⁸¹

Out of all the transformations, $m \leftrightarrow t$ phase change is most crucial, since sintering (an imperative step in the densification of ceramics) requires temperatures around 1500 °C, making

phase transformation of zirconia inevitable during heating and cooling. Transformation of $t \rightarrow m$ results in a 3%-4% volumetric expansion, which can lead to crack formation due to compression, significantly altering zirconia's mechanical integrity. To stabilize this transformation, alloys of zirconia have been fabricated using oxides of various elements such as yttria, calcium, cerium, and magnesium.⁸²

2.4.2. Significance of Zirconia in implantology

Due to its martensitic nature, for all medical applications, 3Y-TZP (Yttrium stabilized tetragonal zirconia polycrystals) is the most widely used due to its stable structure and high flexural strength of 1100-1800 MPa.⁸³

As a prosthetic component, zirconia offers several advantages over titanium. In terms of aesthetics, zirconia does not exhibit the “black line” effect on gingival tissues like titanium, making it a superior choice. Zirconia also promotes better interaction with soft tissues, encouraging superior epithelial attachment due to well-organized collagen and shallow sulcular depth. Additionally, studies have shown reduced microbial adhesion, making it a suitable material to fabricate prosthetic components.⁸⁴⁻⁸⁶

Through several pre-clinical animal trials, zirconia has showed promising results in terms of osseointegration as a viable alternative to titanium, as well as excellent biocompatibility and osteoconductive properties.^{14, 87, 88} Retrospective studies in humans with long-term observations are scarce. However, studies with short-term (minimum 6 months) to long-term (up to 5 years) follow-up periods report over a 93% survival rate, stable marginal bone and soft tissues, a low incidence of peri-implantitis, and a more favourable alveolar response in comparison to titanium.⁸⁹⁻⁹²

One reason for zirconia's high level of biocompatibility could be attributed to its ability to modulate cellular response. Carinci *et al.*⁹³ reported that zirconia influences the cell cycle, vesicular transport, and upregulates several genes that modulate extracellular matrix turnover and subsequent osteoblastic proliferation, resulting in superior bone apposition.

On the other hand, some studies have reported inferior properties for zirconia, as compared to titanium, in terms of its osseointegration, marginal bone loss, bone-implant contact, and overall survival, due to mechanical complications.⁹⁴

Regardless of the findings, zirconia has witnessed tremendous growth in the field of research, from *in-vitro* testing to preclinical and clinical trials.⁷⁸

2.4.3. Limitations of zirconia

The primary drawback of zirconia lies in its mechanical properties. It is prone to a phenomenon known as low-grade degradation, where $t \rightarrow m$ transformation can occur in the

presence of a wet environment, causing compressive forces and microcracking. This leads to surface degradation and subsequent mechanical failure. Additionally, since phase stability is a manufacturing-dependent feature, the high cost of zirconia adds to its limitations in routine practice.⁹⁵

Zirconia also has low fracture toughness (6-8 MPa), which consequently compels the optimal design of the implant to dissipate forces evenly and avoid mechanical complications, thereby improving survival rates. Optimal surgical protocols also become necessary, especially when implants are placed in dense cortical bone, which requires higher torque values for placement.^{71, 96}

The lack of long-term clinical follow-up also serves as a deterrent to the widespread adoption of zirconia in routine practice.⁹⁷

3. Bone quality and implants

3.1. Bone quality in implantology

As discussed in the earlier section, an important biological dependence in the success of implant therapy is its dependence on bone quality. Since bone volume and bone type play key roles in ensuring predictable outcomes for implant therapy, it is imperative to shed light on the microenvironment of peri-implant osteogenesis.⁹⁸

Several classifications of bone quality exist, however, encompassing the field of implantology, one of the oldest and most frequently used classifications for diagnosis of bone quality for implant placements was proposed by Lekholm and Zarb in 1985. Accordingly to this classification, the alveolar complexes of the maxilla and mandible were divided into four types based on the varying proportions of cortical and cancellous bone.⁹⁹

- Type I: Bone is mostly cortical, typically found in the anterior mandible.
- Type II: Thick cortical bone surrounding the marrow, found commonly in the anterior maxilla and anterior and posterior mandible.
- Type III: Thin cortical bone surrounding thick cancellous bone, commonly found in the anterior and posterior maxilla and posterior mandible.
- Type IV: Bone is primarily cancellous, mostly found in the posterior maxilla.

Although this classification formed the basis of diagnosis and treatment planning, it is highly subjective since the tactile sensation of every operator varies, often leading to different categorizations based on the operator's perspective. Additionally, 2D radiographic images often fail to corroborate the apparent bone quality based on this classification, which can lead to misdiagnosis.¹⁰⁰

Further elaborating on the bone classification by Lekholm and Zarb, each bone type exhibits a different remodelling pattern. In type I bone, peri-implant bone regeneration is slower owing to the deliberate remodelling of the cortical lamellar bone. In turn, it is relatively faster in type II bone, since this type also contains trabecular bone that assists on a speedy recruitment of osteogenic cells from both endosteal surfaces and bone marrow, owing to its richer vascular network. This causes the rapid turnover of bone tissue, making type II bone as the more optimal bone type for implant placement.³⁰

3.2. Peri-implant osteogenesis

Peri-implant osteogenesis is a cascade of complex biological processes involving multiple steps of both cellular and extracellular activities at the interface of an implant and the host bone.

Although peri-implant osteogenesis is very similar to bone healing, in terms of inciting a similar host response,¹⁰¹ it is influenced by several factors that are not only determined by the host but also by the characteristics of the implant. Physical properties such as implant material and implant design play a vital role in influencing osseointegration. Among these, surface topography, in particular, is a key determinant. Literature reveals that platelets and osteoblasts have better adhesion and spreading on surfaces with microtopography, resulting in improved proliferation and differentiation.³⁴ Since fibrin adhesion, along other matrix molecules, is also fundamental for directing adhesion and subsequently guiding the migration of osteogenic cells, surface topography holds significant importance in osteointegration, alongside factors such as surface treatments and coating modification.^{102, 103}

The formation of bone starts from day 1 in two ways (Figure 12): in *distance osteogenesis* (bone grows from the host bone cavity towards the implant) and in *contact osteogenesis* (bone grows from the implant towards the bone cavity). In both cases, vascularization and osteoconductivity are key determinants of successful peri-implant osteogenesis.¹⁰⁴

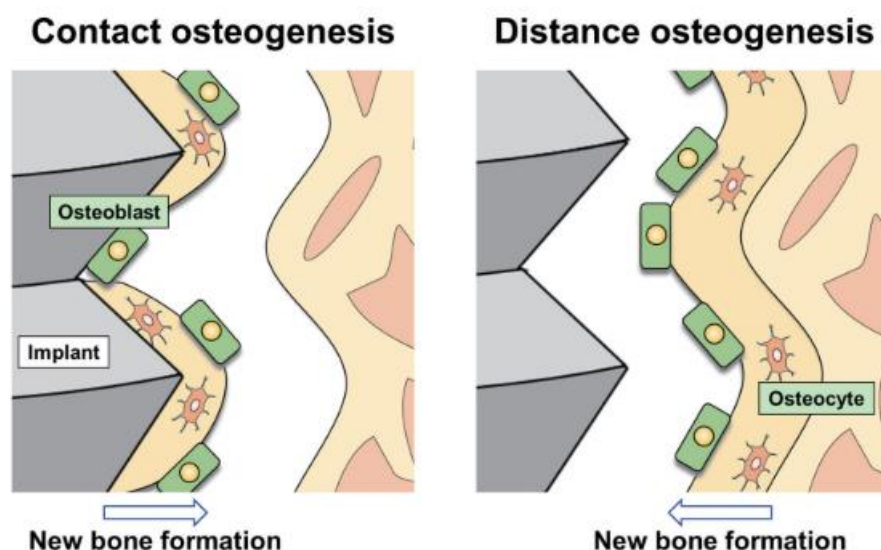


Figure 12: Schematic representation of peri-implant osteogenesis showing two distinct ways of bone formation.

Adapted from Kondo *et al.*¹⁰⁵

From the first day, activated blood cells, mainly platelets, undergo biochemical changes (including induction of phosphotyrosine, increased calcium levels, and phospholipid hydrolysis) and morphological changes (adhesion, spreading, aggregation) in response to the contact with the implant surface.¹⁰⁶ These changes create an osteoconductive scaffold formed by a transient biological matrix known as fibrin. Fibrin, the reaction product of thrombin and fibrinogen, plays a vital role in inducing the migration of osteogenic cells, thereby holding osteoinductive potential.¹⁰⁷

Migrated osteogenic cells secrete an organic matrix where the Ca-P nucleation occurs. Bone proteins such as osteopontin and sialoprotein are important biomarkers of this stage. It is only after the nucleation of the Ca-P complex that collagen fibres begin to express calcify, and lay down a foundation of woven and trabecular bone. This bone is eventually remodelled into lamellar bone around 3 months after implantation.^{76, 106}

4. Bone graft substitute

4.1. Ideal properties of a bone graft substitute

As explored in the earlier section, bone is a complex tissue and its remodelling and healing involve intricate processes. In most instances bone is able to regenerate itself unless clinical scenarios such as developmental defects, infections, diseases, high impact trauma and tumours hinder this process. As a consequence, bone develops defects that can be critical, classically defined by its loss of ability to heal spontaneously, along with the size of the bone volume that is lost in the process.⁴⁹ Bone regeneration, in these critical defects, must harness and employ complex tissue-engineering techniques in enhancing bone regenerative procedures. The bone graft substitute must allow for bone growth and provide mechanical support to optimize implant therapy.

The ideal properties of a bone graft substitute include the following features:^{19, 108}

- Osteogenic potential;
- Promotes osteoconduction;
- Promotes osteoinduction;
- Does not trigger an immune response;
- Provides mechanical support;
- Is fully replaced by host bone;
- Is readily available.

These properties are often dependent on the source of the bone graft, particle porosity, the presence of bioactive substances, the ratios of various compounds employed, among other features.¹⁰⁸

4.2. Classification of bone graft substitutes

The three imperative biomechanisms under which the bone graft substitutes operate are: osteogenic, where the bone graft is able to form new bone; osteoinductive, where the bone graft can recruit osteogenic cells and finally, osteoconductive, where grafts create a favourable microenvironment that can support the bone formation.¹⁹

Bone graft substitutes are often classified based on their origins.²¹

4.2.1. Autogenous bone grafts

Autogenous bone grafts are harvested from the same patient, commonly from the anterior ramus and mandibular symphysis regarding intra-oral locations. These grafts are considered

the gold standard for grafting, majorly attributable to their osteogenic properties, as they contain osteoprogenitor cells. Autogenic bone grafts are used in either block form or particle form, albeit block grafts often lead to higher patient morbidity and potential nerve damage.^{22, 109}

There are several ways to harvest bone, each of which can significantly affect its cellular integrity and subsequent bone formation capabilities. Commonly used techniques include using a bone mill, bone scraper, trapping bone from the aspirator during osteotomy preparation, and using piezo-surgery devices.¹¹⁰

Major advantages of autogenous bone grafts include osteogenic, osteoinductive, and osteoconductive properties, reliable bone quality, good mechanical stability and ease of availability. However, it doesn't always make for an ideal choice owing to its technique sensitivity, iatrogenic failures, limited volume, patient morbidity and risk of nerve damage, as well as additional costs.^{49, 110}

4.2.2. Allogenic bone grafts

Allogenic bone grafts are harvested from a donor and transplanted to a different host of the same species after being processed and decontaminated. Due to the presented limitations of autogenous grafts, allogenic grafts provide a viable alternative since allogenic bone grafts also possess osteoinductive potential.⁴⁹ Currently, allogenic bone grafts are available in various forms, including freeze-dried (mineralized or demineralized), frozen, or fresh. The composition and manufacturing techniques can alter the properties of the graft in terms of resorption rates, biological activity, radiolucency, and clinical indications.¹¹⁰

Allogenic bone grafts present advantages in patients with systemic conditions, poor healing capacities, or inadequate bone in terms of quality and volume. However, the risk of cross-infection, immunogenic incompatibilities (especially in fresh grafts), moderate absorption, variability in tissue, and lack of standardized harvesting and storage protocols are compelling limitations of allogenic grafts.^{109, 111}

4.2.3. Xenografts bone grafts

Xenograft bone grafts are harvested from organisms of different species. These grafts are derived from several origins such as bovine; porcine; equine; silk; corals; algae; and exoskeletons of crustaceans.¹¹² These are available in various forms such as blocks, granules and particulates.²⁰

Depending on their source, xenografts possess several characteristics such as osteoconductivity, low immunogenic response, good resorption rates and moderate mechanical stability. These features, along with good material handling and ease of access and availability

in comparison to autogenous and allogenic grafts, justify their frequent use in clinical practice. However, limitations such as loss of osteogenic and osteoinductive properties during processing stages, the possibility of cross-infection of zoonotic and prion diseases, and cultural and religious limitations furthered the research into synthetic grafts to overcome these limitations.^{112, 113}

4.2.4. Synthetic/alloplastic bone grafts

To overcome the aforementioned limitations of bone grafts of natural origin, advances in tissue engineering led to the exploration and development of synthetic bone grafts, where bone substitutes are artificially created using various combinations of CaP ceramics with varying proportions of HA and α/β -tricalcium phosphate (TCP); calcium sulphates; bioactive glasses; metals such as nickel-titanium and magnesium alloys; and polymers, such as polymethylmethacrylate (PMMA), and polyglycolides, as well as the combination of the former.^{20, 21} Synthetic bone grafts are available in several forms such as blocks, powders, beads, injectable syringes, pastes, sponges, putty, strips, moldable packs, and morsels.¹¹⁴

Depending on their composition, synthetic bone grafts possess several advantages, such as osteoinduction, osteoconduction, ease of handling due to their various available forms, radiopacity that allows easy monitoring of healing, good resorbability, low immunogenicity, a microstructure comparable to human bone, excellent hydrophilicity, and antimicrobial activity. However, poor mechanical stability and a slow rate of bone formation still pose challenges.^{20, 115, 116}

4.3. Microenvironment of bone graft substitutes

At the microscopic level, bone graft substitutes behave differently depending on their morphological features such as size, microtopography, surface characteristics, porosity, and their chemical composition.¹¹⁷

Different porosities of bone grafts significantly influence their osteoconductive and osteoinductive potential. Porosities less than 5 μm allow the permeation of cell metabolites, biochemical signals, and greater accumulation of bone-inducing proteins, thereby forming an inductive scaffold. Larger porosities, between 20 μm and 40 μm , also facilitate cell migration and subsequent bone formation. High interconnected porosity within a porous scaffold enhances bone regeneration through superior vascularization and increased bone volume.¹¹⁸⁻¹²⁰

The size of particles also exerts considerable influence on bone regeneration. Studies have shown that particle sizes of 50 μm reduce the collagen and DNA expressions in cells and may not be suitable for regenerative procedures.¹²¹

Studies reveal that particle dissolution also greatly influences osteogenicity and osteoinductivity of the cells. Ionic dissolution from the bone graft substitutes modulates the pH of the cellular environment that in turn influences cellular proliferation and differentiation and upregulates and downregulates genetic expression as well. Additionally, it affects the vascularization, protein absorption, stimulation of collagen I production, and the formation of biological apatite layers.^{122, 123}

II. OBJECTIVES

In the biological aspects of implant dentistry, this research aims to explore key biological processes pertaining to zirconia in combination with bone graft materials, with the goal of optimizing regenerative processes. Specifically, this study seeks to investigate how different bone graft substitutes influence the cellular response of osteoblastic cells grown over zirconia. Although several studies have been conducted to shed light on the biocompatibility of zirconia with hard and soft tissues, no literature explores the biological response over zirconia in the presence of various biomaterials, particularly through *in vitro* models.

- To evaluate the biological response of osteoblastic cells exposed indirectly to various bone graft substitutes.
- To evaluate the influence of various bone-graft substitutes (bovine origin, synthetic biphasic calcium phosphate and bioglass) on the metabolic activity and ALP expression of osteoblast-like cells grown over zirconia substrates and placed in closed proximity.
- To determine whether there is a difference in the biological response of osteoblast-like cells towards various bone graft substitutes in the presence and absence of zirconia.

The hypothesis was therefore defined as follows:

H₀: There is no difference in the cellular response towards bone substitutes, in the presence of zirconia;

H₁: There is a difference in the cellular response towards bone substitutes, in the presence of zirconia.

III. MATERIALS AND METHODS

1. Materials

In this study, three types of bone graft substitutes were used:

- Xenograft (XENO): a low-temperature bovine-origin xenograft, Cerabone™ (Botiss, Germany; Lote no. 23DA20360) (Figure 13A)
- Biphasic calcium phosphate (BCP): a synthetic biphasic composite with 60% HA and 40% β -TCP, Maxresorb™ (Botiss, Germany; Lote no. 2337-2) (Figure 13B)
- Bioactive Glass (BG): a synthetic bioactive glass composed of 100% phosphosilicate particles in a glycol-ethylene-glycerine binder, Novabone Dental Putty™ (Osteogenics, USA; Lote no. 1923B5) (Figure 13C).



Figure 13: Macroscopic images of the bone graft substitutes used in the study: (A) bovine-origin xenograft - Cerabone™; (B) synthetic biphasic calcium phosphate with 60% HA and 40% β -TCP - Maxresorb™; and (C) synthetic bioactive glass - Novabone Dental Putty™. Scale bar 1 mm.

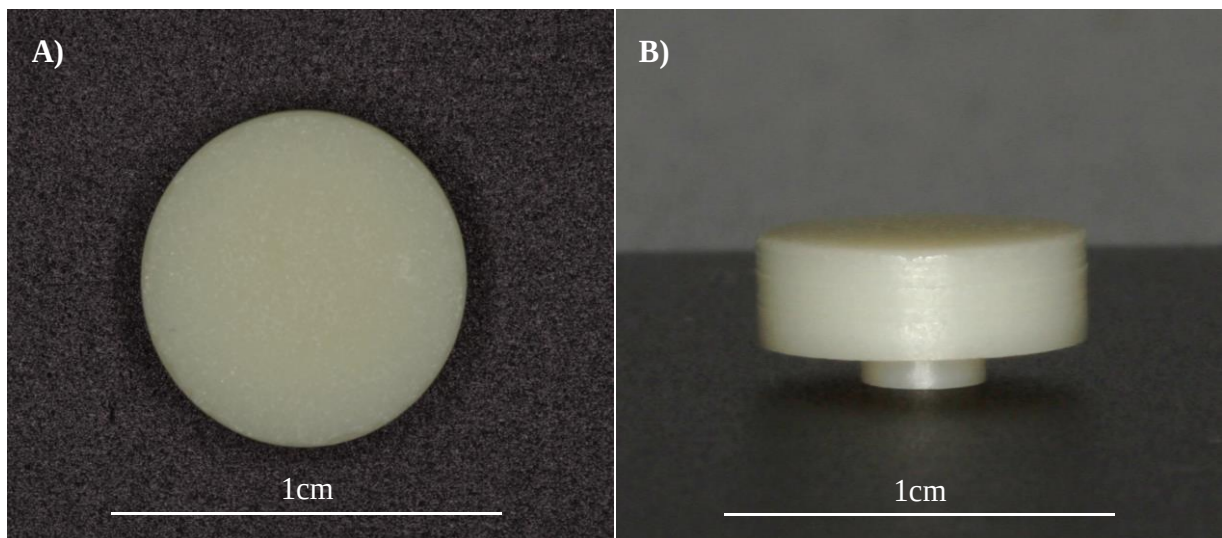


Figure 14: Macroscopic images of custom-made zirconia discs used in the study. (A) top view; (B) side view

Additionally, custom-made zirconia (Zr) discs of 7 mm in diameter, surface roughness of 4.9 μm , manufactured using the same process as dental implants (Patent™ (Zircon Medical

Management AG, Germany), were used (Figure 14). All the materials were supplied by the manufacturers. The bone graft substitutes were used as received. Zirconia discs were cleaned with 70% ethanol, followed by sterilization under UV light for 15 minutes per side.

2. Materials characterization

The morphology and elemental composition analysis of the bone graft substitutes and zirconia discs were performed by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS), respectively. Briefly, the samples were mounted on aluminium stubs with carbon tape, sputter-coated (SPI-Module) with a thin film of gold/palladium, and subjected to high-resolution SEM analysis, followed by EDS analysis (FEI Quanta 400 FEG ESEM/EDAX Genesis X4M).

3. Cell culture

Human osteoblast-like cells (MG63, ATCC) were used in this research. The cells were cultured in alpha minimum essential medium (α -MEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL Amphotericin B (all reagents from Gibco). The cell cultures were maintained at 37 °C in a humid atmosphere with 5% CO₂. At 70-80 % confluence, cells were detached using TrypLE Express (Gibco) for 5 minutes at 37 °C and subsequently used.

3.1. Bone graft substitutes cytocompatibility - Indirect assay

MG63 cells were seeded at a density of 10⁴ cells/cm² in 24-well plates and incubated for 24 hours. Equal amounts of bone graft substitutes (ca. 30 mg) were placed on 24-well inserts (6.5 mm Transwell[®] with 0.4 μ m pore membrane, Costar) and incubated in α -MEM for 1 hour. The inserts were then transferred to the wells containing the 24-hour seeded cells, allowing the indirect contact between the bone grafts and the cell monolayer. Cells exposed only to the culture medium alone served as control. Cell metabolic activity was evaluated at various time points over 7 days using the AlamarBlue[®] assay, and osteoblastic activity was assessed on the 7th day through alkaline phosphatase (ALP) activity measurement, as follows.

3.1.1. Metabolic activity

After 1, 4, and 7 days of exposure to the bone grafts, cell metabolic activity was measured using the AlamarBlue[®] assay. This assay is based on the reduction of resazurin to the

fluorescent byproduct resorufin by metabolically active cells. Briefly, the inserts containing the bone grafts were removed from the wells, and the medium was replaced with fresh medium containing 10% (v/v) resazurin (0.1 mg/mL, Sigma-Aldrich). The cell cultures were then incubated for 3 hours at 37 °C. Fluorescence values were subsequently measured in a microplate reader (Synergy HT, Biotek) with excitation at 540 nm and emission at 570 nm.

3.1.2. Alkaline phosphatase activity

On the 7th day of culture, the medium was aspirated from the wells, and the cells were rinsed with sterile phosphate-buffered saline (PBS, Sigma-Aldrich). The cells were then lysed with 0.1% Triton X-100 for 30 minutes. The substrate p-nitrophenyl phosphate (pNPP, Sigma-Aldrich) prepared in an alkaline buffer was added. The hydrolysis of the substrate by intracellular ALP enzymes was allowed to proceed for 1 hour at 37 °C. The absorbance of the hydrolysed product, p-nitrophenol (yellow), was measured at 400 nm using a microplate reader (Synergy HT, Biotek). ALP activity was normalized to total protein content (nmol/μg), measured using the *DC*TM protein assay (Biorad), as per the manufacturer's instructions.

3.2. Zirconia-bone graft substitutes synergic cytocompatibility

Zirconia discs were pre-incubated in supplemented α-MEM for 1 hour. Following this, MG63 cells were seeded on the surface of the discs at a density of 2×10^4 cells/cm² and allowed to adhere for 24 hours. The discs were then transferred to new wells containing equal amounts of bone graft substitutes (ca. 120 mg), which had been pre-incubated for 1 hour in α-MEM. The plates were further incubated to monitor the effects of bone graft substitutes on the cell monolayer present on the zirconia surface. Cells seeded on zirconia surfaces not exposed to bone graft substitutes served as control. Metabolic activity was evaluated at various time points over 15 days using the AlamarBlue[®] assay. Osteoblast morphology was examined through SEM after 4 and 15 days of culture, while osteoblastic activity was assessed on the 15th day through ALP activity measurements and cytochemical staining of ALP.

3.2.1. Metabolic activity

On days 1, 4, 7, 11, and 15, zirconia discs were transferred to new wells, and cell metabolic activity was measured using the AlamarBlue[®] assay, as previously described. After each measurement, the zirconia discs were returned to their original wells to continue incubation in close contact with the bone graft substitutes.

3.2.2. Cell morphology

On the 4th and 15th days of culture, cells on the zirconia disc surface were rinsed with sterile PBS and fixed using 1.5% glutaraldehyde (TAAB) for 15 minutes. The samples were

dehydrated in increasing concentrations of ethanol aqueous solutions for 30 minutes each and subsequently critical point dried (CPD 7501, Polaron Range). They were sputter-coated with a thin film of gold/palladium and subjected to high-resolution SEM (FEI Quanta 400 FEG ESEM/EDAX Genesis X4M).

3.2.3. Alkaline phosphatase activity

On the 15th day of culture, after removing the medium from the wells and rinsing the zirconia discs with sterile PBS, ALP activity was assessed and normalized as previously described.

3.2.4. Cytochemical staining of alkaline phosphatase

At day 15, zirconia discs were washed twice with PBS and fixed with 1.5% glutaraldehyde for 10 minutes. Subsequently, the samples were immersed in Tris buffer (pH 10) containing sodium naphthyl phosphate and Fast Blue RR salt (both from Sigma-Aldrich) and incubated for 1 hour at room temperature. After incubation, the samples were rinsed with distilled water and air-dried. ALP staining, indicative of enzyme activity, ranges from brown to black. Stained cultures were photographed using an inverted phase contrast microscope (Primovert, Zeiss).

4. Statistical analysis

Results are presented as mean $\pm$ standard deviation. Statistical analysis was performed with GraphPad Prism version 8.0.2. After passing the normality test, the values were subjected to a one-way analysis of variance (ANOVA) followed by Tukey as the post hoc test. Statistically significant differences were considered at α -values < 0.05 .

IV. RESULTS

1. Materials characterization

The surface morphology and elemental composition of Zr and bone graft substitutes were analysed using SEM-EDS, as shown in Figure 15 and Figure 16, respectively. At higher magnifications, Zr samples revealed highly irregular surface topography (Figure 15), with high traces of zirconium in the EDS analysis (Figure 16A).

Both XENO and BCP revealed a granular structure with highly irregular surfaces. Additionally, both materials displayed varying degrees of microporosity, wherein BCP exhibited a more porous microstructure (Figure 16). Elemental analysis confirmed high levels of calcium (Ca) and phosphorus (P) in both materials (Figure 16B, 16C). In contrast, BG displayed a more smooth and uniform appearance (Figure 15), and its elemental analysis revealed peaks for Ca and silicon (Si), along with significant levels of C, likely from its binder components, *i.e.*, polyethylene glycol and glycerine (Figure 16D). This comparative analysis highlights the distinct physical and chemical properties of each bone graft substitute.

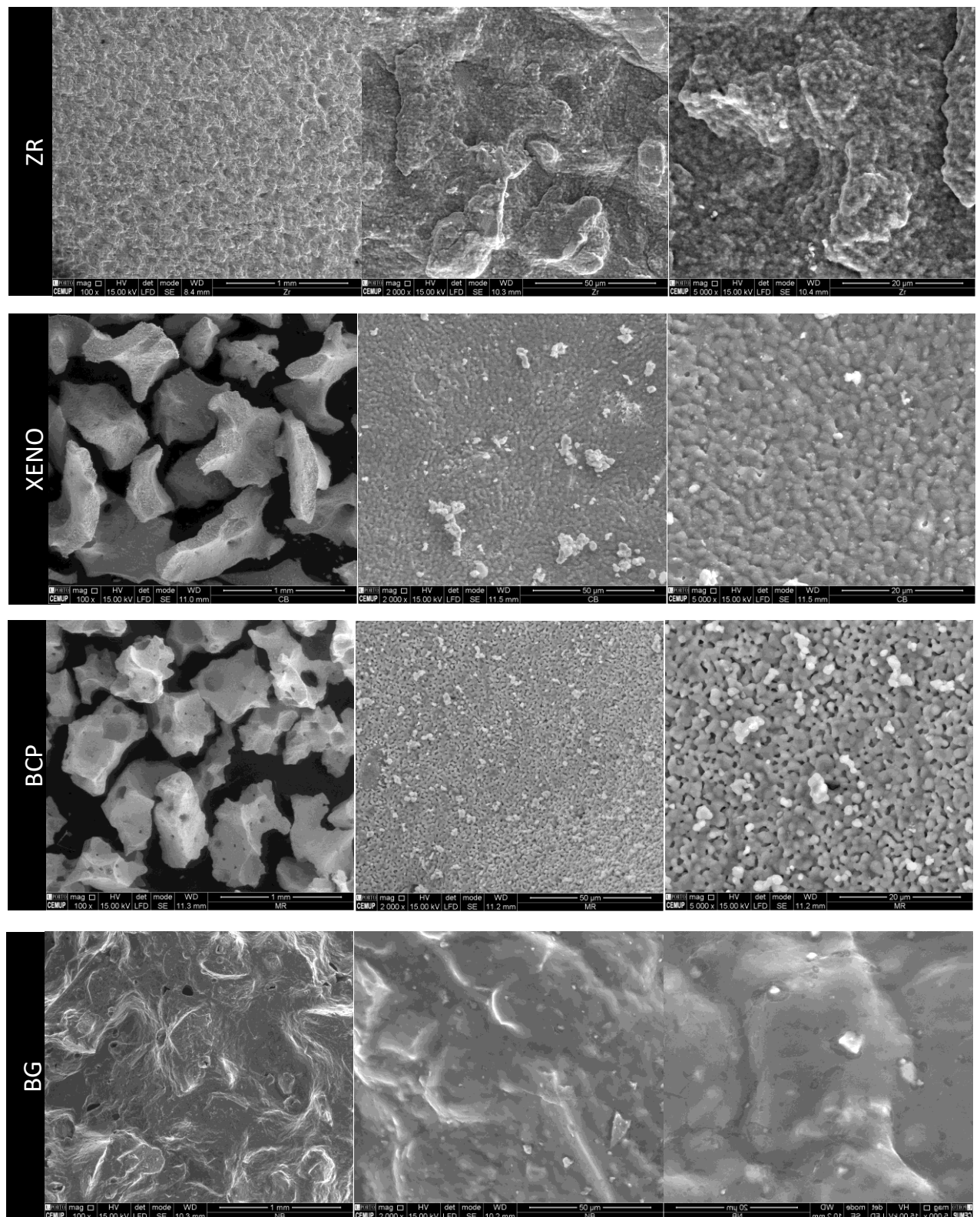


Figure 15: Surface morphology of zirconia and bone graft substitutes at micro- and nano-scale observed under SEM.

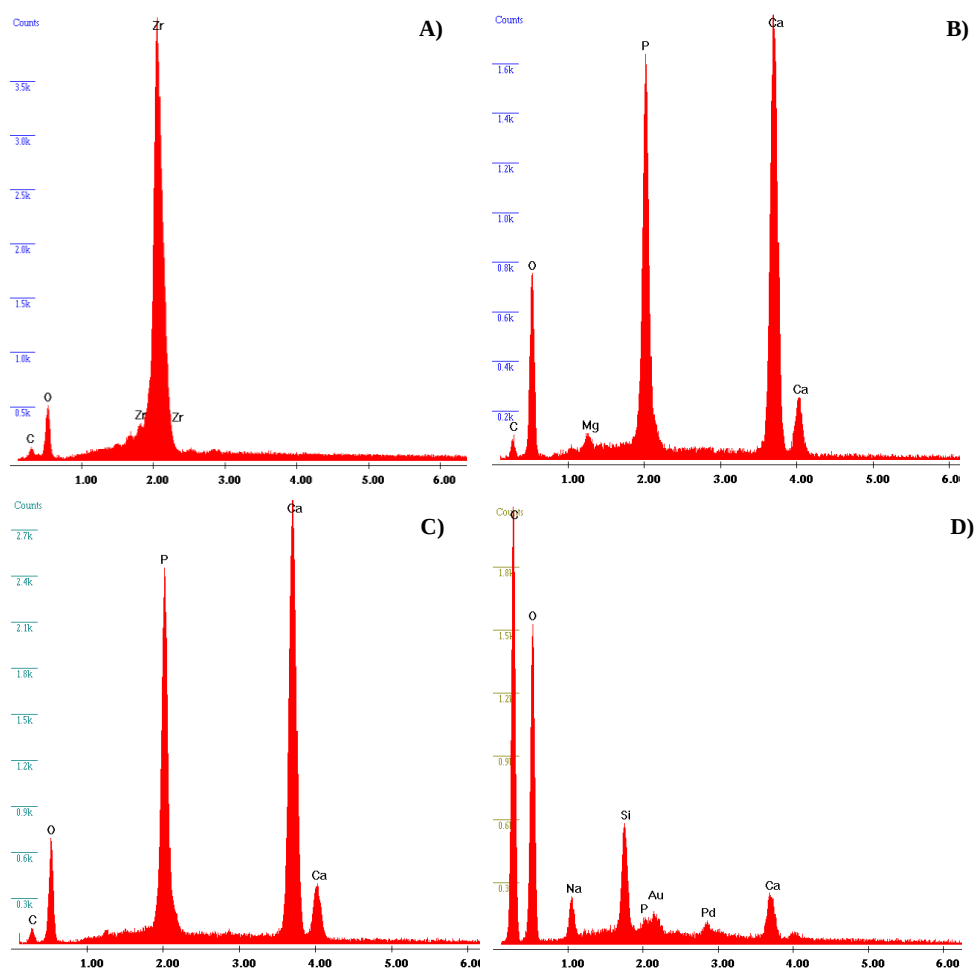


Figure 16: EDS analysis of (A) zirconia and bone graft substitutes: (B) XENO, (C) BCP, and (D) BG.

2. Bone graft substitutes cytocompatibility- Indirect assay

2.1. Metabolic activity

Cells were indirectly exposed to the bone graft substitutes, and their metabolic activity over time is depicted in Figure 17. The results revealed a general upward trend in metabolic activity across all experimental groups over 7 days. Statistical analysis indicated no significant difference between the bone graft substitutes and the control group on day 1, indicating a comparable initial cellular response. However, on days 4 and 7, distinct patterns emerged.

While most biomaterials exhibited similar results to the control, BG induced significantly lower metabolic activity than the control during these periods.

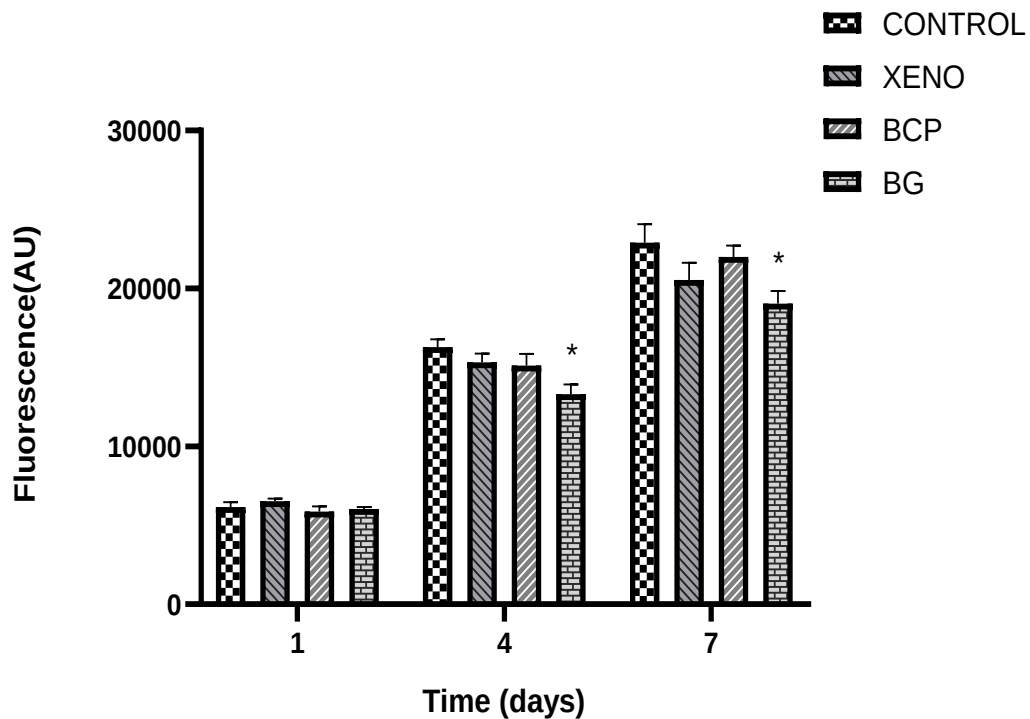


Figure 17: Metabolic activity of MG63 cells cultured in indirect contact with bone graft substitutes over 7 days, as measured by the resazurin assay. * $p < 0.05$, compared to the control group.

2.2 Alkaline phosphatase activity

On the 7th day of cell exposure to the bone graft substitutes, the biochemical assessment of ALP activity was conducted. As shown in Figure 18, the control condition and BCP yielded similar levels of ALP activity, whereas XENO and BG exhibited significantly lower values than the control.

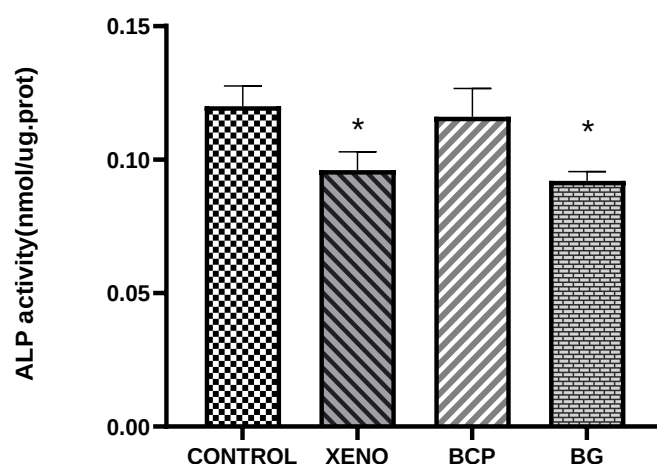


Figure 18: ALP activity of MG63 cells cultured for 7 days in indirect contact with bone graft substitutes. * $p < 0.05$, compared to the control group.

3. Zirconia-bone graft substitutes synergic cytocompatibility

3.1. Metabolic activity

In this phase, cells were seeded onto zirconia samples and indirectly exposed to the bone graft substitutes to assess cell response in this co-culture condition. As shown in Figure 19, all groups exhibited an overall increase trend over the 15 days of culture period, except for the control group, wherein cell metabolic activity peaked on day 11. No significant differences were observed among the conditions until the 7th day of culture. However, by the 11th day, significantly lower metabolic activity was observed in the co-culture conditions compared to the biomaterial-free control. These differences diminished by day 15.

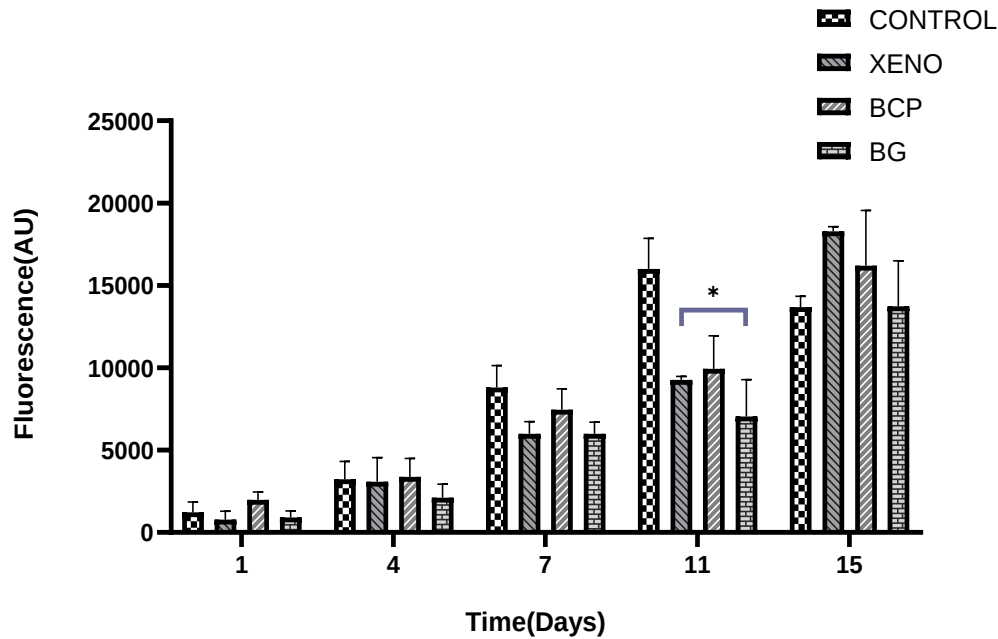


Figure 19: Metabolic activity of MG63 cells cultured on zirconia samples and indirectly exposed to bone graft substitutes over 15 days, as measured by the resazurin assay. * $p < 0.05$, compared to the control group (biomaterial-free).

3.2. Cell morphology

On day 4 of the culture, SEM analysis revealed distinct variations in osteoblastic morphology across all groups (Figure 20). The control group exhibited well-defined and well-spread cells, while cells exposed to XENO and BCP developed a polygonal and triangular shape. Cells exposed to BG on zirconia exhibited a more rounded morphology.

On day 15 of the culture (Figure 21), all groups exhibited well-defined and well-spread osteoblastic cells, covering the entire surface of the zirconia disc. As a notable observation, cells were densely attached to a loose BG crystal, with several cell extensions visible.

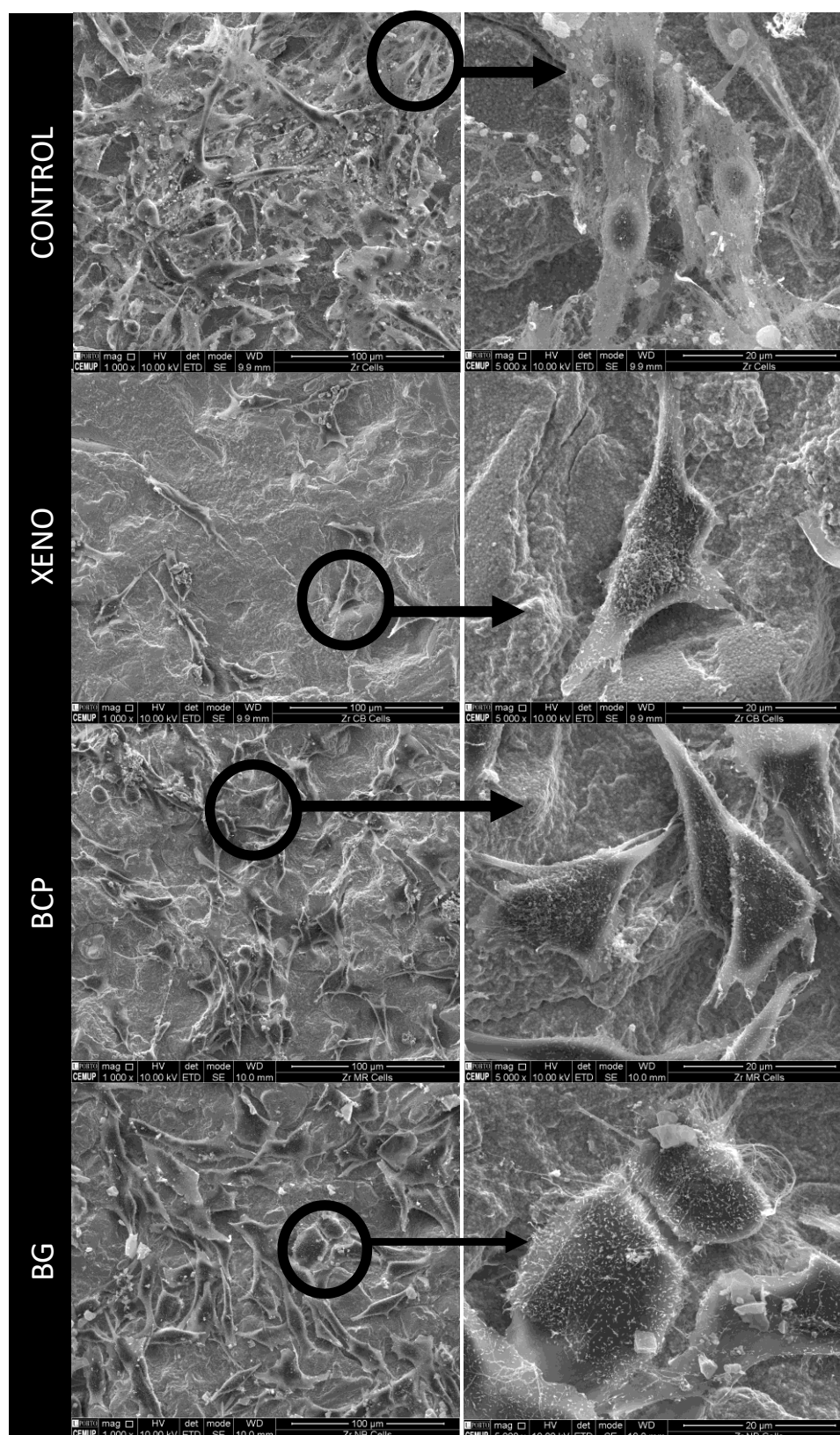


Figure 20: SEM images of MG63 cells cultured on zirconia samples and indirectly exposed to bone graft substitutes on day 4, highlighting cell distribution on the zirconia surface, followed by a magnified view to detail cell-material interaction.

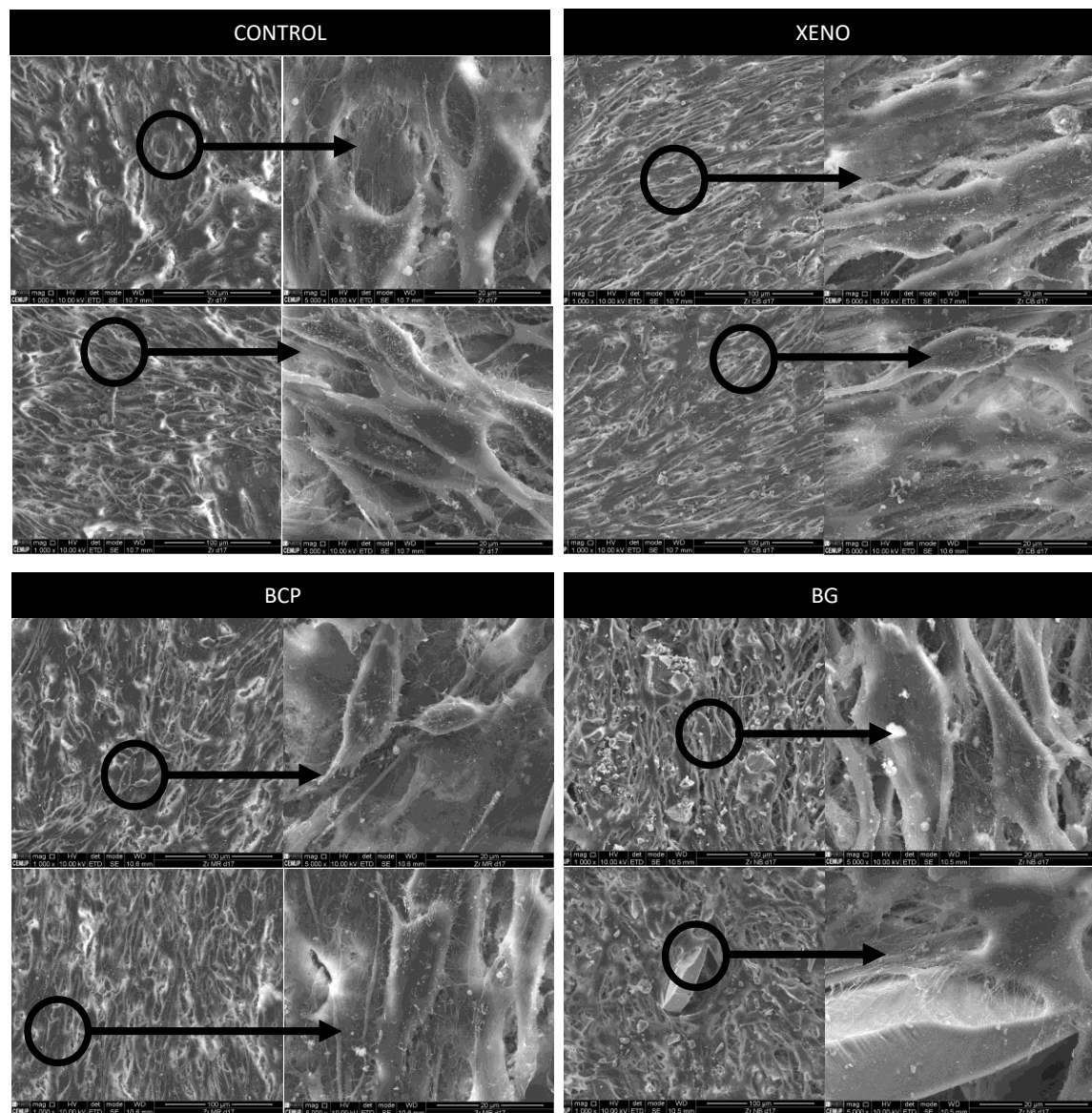


Figure 21: SEM images of MG63 cells cultured on zirconia samples and indirectly exposed to bone graft substitutes on day 15, highlighting the cell distribution on the zirconia surface, followed by a magnified view to detail cell-material interaction.

3.3. Biochemical activity and cytochemical staining of alkaline phosphatase

A biochemical assessment of ALP activity was conducted on the 15th day of cell exposure to the bone graft substitutes. As shown in Figure 22A, XENO yielded levels of ALP activity similar to those of the control condition. BCP and BG exhibited higher ALP activity, with BG being significantly different from the control. Additionally, cytochemical staining of ALP was performed at the same time point, revealing varying degrees of staining intensity across the different conditions tested (Figure 22B). Darker spots were noted on BG, owing to the higher ALP activity.

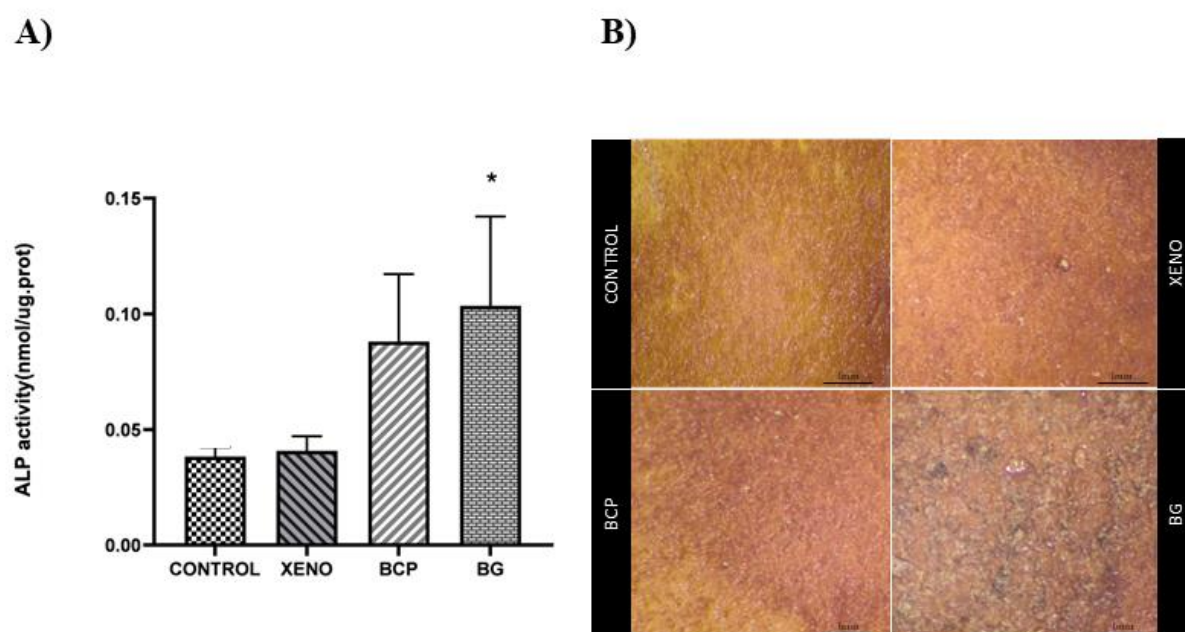


Figure 22: ALP activity of MG63 cells cultured on zirconia samples and indirectly exposed to bone graft substitutes for 15 days, measured (A) biochemically and (B) cytochemically. * $p < 0.05$, compared to control.

V. DISCUSSION

Bone defects are frequently present at the time of implant placement, indicating the need for regenerative procedures. Cha *et al.*,¹⁸ reported that 50.3% of the patients undergoing implant therapy require bone grafting, with sinus floor elevation procedures using the lateral approach (22.1 %) and guided bone regeneration (22.7 %) being the most frequently performed procedures. Concomitant implant placement with grafting opens various possibilities for optimizing the procedure, where several aspects ranging from the choice of implants to the selection of materials, serve as variables.

Narrowing it down to the biological variables, one approach to enhance outcomes is to analyse how different bone graft substitutes modulate the cellular response to other implant materials. Considerable literature exists on the exploration of bone graft substitutes' influence on the biological response to titanium. In 2021, Gunes *et al.*,¹²⁴ analysed the biomechanical osseointegration of titanium implants after guided bone regeneration with multiple bone graft substitutes (human-derived allograft, deproteinized bovine bone graft, HA, and calcium sulphate bio-ceramics) in Sprague Dawley rats. Bone defects were created in the tibia of the rats, followed by the placement of the aforementioned grafts in the defects. After a 90-day healing period, machined titanium implants were placed in the grafted sites, followed by another 90-day healing period. Biomechanical analysis of the new bone revealed no significant differences in any of the experimental groups.

Recent studies, such as that by Guler *et al.*,¹²⁵ conducted an *in vivo* study in Sprague Dawley rats to compare bone graft substitutes of various origins (human-derived allograft, bovine origin xenograft, and a biphasic calcium phosphate synthetic graft) placed in conjunction with titanium implants. After an 8-week healing period, the authors reported no significant difference in the implant-bone contact among the experimental groups. Similarly, Gokhan *et al.*,¹²⁶ conducted a study to assess the results of guided bone regeneration with titanium implants placed in conjunction with various bone graft substitutes (deproteinized bovine bone, human-derived allogenic bone, HA, and calcium sulphate bio-ceramics), using a titanium mesh. After a 90-day healing period, new bone regeneration was analysed through histological staining and by the expression of vascular endothelial growth factor, with no significant differences reported among the groups.

These findings indicate that the osteointegration of titanium is not significantly influenced by the type of bone grafts used.

However, study models analysing the influence of bone graft substitutes in the presence of zirconia have yet to be developed. In 2017, a pilot study conducted by Benic *et al.*,¹²⁷ compared the bone-implant contact of zirconia and titanium implants with simultaneous guided

bone regeneration. Maxillary premolars and molars in dogs were extracted. After a 5-month healing period post-extraction, semi-saddle defects were created in these regions, followed by implant placement with guided bone regeneration. Zirconia implants were placed with concomitant grafting consisting of either deproteinized bovine bone mineral granules (with or without 10% collagen matrix), or deproteinized bovine bone mineral block. Titanium implants were also placed with concomitant grafting using deproteinized bovine bone mineral granules. All experimental groups employed collagen membranes for guided bone regeneration. The results revealed no significant differences among the experimental groups, suggesting that both zirconia and titanium have a comparable response to bovine-origin xenografts.

This *in vitro* pilot research addresses this gap by evaluating the biological response of osteoblastic cells cultured on zirconia in the presence of bone graft substitutes of different origins.

To ensure the clinical relevance of this study, the quantity of bone grafts used was subjectively rationalized based on a common clinical scenario. When grafting is imperative during implant placement, it is recommended that the defect must not exceed 1/3rd of the implant length of a single wall, at the sub-osseous level. To quantify this defect, an implant from the same manufacturer (Patent™, Zircon Medical Management AG, Germany) (4 mm in diameter and 10 mm in length) was subjected to microtomography to measure 1/3rd of the total implant height with half the diameter (Annex 1). The surface area was subsequently calculated and correlated with the surface area of the zirconia substrate to determine the final quantity of the bone graft necessary to cover the defect site. According to the literature, the ideal thickness of the cortical bone where an implant is placed should be between 1-2 mm in width, to withstand occlusal forces.¹²⁸ Based on this criterion, the quantity of the bone graft substitutes was therefore determined to be able to regenerate bone of sufficient thickness in a hypothetical clinical situation.

The results of the present study highlight key insights into the biological processes involving zirconia and bone graft substitutes. In the first set of experiments, cells were indirectly exposed to bone graft substitutes (in the absence of zirconia) and subjected to cytocompatibility assays to assess their biological response. For this, osteoblast-like MG63 cells were seeded at the bottom of a tissue culture plate (Tissue-CP), while bone graft substitutes were placed in inserts, maintaining constant indirect contact with the cells for up to 7 days. The metabolic activity of MG63 cells followed a similar upward trend across all groups, wherein cells exposed to BG on days 4 and 7, showed significantly lower metabolic activity

compared to the control. These results align with previous findings by Mayr-Wohlfart *et al.*,¹²⁹ in which osteoblast-like cells, SAOS-2 line, cultured in the presence of multiple bone grafts (pure α -TCP, porous neutralized glass ceramic, bioactive glass, and solvent-dehydrated bone) showed lower metabolic activity compared to the control group, with a significant difference observed in the bioactive glass experimental group.

To further elucidate the biological effects of bone graft substitutes on MG63 phenotypic behaviour, ALP activity was determined. ALP is a well-known marker of early osteoblastic differentiation.¹³⁰ After 7 days, cells cultured in indirect contact with XENO and BG exhibited significantly lower ALP activity compared to the control group. These results are in agreement with the findings of Kubler *et al.*,¹³¹ who evaluated multiple bone graft substitutes (synthetic HA, HA with traces of Ca-P, low-temperature bovine-origin xenograft, high-temperature bovine-origin xenograft, and high-temperature bovine-origin xenograft with p-15 peptides) on cultured cells derived from human iliac cancellous bone. The authors reported the highest ALP activity in the control group (culture without bone graft substitutes) and the lowest in the low-temperature bovine-origin xenograft. Similarly, Mayr-Wohlfart *et al.*,¹²⁹ reported lower ALP activity in the bioactive glass group compared to the pure α -TCP group and the control group. In contrast, Matsumoto *et al.*,¹²¹ reported that bovine-sourced xenograft and synthetic HA showed significantly higher ALP activity compared to the control.

The effect of BG on cells can be co-related to the works of Silver *et al.*,¹³² wherein BG increased the alkalinity of the cellular medium due to ionic dissolution and influenced the metabolic and ALP activity of the cells. The authors exposed murine osteoblasts directly and indirectly to 45S5 Bioglass® and reported a significant increase in metabolic activity under both conditions compared to control (cells without exposure to bioglass) at several time points from day 1 to day 6. A rise in pH improves glycolysis in cells, thereby increasing ATP production, which subsequently improves metabolic activity. Their results also indicated a high concentration of Ca particles, induced by bioglass, in the cellular medium. Ca particles are known to downregulate the ALP activity in osteoblasts as reported by Anh *et al.*,¹³³ which could explain the lack of upregulation of ALP activity in murine osteoblasts in the alkaline medium as well as the delayed ALP activity on day 7 of the present study. The significantly lower ALP activity after exposure to BG can be further explained by the work of Ghannam *et al.*¹³⁴ The authors reported that variations in the pH of the cellular environment affect the ALP expression in cells cultured with bioglass. This study also highlights the difference between the dynamic *in vivo* environment and the stagnant *in vitro* environment. The high concentration of

corrosion products due to Si-Ca particle dissolution, along with an alkaline pH, although beneficial *in vivo*, can negatively affect osteoblastic differentiation, by delaying it *in vitro*.

Therefore, in the present study, the lower metabolic activity and ALP activity observed in the BG group can be associated with the ionic dissolution rate and change in pH of the cellular medium.

Significantly lower ALP expression after exposure to XENO can be attributed to the rate of Ca-P particle dissolution, influenced by factors such as sintering temperature, particle porosities, and other variables, which in turn significantly affect cellular differentiation.¹³⁵ Another justification for the poor performance of xenografts can be corroborated by the findings of Beloti *et al.*¹³⁶ In their study, an organic bovine-origin bone graft was placed in patients to repair a bone defect. After a healing period of 6 months, bone was harvested from the grafted and ungrafted sites of three patients and subsequently cultured. ALP activity was measured at several time points, and significantly lower ALP was noted in cells from the grafted site, suggesting that xenografts can downregulate cellular proliferation.

In the second set of experiments, zirconia discs with 24-hour adhered cells were incubated in close proximity to the bone graft substitutes, and the cellular response induced by this co-exposure was evaluated over 15 days. Cells showed a general ascending trend in cellular metabolic activity across all experimental groups throughout the 15 days. Only on day 11, a statistically significant difference was noted, with all the experimental groups exhibiting lower metabolic activity compared to the control group.

Regarding ALP activity, a similar trend was observed between the control group and the XENO group, while cells exposed to BCP and BG showed enhanced ALP activity on day 15, with significant differences in activity between control and BG, as additionally confirmed by cytochemical staining.

These contrasting results with those reported in the first set of experiments highlight the importance of the implant material in modulating the biological response of bone graft substitutes; suggesting a possible synergistic effect of these biomaterials with zirconia, especially BG, in promoting more favourable cell behaviour.

The results can be correlated with the work of Kauffman *et al.*,¹³⁷ who cultured osteoblast-like cells (ROS 17/2.8) on active bioglass discs and analysed expression of various biomarkers such as ALP, osteopontin, osteonectin, osteocalcin and bone sialoprotein of cells cultured over the bioglass discs as well cells surrounding the disc, present in the culture media. Cells over the discs expressed significantly lower ALP expression compared to the cells in the

polystyrene (control) during the first week (days 4 and 7), followed by a significant increase in the second week (day 14). These cells also expressed the presence of osteopontin, osteonectin, osteocalcin on days 4, 7 as well as 17 however bone sialoprotein was only detected on days 7 and 17. On the contrary, cells in the culture media, surrounding the bioglass discs expressed significantly higher ALP activity on day 7, along with the expression of osteopontin, osteonectin, osteocalcin, and bone sialoprotein in the first week, with a downregulation of ALP, osteonectin and bone sialoprotein on day 14. These findings provide insightful corollaries regarding the effects of ionic dissolution, pH, and material leaching on surrounding cells concerning cellular differentiation. Ionic overspill from the materials can enhance the osteogenic behaviour of the surrounding cells by altering the medium, which provides a nest for cellular differentiation, as observed in the first week. The downregulation of these biomarkers in the second week suggests the onset of the late stage of maturation. Alternatively, supersaturation of ions over the disc results in the delayed differentiation of cells as evident by the expression of osteoblastic biomarkers.

The results can be further corroborated with the findings reported by Shinn-Jyh Ding *et al.*,¹³⁸ who developed a 20% Ca-Si formulation coated over zirconia to form a Ca-Si-Zr composite. The ALP activity of HMSCs on days 7 and 11, cultured on the composite, was significantly higher to the untreated zirconia surface. A possible explanation to this mechanism could be attributed to the findings of Stanic *et al.*,¹³⁹ where a superficial infusion of Si particles into Zr surfaces promoted superior adhesion of osteoblasts, which could further elucidate the synergistic effects of Zr and Si.

Zirconia's superior osteoconductive properties are evidenced by significantly high expression of ALP, biglycan, osteocalcin, as well as modulation of ECM, as reported by Carinci *et al.*,⁹³ Zhang *et al.*,¹⁴⁰ Kohal *et al.*,¹⁴¹ and Gapski *et al.*,¹⁴²; along with its favourable interactions with calcium-silicate-based materials. This may explain the enhanced cellular response towards bone graft substitutes in the present study since these findings suggest that zirconia's surface properties, combined with specific bone graft materials, could play a crucial role in optimizing cell behaviour.

Surface modifications on zirconia are also an ongoing area of research due to its limitations related to low-temperature degradation and manufacturing difficulties. Processes such as sandblasting, acid-etching, anodising, laser treatments, UV treatments, and bio-coatings with various compositions (Ca-P, dopamine, etc) are some of the beneficial approaches that not only help overcome manufacturing challenges but also improve cell adhesion and proliferation.¹⁴³⁻¹⁴⁵

VI. CONCLUSIONS

Through this *in-vitro* study, it can be concluded that bone grafts can have varying degrees of influence on cell proliferation and differentiation in the presence of zirconia materials. Therefore, the null hypothesis was rejected.

The results underscore interesting insights that could additionally implicate that the composition of bone graft substitutes plays a vital role in influencing cellular response in the presence of zirconia.

Despite its *in vitro* nature, this study holds clinical significance. With the numerous choices of bone substitutes available on the market today, this study can help informed decisions when selecting from various options to better optimise clinical outcomes during regenerative procedures at the time of implant placement.

VII. LIMITATIONS AND SCOPE FOR FUTURE

This pilot study addressed the metabolic activity and ALP expression in osteoblast-like cells to assess the cytocompatibility and osteoblastic proliferation. However, to have a more comprehensive understanding of the bone formation process and the effectiveness of different bone graft substitutes and implants, future studies should address a broader range of differentiation markers. Additionally, using other cell types that play a role in osteointegration, such as osteoclasts and BMSCs, could provide a complete picture of how different materials influence the overall bone healing and integration process.

Furthermore, *in vivo* models are required to validate the results of the present study by assessing various parameters such as bone-implant contact and removal torque. These parameters provide insights into the mechanical stability and integration of Zr implants. Given that current *in vivo* research on Zr is sparse, further studies are necessary.

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IX. APPENDICES

1. Micro CT image of implant

Micro Ct image of zirconia implant with facial and coronal view respectively, used for measuring the area requiring grafting, to determine the quantity of bone graft substitute to be used.

