



Journal of
Functional Biomaterials

Special Issue Reprint

Biomaterials Applied in Dental Sciences

Edited by
Gustavo Fernandes

mdpi.com/journal/jfb



Biomaterials Applied in Dental Sciences

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Guest Editor

Gustavo Fernandes



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This is a reprint of the Special Issue, published open access by the journal *Journal of Functional Biomaterials* (ISSN 2079-4983), freely accessible at: https://www.mdpi.com/journal/jfb/special_issues/9K8845KAH9.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. <i>Journal Name</i> Year , Volume Number, Page Range.
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ISBN 978-3-7258-8359-2 (Hbk)

ISBN 978-3-7258-8360-8 (PDF)

<https://doi.org/10.3390/books978-3-7258-8360-8>

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About the Editor

Gustavo Fernandes

Gustavo Fernandes is an Associate Professor, Assistant Director of Periodontics at A.T. Still University (ATSU) of the Missouri School of Dentistry, and an ATSRI Scientist II. He ranks as the #1 researcher in Dentistry at ATSU and among the top 2% of researchers overall at the institution.

With over 21 years in research and 18 years of clinical practice, Dr. Fernandes has spent 14+ years in academia across Brazil, Portugal, and the U.S. He previously served as Head of Periodontics, Implant Dentistry, and Regenerative Medicine in both Brazil (2014–2018) and Portugal (2018–2021). He also serves on the IADR Constitution Committee.

A prolific innovator, he has introduced several novel methodologies to the scientific community: (1) MiTT: a mixed thickness tunnel access technique for periodontal plastic surgery; (2) GF-PreDImp – an algorithm tool to analyze the predictability of a dental implant; (3) GF-PeDRA® – advanced algorithms for dental implant success predictability and periodontal risk assessment; (4) GFsa – a new index system for assessing researcher productivity; (5) GF-MoR's complexes – novel bacterial clusters improving upon Socransky's complexes; (6) iMPACT and quadrant protocol – standardized protocols to treat peri-implantitis; (7) GF-PAPro – a comprehensive diagnostic periodontal assessment protocol; and (8) Periodontitis staging review: a seven-point critical modification for classification.

His extensive scholarly output includes 230+ peer-reviewed articles, mentoring 30+ graduate students, and involvement in 5 international grants. He reviews for 150+ journals and serves on 24 editorial boards. His teaching and research accolades include the 2022 Academic Innovation Fund Prize; 2023 Professor of the Month (University of Michigan); 2024 ATSU Innovation in Teaching Award (third place); and 2025 honors, including Outstanding Faculty of the Year and first/third place for Research Day prizes for his clinical algorithms.



Editorial

From Fundamental Biointeractions to Advanced Clinical Therapies in Dentistry Using Biomaterials and Biological Components: An Editorial

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1. Introduction

The field of dental biomaterials has undergone a monumental shift, evolving from simple inert fillers to complex, bio-instructive systems capable of orchestrating tissue regeneration. This Special Issue, titled “Dental Biomaterials”, explores this journey, framing the progress of biomaterials within the context of bioengineering and clinical application. The rapid advancement of materials for grafting procedures, reconstructions, and prosthetic applications has outpaced many practitioners’ fundamental biological understanding, creating a gap between technical capability and biological rationale. This editorial synthesizes high-impact research on molecular signaling, hard and soft tissue substitutes, and updated microbiological paradigms. The recent literature demonstrates the efficacy of nanostructured bone grafts, such as hydroxyapatite (HA), and recombinant proteins (rhBMPs) in enhancing osseointegration, the clinical reliability of short/extra-short implants, and the regenerative potential of autologous platelet concentrates (APC) in the healing process. Furthermore, the role of cellular dynamics (ROS, LMW-PTP) and the macrophage-driven immune response are identified as critical factors for success. While the evolution of dental technology offers unprecedented opportunities, clinical success remains tethered to a deep comprehension of biological fundamentals. This editorial argues that navigating the “biomaterials’ world” requires more than just technical skill; it demands a return to biological literacy and knowledge of basic and advanced concepts to ensure long-term, predictable outcomes for patients.

2. The Technical—Biological Paradox

The progress in biomaterials applied in dental sciences, intrinsically linked to the evolution of bioengineering, has enabled a radical transformation in the materials used for bone and soft tissue grafts, implants, and prosthetic reconstructions. We are currently in an era where “bio-intelligence” is integrated into the biomaterials we use daily. However, as we embark on this journey “traveling through the biomaterials world”, we must confront a disturbing paradox in modern dentistry.

There is an undeniable fervor among dental professionals to adopt advanced procedures—be it guided implant surgery, the use of growth factors (GFs), or the application of complex monolithic ceramics. Yet, this enthusiasm for the “new” and “technological” is frequently accompanied by a significant lack of basic understanding regarding the biological foundations that dictate the success or failure of these materials. Many clinicians seek to master the “how” of a procedure while neglecting the “why” of the tissue response.

Choosing a biomaterial should not be a decision based on marketing or simplified clinical protocols; it must be an exercise in clinical reasoning rooted in cell biology, immunology, and biomechanics. Understanding advances in biomaterials enables appropriate applications and effective strategies to improve dental treatment outcomes.

This editorial aims to address this gap, providing a comprehensive overview of how biomaterials have evolved from passive structural replacements to active participants in tissue regeneration.

3. The Foundation: Molecular Signaling and the Cellular Interface

The “travel” begins at the subcellular level. A recurring criticism of contemporary clinical practice is the disregard for the initial events of the host–biomaterial interface. Before a clinician observes “osseointegration” on a radiograph, a complex series of molecular interactions must occur.

Early research into osteoblast adhesion dynamics revealed that cellular anchoring is not a static event but a highly regulated signaling process. The roles of Reactive Oxygen Species (ROS) and Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) have been highlighted as potential pathways for controlling osteoblast behavior. This suggests that the surface of a dental implant or a bone graft is not merely a physical scaffold but a chemical messenger that can trigger or inhibit bone formation.

Furthermore, we must reconsider the role of the immune system in the success of biomaterials. For years, the “foreign body reaction” was viewed negatively. However, modern perspectives emphasize that macrophages are central orchestrators of bone remodeling and osteogenesis. The transition from a pro-inflammatory (M1) to a pro-healing (M2) macrophage phenotype is the biological pivot upon which regenerative success turns. Clinicians who overlook this immunological component may struggle to explain why technically perfect graft procedures occasionally fail due to “unexplained” inflammation.

4. “Hard” Biomaterials: Navigating Bone Regeneration and Osseointegration

The evolution of “hard” biomaterials has moved toward biomimetics (materials that mimic the physical and chemical properties of natural bone). This section of our journey explores how we can manipulate the bone bed to accept and integrate various substitutes.

4.1. Advanced Bone Substitutes and Bioactive Enhancements

The development of nanostructured biomaterials represents a significant leap forward. Recent advancements emphasize the dual function of implant surfaces: supporting bone integration while actively limiting bacterial colonization. For instance, Martín-Santana et al. demonstrated that zinc-doped biomimetic calcium phosphate (CaP-Zn) coatings on surface-activated Ti6Al4V implants provide a multifunctional interface. Their work confirms that these biomimetic depositions significantly enhance bioactivity and osteoconductivity while introducing essential antimicrobial properties [1].

Similarly, addressing the critical clinical reality that bacterial infection is a primary driver of implant failure, Alenezi’s comprehensive systematic review and meta-analysis highlight the efficacy of modifying metallic implants with silver (Ag) coatings. The findings confirm that these targeted coatings not only boost antibacterial performance but also actively promote bone formation and osseointegration in animal models [2].

A comparison of biodegradable and ceramic hydroxyapatite (HA)-based grafts using synchrotron analysis and scanning electron microscopy (SEM) has shown that the synthesis process and resulting nanomorphology directly influence the biological response. This is further enhanced by the association of bone morphogenetic proteins (BMPs). For instance, recombinant human BMP-7 (rhBMP-7) associated with nanometric HA coatings on

titanium surfaces has been shown to significantly improve osseointegration in preclinical models. Furthermore, the use of rhBMP-2 has been clinically validated to reduce volumetric contraction in post-extraction sockets (humans), a crucial consideration for ridge preservation [3].

In clinical practice, the choice among autologous, allogeneic, xenogeneic, or synthetic grafts remains a daily decision. A study comparing four bone grafts with expanded polytetrafluoroethylene (e-PTFE) membranes in critical-size defects in rats emphasizes that histomorphometric outcomes are highly dependent on the graft's ability to maintain space and support cellular infiltration.

4.2. Pharmacological Interactions in Bone Healing

A critical area often overlooked by technically focused clinicians is the systemic influence of medications on biomaterial performance. The interaction between bisphosphonates (such as Zoledronate and Clodronate) and nanostructured HA grafts has been studied to foster better clinical reasoning. Understanding how these systemic modifiers affect local bone repair is essential for treating the modern, medically complex patient. We cannot treat the mouth as an isolated environment; it is a biological system influenced by systemic pharmacology and epigenetics.

4.3. The Evolution of Dental Implants: Materials and Dimensions

The “travel” through implantology reveals a shift toward microstructural optimization and precise biomechanical engineering. While commercially pure (CP) titanium is a staple due to its high biocompatibility, its relatively low strength can limit its use in highly loaded applications. Hlinka et al. evaluated ultrafine-grained (UFG) titanium, demonstrating that reducing the grain size and applying electrochemical surface modifications drastically improve the biological response, corrosion resistance, and overall technological properties of the implants [4].

Beyond material composition, the implant's macrogeometry plays a profound role in tissue response. Gehrke et al. conducted an extensive *in vitro* and *in vivo* study on implant macrogeometry, revealing that controlling cortical bone compression during insertion is crucial. Their findings show that minimizing excessive compression yields a more favorable biological reaction and accelerates peri-implant bone healing, which is vital for long-term primary stability [5].

Furthermore, clinical success is tightly linked to patient-specific biomechanics. Abe et al. provided a retrospective analysis of female patients, demonstrating that the magnitude of occlusal force and masticatory performance strongly influence both patients' selection of implant treatment for a missing mandibular second molar and the restoration's long-term success [6].

In addition, the debate between titanium and zirconia remains a focal point. Meta-analyses of preclinical studies indicate that both materials achieve high levels of histological osseointegration, though zirconia offers specific advantages in soft tissue response and esthetics. Clinically, monolithic CAD/CAM zirconia restorations have proven to be robust and successful in tooth-supported applications.

Perhaps the most significant “technology applied to dentistry” in recent years is the refinement of implant dimensions. To avoid complex and morbidity-prone grafting procedures, the use of short and extra-short implants has gained traction. Robust evidence now supports the view that extra-short implants (≤ 6 mm) perform comparably to longer implants in terms of survival while significantly reducing the need for bone augmentation. Long-term retrospective studies with follow-ups exceeding 74 months have further con-

firmed the reliability of short implants (>6 mm and ≤ 8.5 mm) in the posterior segment. This evolution allows for a more patient-centric approach, prioritizing biological conservation.

5. Restorative Biomaterials: Interfaces and Environmental Resistance

The biological success of an implant or graft is intrinsically linked to its restorative counterpart. The durability and integration of modern prosthetics require a deep understanding of material interfaces and their behavior over time.

Şentürk et al. comparatively evaluated the shear bond strength (SBS) between monolithic zirconia (MZ) and various core build-up materials. The in vitro study provides essential guidelines for clinicians, proving that the choice of core material significantly impacts the mechanical retention and stability of highly esthetic zirconia reconstructions [7]. Moreover, these restorative materials are constantly challenged by the oral environment. Degirmenci et al. highlighted this by assessing CAD/CAM resin nanoceramics and demonstrating that aging factors such as thermocycling and ultraviolet (UV) exposure significantly alter surface characteristics and reduce repair bond strength over time [8].

Even in the transitional phase of treatment, provisional materials require bio-intelligence. Nazemi Salman et al. introduced a nanoclay-infused Cavit temporary filling, establishing its superior antibacterial characteristics. This innovation is crucial for sealing access cavities and protecting the internal environment during delayed final crown placements in pediatric and endodontic procedures [9].

6. Soft Tissue Biomaterials: From Hemostasis to Regeneration

Our journey continues into the realm of “soft” biomaterials, which are crucial for protecting the bone–implant interface and achieving esthetic harmony.

6.1. Autologous Platelet Concentrates (APC)

The shift from first-generation platelet-rich plasma (PRP) to second- and third-generation platelet concentrates—platelet-rich fibrin (PRF)—represents one of the most successful integrations of bioengineering into daily practice. These materials act as reservoirs for GFs, facilitating tissue repair and regeneration. Recent in vitro models have deeply expanded our understanding of these dynamics. Kirilova et al. evaluated the effects of various biomaterials derived from autologous blood on human dental pulp stem cells (hDPSCs). Their study confirmed that these advanced platelet concentrates significantly enhance stem cell migration, acting as bioactive scaffolds that recruit progenitor cells to the site of injury, thereby accelerating soft tissue and pulp regeneration [10].

Systematic reviews have highlighted the efficacy of these concentrates in many dental procedures, including the surgical treatment of gingival recessions. Specifically, the use of L-PRF in combination with coronally advanced flaps (CAFs) has shown acceptable results in treating Type 1 gingival recessions. Beyond periodontal surgery, liquid PRF has been used to coat implant surfaces to enhance the early stages of osseointegration through bioactive “bio-functionalization” of the titanium surface. This is a prime example of how simple biological technology can be applied to enhance mechanical systems.

6.2. Ridge Preservation and Wound Healing

The management of extraction sockets has also evolved to include soft materials, bone grafts, and hemostatic biomaterials. Assessment of collagen membranes and sponges in alveolar sockets has shown their prospective clinical value in stabilizing the initial clot and facilitating subsequent healing. Additionally, the use of autologous demineralized dentin matrix (DDM) for ridge preservation has emerged as a promising systematic alternative, utilizing the patient’s own tooth structure as a bioactive bone substitute.

7. Rethinking Failure: Microbiology, Peri-Implant Condition, and New Device for Treatment

No journey through the world of biomaterials is complete without acknowledging the threats to success. The lack of basic understanding regarding the oral microbiome is a major contributor to the rise in peri-implant diseases.

Modern research suggests we must move beyond the classical Socransky complexes. New bacterial clusters, proposed as the GF-MoR complexes, provide a summarized framework for understanding the polymicrobial nature of periodontal and peri-implant diseases [11]. Furthermore, the molecular pathways involving sclerostin and its inhibition (antisclerostin) are being explored to improve the bone remodeling environment around dental implants.

As we revisit peri-implant diseases, it is clear that we must rethink the future of compromised dental implants. The technology of tomorrow will not just involve better materials but better ways to manage the host–biofilm–biomaterial triad, utilizing scientifically validated protocols for surface refinement and re-osseointegration to salvage failing implants. Advancing the therapeutic frontier of peri-implantitis, the novel iMPACT Tool and Quadrant Protocol provide a scientifically validated pathway for implant surface refinement and successful re-osseointegration, effectively bridging high-resolution SEM/EDS analysis with long-term clinical predictability.

8. Conclusions: The Compass for the Journey

Traveling through the world of biomaterials is a journey of immense potential. The evolution from basic plates and screws to nanostructured, bioactive, and GF-enhanced systems has redefined what is possible in dental rehabilitation. However, the recurring point is to call readers to “Biological Responsibility”.

The modern professional must resist the urge to be a mere technician. To truly serve patients and improve treatment outcomes, one must master the biological principles that govern these materials. Technology applied to the dental area—be it CAD/CAM, short/extra-short implants, new devices, or APC—is only a tool. The true driver of success is the clinician’s ability to apply these tools within a framework of scientific reasoning. It is our hope that the diverse and rigorous research presented here serves as a compass for clinicians and researchers alike, guiding them toward a more integrated, biologically sound future in dental biomaterials.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

The Influence of Thermocycling and Ultraviolet Aging on Surface Characteristics and the Repair Bond Strength of CAD/CAM Resin Nanoceramics

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Abstract: Background: The durability of computer-aided design/computer-aided manufacturing (CAD/CAM) resin nanoceramics in the oral environment is influenced by aging factors such as thermocycling and ultraviolet (UV) exposure. This study investigates the impact of these aging processes on surface characteristics and repair bond strength. Methods: CAD/CAM resin nanoceramic samples were divided into the following five groups: control (non-aged), 1-year and 5-year thermocycling, and 1-year and 5-year UV aging ($n = 12$). For the thermocycling procedure, the parameters employed were a temperature range of 5–55 °C with dwell times of 20 s per bath and 10,000 and 50,000 cycles; for the ultraviolet aging process, the parameters were established at a wavelength of 340 nm, an intensity of 0.55 W/m², and durations of 300 h and 1500 h. Surface roughness, microhardness, and repair bond strength were analyzed through profilometry, Vickers microhardness testing, and shear bond strength assessment, respectively. SEM, AFM, and XRD analyses were performed for structural evaluation. Results: Both thermocycling and UV aging significantly increased surface roughness ($p < 0.001$) while reducing microhardness and repair bond strength ($p < 0.001$). UV aging had a more pronounced effect, particularly after five years, leading to the highest surface roughness (Ra: 61.77 µm; Rz: 271.57 µm) and lowest microhardness properties (63.13). EDAX analysis indicated matrix degradation and an increase in inorganic filler exposure. Conclusions: Aging significantly affects the surface characteristics of CAD/CAM resin nanoceramics, with UV aging exhibiting the most detrimental impact. These findings highlight the necessity of considering long-term material stability in dentistry.

Keywords: CAD/CAM resin nanoceramic; UV aging; surface properties

1. Introduction

Recent advancements in computer-aided design and computer-aided manufacturing (CAD/CAM) systems have facilitated the creation of aesthetic CAD/CAM restorations that demonstrate improved clinical outcomes [1]. Moreover, the incorporation of intraoral cameras, advanced design software, enhanced biomaterials, and digital modeling within these systems has substantially influenced the field of prosthetic and restorative dentistry [2]. This integration has contributed to the growing prevalence and acceptance of CAD/CAM systems in contemporary dental practices [3]. In response to patient preferences and the requirements of dental professionals, ceramics have emerged as the preferred materials

for aesthetic restorations, applicable in both traditional production techniques and fully digital workflows [4]. The aesthetic qualities, biocompatibility, durability, and resistance to discoloration inherent in ceramic materials are the primary factors underpinning this selection [5]. However, Kassem et al. have identified several significant disadvantages associated with ceramics. These include their propensity to cause considerable wear on opposing dentition, the increased visibility of defects on the intaglio surfaces due to their brittle characteristics, and their tendency to fracture [6]. In response to prevailing challenges in the field, the market has recently introduced new CAD/CAM materials known as hybrid-resin ceramics. These innovative blocks are designed to integrate the benefits of both resin and ceramic restoration materials, thereby enhancing the performance and effectiveness of dental restorations [7].

Hybrid-resin ceramics represent advanced materials characterized by a nanohybrid structure that incorporates inorganic ceramic fillers, which have been developed utilizing cutting-edge nanotechnology [8]. The materials incorporate micro, hybrid, and nano fillers in addition to a resin matrix [9]. The resin matrix structure is composed of various dimethacrylate thermosets, including ethoxylated bisphenol-A dimethacrylate (BisEMA), ethylene glycol dimethacrylate (EGDMA), bisphenol-A-diglycidyl methacrylate (BisGMA), and urethane dimethacrylate (UDMA). The filler component consists of silica fillers in nanoscale sizes, nanocluster particles derived from these fillers, as well as zirconia and barium glass fillers [10]. Cerasmart (GC), commonly referred to as resin nanoceramics, is one of the most prominent hybrid-resin ceramic materials available. This innovative material comprises 71% silica along with barium glass nanoparticles, showcasing its advanced compositional structure [11]. This material presents several significant advantages for clinicians, including low abrasion and superior flexural properties, as well as enhanced resistance to chipping and cracking during the milling process. Moreover, it allows for accelerated milling procedures and reduces wear on milling instruments due to its low microhardness. Its structure is less brittle compared to glass ceramics, and it does not require a post-production firing process. Additionally, the material is equipped with a dedicated staining kit that satisfies high aesthetic standards, and it facilitates the efficient finishing of restorations through polishing [12–14]. Nevertheless, it is well recognized that CAD/CAM materials containing a resin matrix are considerably influenced by the moist environment found within the oral cavity [15]. In addition to intraoral conditions, insufficient interconnection, improper occlusal adjustments, or the presence of parafunctional habits in the patient may render the fracture of resin nanoceramic restorations inevitable [5]. Lohbauer et al. highlighted this concern in their research, noting that the initially assessed mechanical properties may not serve as a reliable criterion for evaluating the clinical performance of these materials [16].

Aging methodologies can be utilized to assess the mechanical resistance and physical properties of dental materials within the oral environment in a timely manner under standardized laboratory conditions. This approach effectively simulates long-term clinical scenarios, enabling a more accurate evaluation of material performance [17,18]. The predominant aging method favored in contemporary literature remains thermocycling [19]; however, various methods exist for treatment and degradation, including soaking in water, boiling in water, exposure to acid, autoclave processing, immersion in a water–ethanol mixture, and ultraviolet (UV) aging [20]. In the seminal research conducted by Heydecke et al., it was underscored that the incorporation of aging procedures involving light exposure and variations in moisture—referred to as the weathering process—are critical factors for the accurate assessment of the performance of non-metallic materials [21]. In the current UV aging methodology, samples are subjected to continuous variations in temperature, heat, and humidity while being exposed to visible light that emits UV radiation. This approach

accounts for these additional parameters in the assessment process [1]. It is anticipated that the aging process will induce intrinsic physical or chemical modifications in materials [22]. However, it is imperative to recognize that contemporary materials may demonstrate varying degrees of resistance to mechanical and chemical degradation. Such variability is attributable to differences in filler composition and the chemistry governing the interaction between the matrix and the filler [23]. The biomechanical behavior characteristics of resin nanoceramic materials have not been extensively studied to date. The limited research findings that do exist exhibit considerable variability, which is believed to be primarily attributed to the differing aging methodologies employed in these studies [19,24]. Moreover, the absence of long-term clinical data from this perspective may lead to confusion among clinicians concerning the use of new materials. The mechanical and chemical resistance of CAD/CAM resin nanoceramics plays a crucial role in their long-term efficacy within the oral environment, where they encounter thermal fluctuations, moisture, and ultraviolet exposure. It is imperative to comprehend the degradation mechanisms of these materials over time to accurately predict their clinical durability and reduce the incidence of restoration failures. Therefore, it is crucial to conduct surface analyses, including assessments of microhardness, roughness, elemental changes, and phase alterations that occur in resin nanoceramic materials following various aging methods. Literature plays a crucial role in the selection of durable CAD/CAM materials by elucidating the impact of aging on surface properties and the strength of repair bonds. Materials exhibiting enhanced resistance to ultraviolet aging or thermal cycling are generally preferred for long-term restorations in high-stress environments. Conversely, certain materials may be more suitable for short-term applications. This knowledge aids clinicians in optimizing treatment outcomes while minimizing the frequency of repairs or replacements.

The objective of this study is to assess the impacts of thermocycling and UV aging at various cycles on the microhardness, surface roughness, and repair bond strength of CAD/CAM resin nanoceramic, in comparison to resin nanoceramic that has not undergone aging. The null hypothesis posits that there will be no significant differences in the microhardness, surface roughness, and repair bond strength values of CAD/CAM resin nanoceramics based on the aging method or cycle duration.

2. Materials and Methods

The experimental procedure employed in the current study is illustrated schematically in Figure 1, and the characteristics of the materials utilized are detailed in Table 1.

Table 1. Materials utilized in the research.

Substance	Category	Components
Cerasmart	Resin nanoceramic CAD/CAM block	Bis-MEPP ¹ , UDMA ² , dimethacrylate, 71% silica (20 nm), barium glass (300 nm)
Ceramic Primer II	Silane	Silane, phosphate monomer, methacrylate, ethanol
G-Premio Bond	Universal adhesive	4-MET ³ , phosphate monomer, thiophosphate monomer, dimethacrylate, acetone, water, photoinitiator
G-aenial Universal Flo	Flowable composite resin	Matrix (31% wt): UDMA ² , Bis-MEPP ¹ , TEGDMA ⁴ Filler (69% wt, 50% vol): Silicon dioxide (16 nm), Strontium glass (200 nm), Pigment Initiator: Photo initiator (trace)

¹ Bis-MEPP: bisphenol-A ethoxylate dimethacrylate. ² UDMA: urethane dimethacrylate. ³ 4-MET: 4-methacryloxyethyl trimellitic acid. ⁴ TEGDMA: triethyleneglycol dimethacrylate.

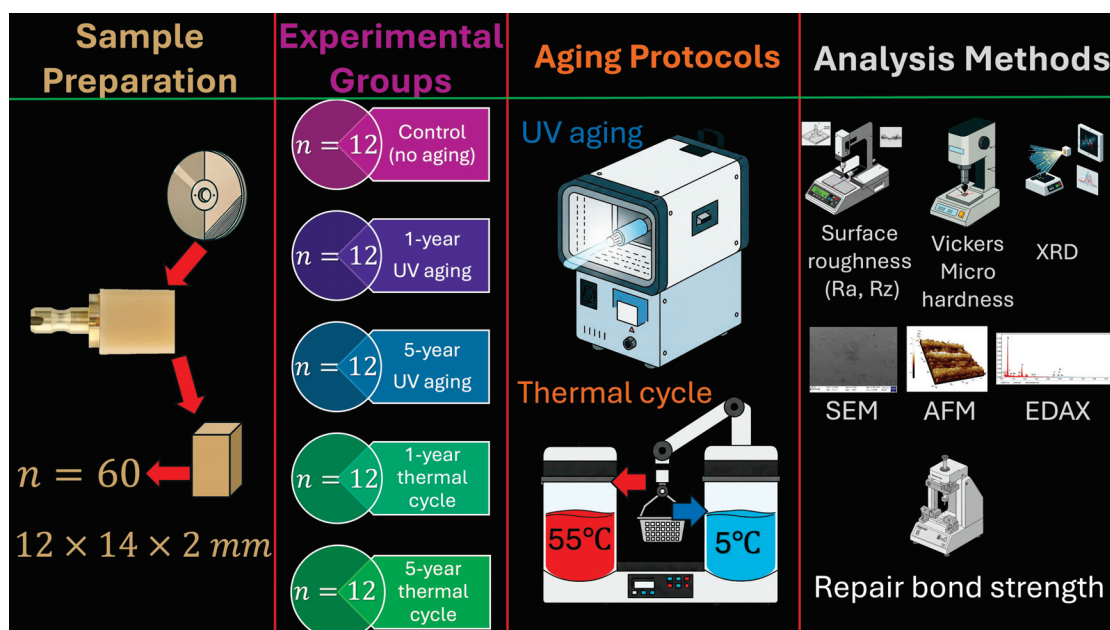


Figure 1. Flowchart of the study.

2.1. Calculation of Sample Size

The sample size for the study was determined using G*Power 3.1 software and was based on data from a previous research study [17]. The analysis, which was conducted using a normal distribution, utilized a significance level of 0.05, an effect size of 0.5, and a power of 0.95. As a result, it was determined that there would be 12 samples in each subgroup, leading to a total sample size of 60.

2.2. Specimen Preparation

Sample preparation was conducted in a climate-controlled laboratory maintained at a temperature of $23 \pm 1^\circ\text{C}$ and a relative humidity of $50 \pm 5\%$ to mitigate environmental variability. Cerasmart block, which is a CAD/CAM resin nanoceramic, was utilized for the study. The blocks were sectioned using a diamond blade attached to a low-speed cutting device (Isomet 5000; Buehler, Lake Bluff, IL, USA) while maintaining continuous water cooling throughout the process. A total of 60 rectangular samples, each with dimensions of $12 \times 14 \times 2 \text{ mm}^3$, were fabricated. The dimensions of all samples were measured with precision using a digital caliper (Absolute Digimatic, Mitutoyo, Kawasaki, Japan), which boasts an accuracy of 0.01 mm. Calibration of the caliper was conducted after every five samples to ensure the integrity of the measurements. Samples that did not conform to the specified size criteria were excluded from the study. The qualified samples were subsequently positioned in a $20 \times 20 \text{ mm}^2$ Teflon mold, which was prepared using a self-polymerizing acrylic resin (SC Acrylic Resin, Imicryl Dental, Konya, Turkey), ensuring that one surface remained fully exposed. To standardize the surfaces and eliminate any potential external scratches or defects, each sample was polished for 30 s with silicon carbide paper of 600, 800, 1000, and 1200 grit, utilizing finger pressure throughout the process. The polished samples were subjected to cleaning in an ultrasonic bath filled with distilled water to ensure the removal of any residual surface contaminants. Subsequently, they were gently dried using a clean paper towel. The dried samples underwent a thorough re-evaluation by two independent researchers, with any samples exhibiting defects being excluded from the study.

The prepared samples were categorized into five subgroups utilizing random numbers generated by a computer program, in accordance with the aging procedure and cycle duration to be implemented ($n = 12$).

2.3. Aging Procedure and Cycle

In the current research, two distinct aging procedures were implemented, accompanied by two varying cycle durations.

1. The samples designated as the control group were not subjected to any aging procedures. They were maintained in a container containing artificial saliva and incubated at 37 °C until the time of measurement ($n = 12$).
2. The samples that were subjected to thermocycling were placed in a thermocycling device (MOD Dental, Ankara, Turkey) containing baths with temperature ranges from 5 °C to 55 °C. The duration of each bath exposure was established at 20 s, while the transfer time between baths was fixed at 2 s [25]. The samples designated for the 1-year aging cycle underwent a testing regimen of 10,000 cycles, whereas the samples allocated for the 5-year aging cycle were subjected to 50,000 cycles, utilizing the same testing parameters.
3. A weathering machine (Ci35 Weather-Ometer; Atlas Electronic Devices Co., Illinois, USA) was used for the UV aging procedure. The specimens were subjected to a controlled exposure using a xenon arc lamp, filtered through borate borosilicate glass, at an intensity of 0.55 W/m²/nm, specifically measured at a wavelength of 340 nm. Aging was carried out based on the parameters established by Silva et al. [26] to replicate one year of clinical use. The conditions included a dry bulb temperature of 47 °C for light exposure and 38 °C for dark conditions, humidity levels of 50% in light and 95% in dark, a black panel temperature of 70 °C for light and 38 °C for dark, and a water temperature maintained at 50 °C. Each test cycle was designed to consist of a standardized duration of 40 min of light exposure, followed by 20 min of combined light exposure and front water spray. This was succeeded by an additional 60 min of light exposure and concluded with 60 min of darkness, accompanied by backwater spray. Due to these cycles, samples that received a total exposure energy of 150 kJ/m² and a cumulative exposure time of 300 h were regarded as having aged for one year. In contrast, the exposure time was established at 1500 h for the five-year aging process. Throughout the aging processes, careful attention was devoted to monitoring the environmental conditions utilizing calibrated sensors.

2.4. Surface Roughness Measurement

The average surface roughness (R_a) and the difference between the highest peak and the deepest valley on each specimen's surface (R_z) were assessed following the aging procedures utilizing a two-dimensional profilometer (Surftest 402, Mitutoyo, Tokyo, Japan). This measurement was conducted with a 5- μ m diamond stylus positioned at an angle of 90 degrees. Three measurements were conducted for each specimen by rotating the specimen by 60 degrees. The tracing length was set at 3 mm, the cutoff length (λC) was specified as 0.25 mm, and the stylus speed was maintained at 0.25 mm/s. The average roughness value for each specimen was calculated and documented in micrometers. The calibration of the profilometer was periodically verified following the assessment of three specimens using a standard calibration apparatus.

2.5. Surface Microhardness Measurement

Following the aging procedures, the hardness values of the samples were assessed using a Vickers Microhardness Tester (HMT-2T; Shimadzu, Kyoto, Japan). Each sample

surface underwent three separate indentations, applied with a load of 9.807 N and a dwell time of 20 s, ensuring that the measurement areas were closely localized. The readings were obtained under $\times 40$ magnification. The average of the three measurements was then calculated and recorded for each respective sample. Furthermore, the calibration of the microhardness tester was rechecked after performing nine measurements on each of the three samples using a standard pretested apparatus.

2.6. X-Ray Diffraction (XRD) Analysis

The effects of aging conditions and applied cycle time on the crystal phases of the samples were assessed using X-ray diffraction (XRD) analysis. For this analysis, an additional sample was prepared for each subgroup, and prior to aging, the samples were polished with 15- μm diamond lapping film to achieve a mirror-like surface, which is crucial for accurate measurement. The surfaces of the aged samples were then scanned for 3 s in continuous mode using a Cu $K\alpha$ tube X-ray diffractometer (Rigaku Ultima IV Multifunctional, Tokyo, Japan). The scan range was set from 5 to 90° (2θ) with X-ray current and excitation voltage maintained at 40 kV and 30 mA, respectively, a step width of 0.01° , and a scanning rate of $1.0^\circ/\text{min}$ ($2\theta/\text{seg}$).

2.7. Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), and Chemical Elemental Analysis

Following the evaluation of surface properties, three samples were randomly selected from each subgroup for analysis utilizing scanning electron microscopy (SEM) and atomic force microscopy (AFM). AFM images were acquired in contact mode, generating three-dimensional (3D) representations with a resolution of 512×512 pixels. These images were produced by scanning areas measuring $20 \times 20 \mu\text{m}$ at a scanning speed of 1 Hz, employing an atomic force microscope (XE-100 E, Park Systems, Suwon, Republic of Korea). Nanosensors PPP-CONTSCR 10M (Park Systems), with a nominal force constant of 0.2 N/m and a resonance frequency of 23 kHz, were employed to image the specimens. The cantilever measured 225 μm in length, 1 μm in thickness, and had an average width of 48 μm . A reflective aluminum coating was applied to the backside to facilitate operation in contact mode. The atomic force microscope underwent calibration through the acquisition of measurements in a three-dimensional view of a reference sample, utilizing software calibration that involved imaging a standard sample. For the purpose of analyzing surface topography based on AFM images, the XEP Data Analysis Program, version 1.8.0, Build 48 (Park Systems), was employed. Subsequent to the completion of AFM analysis, SEM was conducted on the samples. Given the non-conductive nature of the samples, their surfaces were coated with a layer of gold and palladium using a Polaron Sputter Coater (model number SC7620, VG Microtech, West Sussex, England) for a duration of 60 s. Following this process, the samples were placed within the SEM apparatus (Gemini model SEM500, Carl Zeiss, Oberkochen, Germany), where SEM images were captured of the most representative areas at an original magnification of $\times 1500$ and $\times 500$. The analysis was further enhanced through the use of Energy Dispersive X-ray Spectroscopy (EDS), utilizing the Noran Voyager III system (document number M3100, Noran Instruments, Middleton, WI, USA), which was integrated into the SEM equipment.

2.8. Repair Bond Strength (SBS) Test

Following the measurement and analysis of the surface characteristics of the aged samples, the repair procedure commenced. A 2 mm thick green belt diamond fissure bur (837L-014 GL, Bosphorus, Istanbul, Turkey) was employed in accordance with the manufacturer's recommendations. The grinding process was conducted under water cooling at a speed of 40,000 rpm. Throughout the procedure, the bur was maintained

parallel to the sample surface. It was applied to the sample surface five times with minimal pressure, and the bur was replaced after every five samples to ensure optimal performance. Upon the completion of the bur-grinding process, the samples were subjected to a cleaning procedure lasting five minutes within an ultrasonic bath. This step was followed by a careful drying process utilizing a paper towel. Ceramic Primer II was applied in a uniform, thin layer to the surface of the sample and subsequently dried using an air spray technique. The application of a universal adhesive, specifically G-Premio Bond, was performed on the surface utilizing an applicator. The adhesive was permitted to set for 10 s and was subsequently dried using maximum air pressure for an additional 5 s. Following this process, the samples were subjected to visible blue light exposure with a wavelength range from 430 to 480 nm and a light intensity of 1400 mW/cm². This was achieved utilizing an LED light device (D-Light Pro, GC Corporation, Tokyo, Japan), positioned 10 mm from the samples for a duration of 10 s. To ensure the accuracy of the LED light device's intensity, calibration was performed by assessing one out of every five samples, with the assistance of a radiometer (LED Radiometer, SDI Dental Limited, Melbourne, Australia). A flowable composite (G-aenial Universal Flo, GC Corporation, Tokyo, Japan) utilizing the same flexible nanoceramic technology as Cerasmart was selected as the repair material. The composite was applied using a 2 mm thick mold placed over the sample surface and was cured according to the standards outlined in ISO 4049 [27]. The repaired samples were stored in distilled water at 37 °C for a duration of 24 h. The shear bond strength (SBS) test was performed using a universal testing machine (Trapezium X, Shimadzu Corp., Tokyo, Japan) with a customized steel jig. The jig featured a 2 mm diameter chisel-shaped loading head positioned 0.5 mm from the composite CAD/CAM resin nanoceramic interface to ensure uniform shear force distribution. Samples were secured in a rigid metal holder with cyanoacrylate adhesive to prevent movement during testing. Shear force at a cross-head speed of 0.5 mm/min until fracture occurred. The resulting data were recorded in megapascals (MPa). Following fracture, the samples were examined under a light microscope (TM-505, Mitutoyo, Tokyo, Japan), and the fracture patterns were categorized as adhesive, cohesive, or mixed.

2.9. Statistical Analysis

Data analysis was conducted using IBM SPSS V23. The Shapiro–Wilk test was employed to assess the appropriateness of the normal distribution assumption. The effects of the aging procedure and cycle time on microhardness, repair bond strength, and surface roughness values were evaluated using a two-way MANOVA. For data that did not conform to a normal distribution across three or more groups, the Kruskal–Wallis test was applied. One-way analysis of variance (ANOVA) was utilized for normally distributed data across three or more groups, with multiple comparisons carried out using Tukey's HSD and Tamhane's T2 tests. The Chi-square test was implemented to compare failure types between groups, and Spearman's rho correlation coefficient was used to explore the relationship between quantitative parameters that were not normally distributed. The analysis results were presented as the mean $\pm$ standard deviation for quantitative data and as frequency (percentage) for categorical data. A significance level of $p < 0.050$ was established for the evaluation of statistical significance.

3. Results

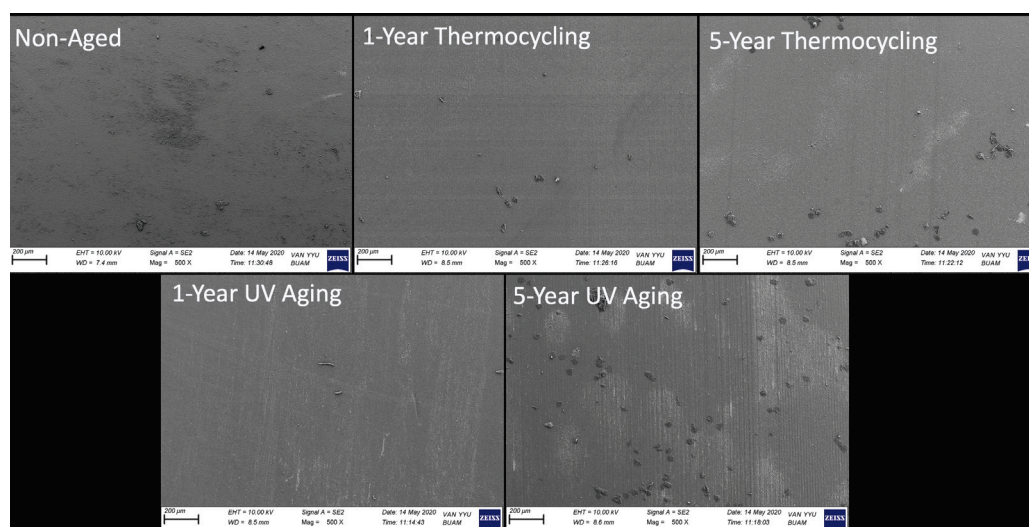
Ra, Rz, microhardness, and SBS values for the samples from both the aged and non-aged (control) groups are summarized as means, standard deviations (SDs), and medians in Table 2.

Table 2. Mean, median, and statistical comparison of Ra, Rz, microhardness, and SBS in aged and non-aged samples.

		5-Year UV Aging	1-Year UV Aging	5-Year Thermo-cycling	1-Year Thermo-cycling	Non-Aged (Control)	<i>p</i>
SBS	Mean $\pm$ SD	63.13 $\pm$ 1.24 ^a	68.12 $\pm$ 0.85 ^b	68.18 $\pm$ 0.95 ^b	71.31 $\pm$ 0.77 ^c	75.63 $\pm$ 1.45 ^d	<0.001 ¹
	Median	63.45 (60.60–64.40)	68.30 (66.50–69.20)	68.35 (67.00–70.10)	71.05 (70.50–72.80)	75.35 (73.90–78.60)	
	Mean $\pm$ SD	6.61 $\pm$ 0.67	8.64 $\pm$ 0.48	10.62 $\pm$ 2.04	11.32 $\pm$ 0.63	11.54 $\pm$ 0.74	<0.001 ²
	Median	6.40 (6.00–7.70) ^b	8.65 (8.10–9.50) ^b	9.75 (9.10–15.80) ^a	11.35 (10.30–12.40) ^a	11.75 (10.30–12.70) ^a	
Ra	Mean $\pm$ SD	61.77 $\pm$ 0.24	40.79 $\pm$ 0.34	37.45 $\pm$ 0.34	36.08 $\pm$ 0.27	0.05 $\pm$ 0.02	<0.001 ²
	Median	61.79 (61.34–62.10) ^d	40.84 (40.12–41.23) ^{cd}	37.38 (37.00–38.00) ^{bc}	36.00 (35.67–36.81) ^{ab}	0.04 (0.02–0.10) ^a	
Rz	Mean $\pm$ SD	271.57 $\pm$ 4.02 ^a	213.90 $\pm$ 3.79 ^b	200.61 $\pm$ 6.22 ^c	187.91 $\pm$ 3.85 ^d	0.22 $\pm$ 0.06 ^e	<0.001 ¹
	Median	271.26 (265.43–281.22)	213.62 (208.77–219.28)	200.03 (189.89–215.81)	188.47 (180.45–193.47)	0.22 (0.14–0.31)	

¹ One-way analysis of variance test statistic, ² Kruskal Wallis test statistics, ^{a–e}: There is no difference between groups with the same letter.

The results from the two-way MANOVA indicated that the aging procedure, cycle duration, and interaction between these two factors had statistically significant effects on the Ra and Rz values of the samples ($p < 0.001$, $F = 27,665.9$; $p < 0.001$, $F = 16,398.5$; $p < 0.001$, $F = 12,616.8$, respectively). The highest Ra value recorded among the groups was 61.77 μm , associated with samples that underwent a 5-year UV aging cycle. This was followed by samples aged for 1 year under UV aging (40.79 μm), 5-year thermocycling (37.45 μm), 1-year thermocycling (36.08 μm), and samples from the control group (0.05 μm), respectively. The trend observed in the Ra values of the samples was similarly noted in the Rz values, with the most pronounced roughness structure measured at 271.57 μm in samples subjected to a 5-year UV aging cycle. As anticipated, the control group exhibited the lowest Rz value at just 0.22 μm . SEM microphotographs taken post-aging procedures, which support the surface roughness measurements, are presented in Figures 2 and 3.

**Figure 2.** SEM image (500 $\times$) of the surface of the CAD/CAM material after aging procedures.

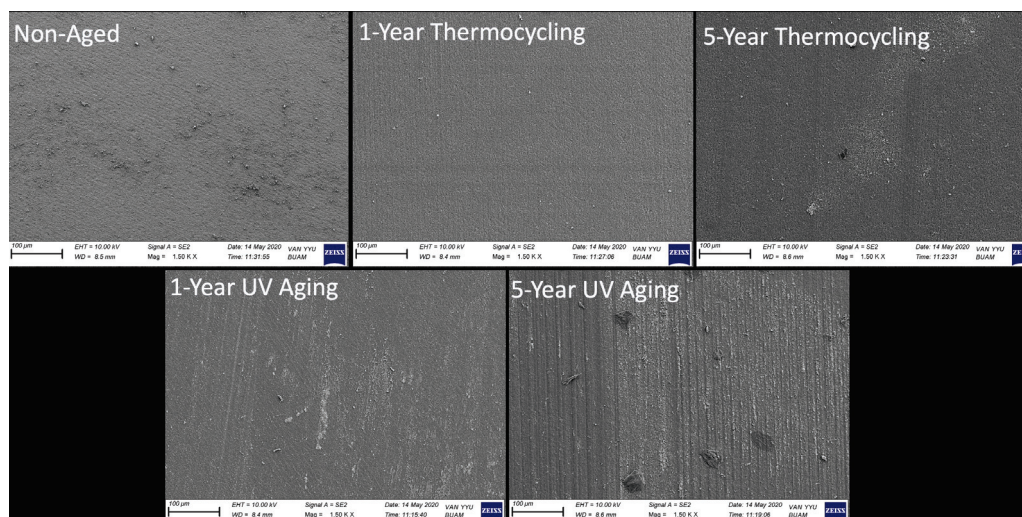


Figure 3. SEM image (1500 $\times$) of the surface of the CAD/CAM material after aging procedures.

As observed in both Figures 2 and 3 at higher magnification, the surfaces of the non-aged control group samples exhibit a relatively uniform structure, though not entirely free of minor irregularities. In contrast, a damage and wear pattern linked to the effects of the procedure was observed on the surfaces of samples subjected to both thermocycling and UV aging. It can be concluded that as the cycle time of the procedure increased, the wear patterns resulting from the damage became more pronounced, with a corresponding increase in crevice depths.

The EDAX analysis identified the presence of carbon (C), oxygen (O), silicon (Si), barium (Ba), and aluminum (Al) elements across all groups. The results indicated that the wear occurred at the matrix level, which was evidenced by the increase in measurable inorganic filler ratios following the aging procedures. In the control group, the Si ratio measured by weight was determined to be 15.02%, while the Ba ratio was 3.83%. In contrast, these ratios increased significantly to 26.83% and 34.88%, respectively, in the samples subjected to five years of ultraviolet (UV) aging. In the samples subjected to a five-year thermocycling, the weight percentages of Si and Ba were constrained to 26.61% and 31.05%, respectively (Figure 4).

The images captured during the AFM analysis correspond closely with the SEM images, as well as the Ra and Rz measurements. Notably, the control group displayed the smoothest and most uniform microphotographs among all groups. In contrast, surfaces that underwent the aging procedure were found to exhibit irregular topography, characterized by cracks and pronounced protrusions. The observation of deep slits aligned with the trajectory of the wear pattern, as evidenced by SEM images following five years of UV aging, constitutes significant data (Figure 5).

Upon examining the microhardness data, a noteworthy finding emerged: the measurements demonstrated a significant decrease in response to the aging procedure, the cycle, and their interaction ($p < 0.001$, $F = 217.3$; $p < 0.001$, $F = 211.2$; $p = 0.002$, $F = 11.0$, respectively). The control group recorded the highest microhardness value at 75.63 VHN, while the samples subjected to 5 years of thermocycling and 1 year of UV aging exhibited statistically similar microhardness values ($p > 0.05$) (Table 2). Furthermore, Spearman's rho correlation coefficient indicates a statistically significant negative correlation between the microhardness values of the samples and the Ra and Rz ($p < 0.001$; $r = -0.918$; $p < 0.001$; $r = -0.899$, respectively) (Table 3).

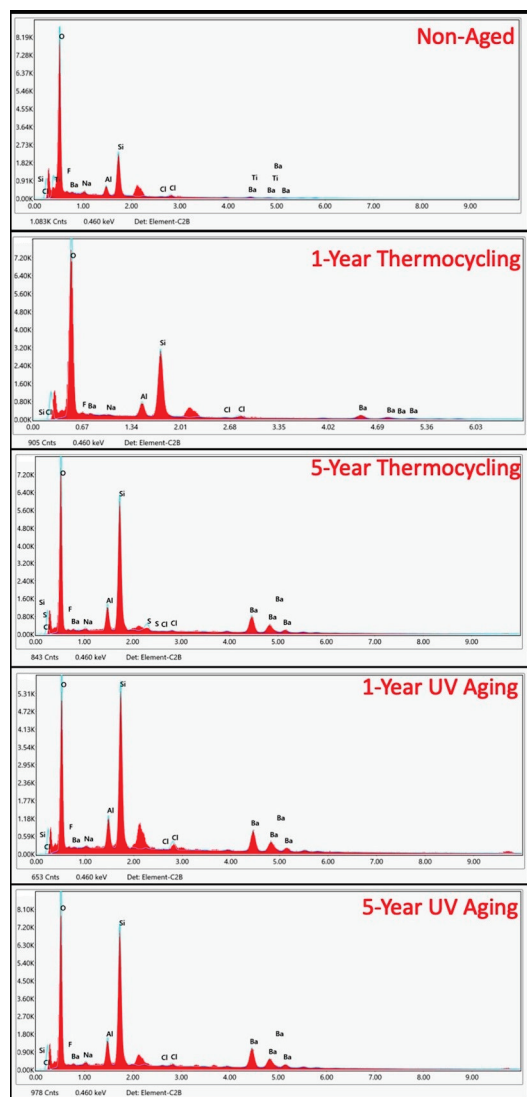


Figure 4. Chemical analysis of the surface of the CAD/CAM material after aging procedures.

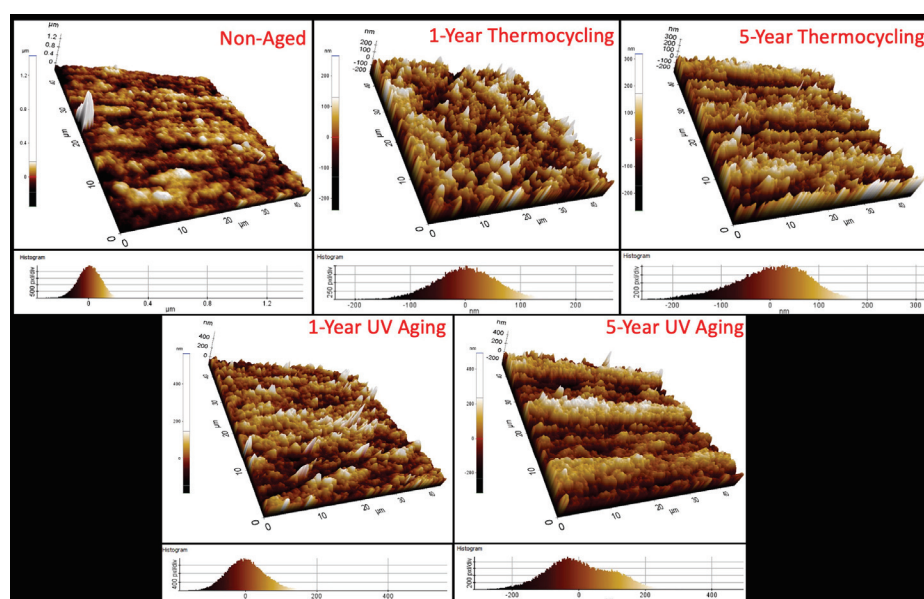


Figure 5. AFM images of the surface of the CAD/CAM material after aging procedures.

Table 3. Correlation analysis results.

		Microhardness	SBS	Ra
SBS	<i>r</i>	0.796		
	<i>p</i>	<0.001		
Ra	<i>r</i>	−0.918	−0.849	
	<i>p</i>	<0.001	<0.001	
Rz	<i>r</i>	−0.899	−0.852	0.954
	<i>p</i>	<0.001	<0.001	<0.001

r: Spearman's rho correlation coefficient.

Additionally, the XRD analysis showed that the samples exhibited a broad peak around 20°, with all samples displaying a similar amorphous phase (Figure 6). This suggests that the effects of aging are not sufficiently pronounced to induce a phase change.

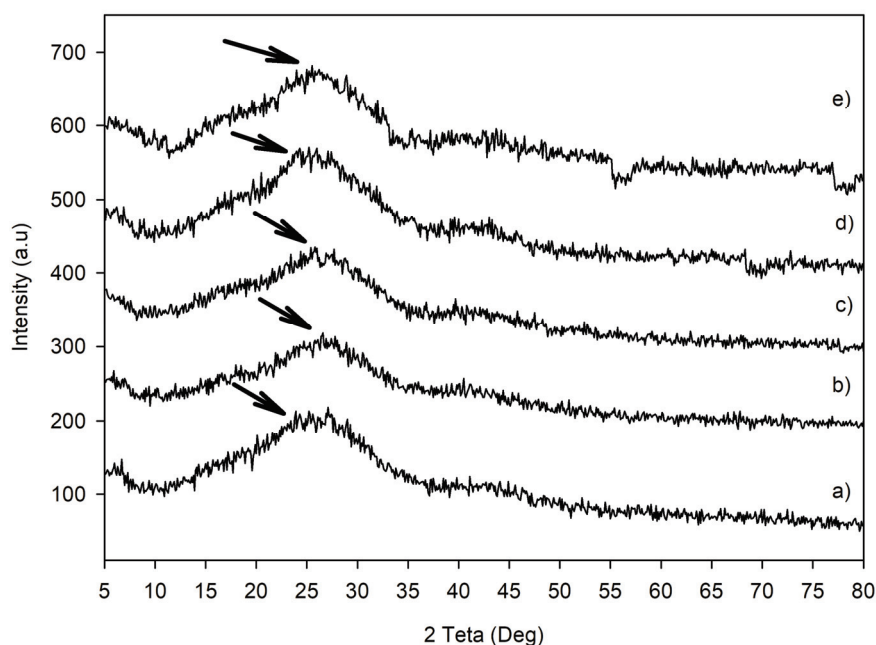


Figure 6. XRD analysis of the surface of the CAD/CAM material after aging procedures: (a) non-aged; (b) 1-year thermocycling; (c) 5-year thermocycling; (d) 1-year UV aging; (e) 5-year UV aging. Black arrows indicate the characteristic peaks of the surfaces.

The SBS values exhibited significant variations based on the preferred aging procedure and cycle ($p < 0.001$; $F = 102.2$; $p < 0.001$; $F = 17.1$, respectively). Notably, the highest SBS value was recorded in the control group, followed by samples subjected to 1-year thermocycling, 5-year thermocycling, 1-year UV aging, and, finally, 5-year UV aging (Table 2).

The failure types of the repaired samples are presented in Table 4. Adhesive failure was the most prevalent, occurring at a rate of 48.3%. Cohesive failure was predominantly observed in samples that underwent 5-year UV aging, with a rate of 75%. In contrast, the mixed failure type was observed infrequently.

Table 4. Distribution of failure modes.

Failure Mode	5-Year UV Aging	1-Year UV Aging	5-Year Thermocycling	1-Year Thermocycling	Non-Aged (Control)	Total	<i>p</i> *
Adhesive	2 (16.7%) ^a	4 (33.3%) ^{ab}	5 (41.7%) ^{ab}	8 (66.7%) ^{ab}	10 (83.3%) ^b	29 (48.3%)	0.01
Cohesive	9 (75%) ^a	7 (58.3%) ^{ab}	5 (41.7%) ^{ab}	1 (8.3%) ^b	1 (8.3%) ^b	23 (38.3%)	
Mix	1 (8.3%)	1 (8.3%)	2 (16.7%)	3 (25%)	1 (8.3%)	8 (13.3%)	

* Chi-square test, ^{a,b}: There is no difference between groups with the same letter.

4. Discussion

Although resin nanoceramic restorations have shown promising mechanical properties *in vitro*, their translation to clinical performance is somewhat limited. The effectiveness of these materials in a clinical context is closely linked to their chemical stability in the oral cavity [28]. To mitigate the risk of catastrophic failures during the use of restorations, it is imperative to thoroughly elucidate the mechanical and chemical degradation processes of the material through an appropriate aging methodology [29]. The findings from the current study indicate that the aging process and the duration of the applied cycle significantly influenced the microhardness, surface roughness, and repair bond strength of the samples ($p < 0.001$). Consequently, the study's null hypothesis was rejected.

In the review of dental literature pertaining to the aging applications of materials, it is important to note that the procedures of soaking in water, exposure to acidic environments, thermocycling, and boiling in distilled water are frequently utilized [30]. All of these methodologies share a common characteristic: they replicate an accelerated aging process by generating a synergistic effect resulting from hydrolytic and thermal degradation [31]. While a consensus among researchers is lacking, certain studies indicate that various methods yield comparable effects on the mechanical properties of hybrid CAD/CAM blocks that incorporate resin [10,19]. Bessa et al. reported that varying water temperatures in the thermocycling method induced repetitive cycles of contraction and expansion stress in the material, thereby facilitating a more practical and effective aging process [32]. Given the gathered information, thermocycling was selected as one of the preferred methods in the present study. However, Oliveira et al. conducted a comparative analysis of the effects of water aging and ultraviolet (UV) aging on resin-based dental materials. Their findings indicated that the UV aging procedure led to intermittent water condensation. Consequently, they underscored that the refractive index of the components comprising the polymeric matrix of resin materials was altered, resulting in a more significant color change occurring in a substantially shorter duration when compared to water aging [33]. The current research team recognized the significance of elucidating the effects of UV aging, a contemporary approach that serves to inform the experimental protocols of researchers. Consequently, this aspect was regarded as essential and subsequently incorporated into the current study.

Surface roughness is a critical characteristic influencing the mechanical properties of polymer-based dental materials. It serves as a vital parameter in the assessment of surface properties [34]. Ardu et al. highlighted that surface roughness serves as a critical indicator of the aesthetic stability and durability of restorations [35]. In the current study, surface roughness measurements were conducted to elucidate the mechanical properties of CAD/CAM resin nanoceramic in relation to aging and repair bond strength. The results indicated a significant increase in both Ra and Rz parameters following the aging procedures ($p < 0.001$). Furthermore, a positive correlation was observed between the measured values and the increase in cycle time. Kim et al. reported that the aging process markedly increased the surface roughness of all materials evaluated in their study. This research

focused on assessing the surface properties and mechanical strength of five distinct dental CAD/CAM restorative materials following exposure to thermocycling and mechanical loading. It was highlighted that the highest surface roughness values (S_a and S_q) among the materials tested were observed in the Cerasmart group. Furthermore, the researchers indicated the presence of localized surface uplift in the AFM images following an aging process, which corroborated the roughness data. Additionally, significant alterations in the microstructure of Cerasmart were noted in SEM images [34]. The findings presented herein align closely with our current research outcomes, both qualitatively and quantitatively. An additional *in vitro* study that examined the effects of various aging protocols on CAD/CAM resin nanoceramics indicated a significant increase in surface roughness compared to the control group, regardless of the alterations made to the aging procedure. Notably, the researchers reported that the R_a value recorded after a 3-month water soaking application, which was preferred as the aging procedure, measured 76.8 nm. This value increased to 88.1 nm after 9 months, highlighting a positive correlation between the R_a value and the duration of exposure. Furthermore, an important finding from the study reveals that the R_a value observed in samples subjected to a 1-year thermocycling application was limited to 69.8 nm, a figure that was statistically comparable to the surface roughness of the control group. This finding suggests that the effects observed will vary depending on the specific aging procedure employed [31]. The findings of the current study substantiate the researchers' observations, revealing that the R_a value of 37.45 μm , measured after five years of thermocycling, is statistically comparable to the R_a value of 40.79 μm observed in samples subjected to one year of UV aging. This phenomenon may be attributed to the underlying mechanisms associated with aging processes. It is well-established that the temperature fluctuations experienced during thermal cycling induce thermal fatigue in restorative materials when exposed to humid conditions. Heat transfer occurs through convection, leading to the formation of thermal boundary layers and uneven temperature distributions. These variations can induce thermal stresses due to differing thermal expansion coefficients or non-uniform material properties. In multi-phase materials such as composites, the expansion behaviors of filler particles can differ, resulting in additional internal stresses, particularly at the matrix–filler interface. Such processes contribute to the deterioration of the internal integrity of the materials. Cyclic temperature fluctuations primarily impact the surface layer, where stress gradients are most pronounced, thus heightening the risk of degradation. This leads to an enhancement in surface roughness [36]. In the UV aging procedure, alongside the thermal effects observed during thermocycling, there exists an interaction with unreacted amines present within the polymer matrix. These unreacted amines have the capacity to absorb UV light, subsequently leading to the formation of molecules that possess elevated energy states [3]. The observed increase in surface roughness during ultraviolet aging may primarily account for the more significant alterations noted. Existing literature indicates that aging processes typically result in an increase in surface roughness; however, these enhancements are generally deemed clinically acceptable, as they remain below the threshold of 0.2 μm , which is critical for *in vivo* bacterial adhesion [19,37]. In the present study, the R_a values measured following the aging process are significantly elevated beyond the established threshold. This variation in findings may be attributed to the differing aging procedures employed in the methodologies of the respective studies, as well as the fact that the cycle durations were restricted to 28 days and 3 months.

Microhardness serves as a relative indicator of a material's resistance to external indentation forces and is recognized as a predictor of the strength of restorations in clinical applications [37]. In the present study, the inclusion of microhardness measurement in the methodology was deemed essential for predicting the clinical applicability of the restora-

tion, detailing the effects of the aging procedures employed, and accurately correlating the results. A previous research assessed the mechanical properties of four distinct CAD/CAM resin nanoceramics, including Cerasmart, following various aging procedures. The findings indicated a significant reduction in microhardness across all experimental groups, with Cerasmart exhibiting the lowest microhardness values following each procedure when compared to the other materials evaluated. Among the findings reported, it is noteworthy that the microhardness value within the Cerasmart group decreased from 64.1 VHN to 59.0 VHN after merely 7 days of immersion in water. Additionally, following the thermocycling procedure, the microhardness further declined to 58.0 VHN. The researchers elucidated that the aging processes typically result in a downward trend in microhardness values. However, the variations in these decreasing values, contingent upon the specific aging procedure employed, may reflect the technical differences inherent in the respective methodologies [10]. In a similar vein, Kim et al. conducted a comprehensive study on the interaction of Cerasmart with water soaking, thermocycling, and mechanical loading procedures. Their results revealed that the lowest microhardness value was observed in the Cerasmart group following all aging procedures. The researchers noted a statistically significant decrease in the microhardness across the sample groups post-aging, which they considered to be a predictable outcome. They emphasized that the significant differences identified among the various aging procedures provided valuable insights for in vitro studies. Furthermore, the study highlighted an increase in the measured rates of the elements O, C, Si, and Ba following aging, which the researchers argued could be correlated with the observed loss of microhardness [34]. The findings of the current study align closely with both the observed reduction in microhardness following aging and the results obtained from the EDAX analysis. The decrease in microhardness identified in Cerasmart is likely attributable to water absorption. Given that all aging procedures employed in this investigation involve a humid environment, it is well established that water absorption leads to swelling within the resin matrix network, subsequently resulting in a reduction in frictional forces among the polymer chains [28]. The process initiates hydrolytic degradation of ester, urethane, amide, and siloxane bonds within the structure of resin nanoceramics. This degradation may lead not only to a reduction in hardness but also to the formation of additional voids on the surface [38]. This situation elucidates the observed increase in the concentrations of O, C, Si, and Ba elements on the surface, as well as the corresponding decrease in microhardness. Nevertheless, there are numerous studies in the literature that indicate that, despite the absorption of water by Cerasmart after aging, a reduction in microhardness was not detected. According to the researchers, the principal explanation for this observation is that the Bis-MEPP and DMA monomers present in Cerasmart mitigate the risk of cross-linking and monomer conversion, thereby enhancing resistance to hydrolytic degradation [28,39]. The divergence in findings may be attributed to the fact that the preferred aging procedure utilized in one study involved dry storage, whereas the thermocycling regimen employed in the other was comparatively short-term.

Restoration fractures may occur as a result of sudden trauma in the short term, or they may manifest after prolonged fatigue associated with masticatory functions over time [40]. The prevailing consensus in the literature indicates that repair should be regarded as a preferred alternative to restoration or replacement for suitable defects. This approach is not only cost-effective but also simplifies the overall process [41]. Upon reviewing the existing studies that assess the efficacy of repair procedures, it becomes evident that the literature predominantly emphasizes surface treatments designed to enhance repair bond strength. Furthermore, there is a paucity of research investigating the variations in repair success rates between aged and non-aged restorations [42]. The current researchers have not identified any literature that evaluates the repair bond strength in samples subjected

to different aging methods. Addressing this gap, the present study assessed the repair bond strength in both aged samples, treated through various procedures, and unaged samples. Bessa et al. similarly investigated the impact of aging on the repair bond strength of CAD/CAM monolithic materials, centering their research on a comparable theme. Their findings indicated that the repair bond strengths of aged samples were consistently lower, independent of the surface cleaning method employed or the surface treatment administered. Notably, the SBS recorded in the analyzed aged samples was 7.70 MPa; this value was statistically comparable to the 6.62 MPa observed in the unaged control group. The researchers indicated that they observed alterations in the Weibull modulus and elevated characteristic strength values of the material following the application of thermocycling, which simulated a duration equivalent to one year of clinical use. They noted that the significant reduction in repair bond strength could be attributed to changes in the material's properties resulting from aging. The study indicated that a mixed mode of failure was frequently identified through the fractographic analysis of the results obtained. This observation was interpreted as a reflection of the repair process in relation to the adhesive procedure, which does not preclude its clinical application [32]. In a recent investigation that included CAD/CAM resin nanoceramic material in the sample group, the repair bond strength of both non-aged and in situ aged samples was assessed. The findings indicated that aging led to a significant reduction in the SBS values, which was attributed to an increase in surface roughness resulting from the aging process and a corresponding rise in the concentration of inorganic fillers as revealed through an EDAX analysis. Furthermore, it was observed that the SBS values achieved through various surface treatments in unaged samples could be elevated to as much as 14.27 MPa; however, the values recorded following aging exhibited a dramatic decline, decreasing to as low as 0.17 MPa. Failure analysis demonstrated a mixed failure rate of 74% [43]. The results of the current study align with the established understanding that aging leads to a significant reduction in SBS values. As presented in Table 2, the repair bond strength values range from 11.54 MPa to 6.61 MPa. The observed variability in the data may be attributable to the classification of the samples as resin nanoceramics. However, these samples possess differing compositions in commercial production, which may result in varying effects of aging. Additionally, a notable distinction in this study is the predominance of adhesive failure as the most frequently observed mode. This phenomenon may be linked to the absence of various surface treatments in the methodology employed, which, subsequently, did not yield an enhancement in repair bond strength. It is widely acknowledged in the literature that adhesive failure is indicative of reduced SBS, while cohesive and mixed failures are suggestive of increased SBS [17].

Every *in vitro* study inherently possesses certain limitations. The most significant limitation of the current research is the reliance on a single resin nanoceramic, with the samples not being produced in an anatomically relevant form and without the use of a CAD/CAM system. Furthermore, as the primary objective of the current study is to investigate the fundamental effects of aging processes on materials in isolation, the replication of complex biomechanical stresses typical of the oral environment would considerably complicate the experimental design and impede the interpretation of the results. Consequently, masticatory forces were not simulated in this study. Although artificial aging methods were effectively employed to approximate intraoral conditions, the impacts of saliva—along with its constituent enzymes, ions, proteins, and even microbiota—were not taken into account. The influence of salivary enzymes and oral biofilms on the acceleration of resin nanoceramic degradation has yet to be thoroughly investigated and warrants focused research attention. Consequently, it is essential that the current results are contextualized with comparisons to both *in vitro* studies and clinical data pertaining to *in situ* aging.

5. Conclusions

In light of the constraints inherent to the current in vitro study, the following conclusions may be formulated:

1. The aging procedures and applied cycle time exert a significant influence on the surface properties and repair bond strength of CAD/CAM resin nanoceramics ($p < 0.001$).
2. An increase in surface roughness, accompanied by a decrease in microhardness and repair bond strength, was observed in aged CAD/CAM resin nanoceramic samples ($p < 0.001$).
3. Samples that underwent a 5-year UV aging process demonstrated the highest surface roughness, accompanied by the lowest values in microhardness and repair bond strength.

Author Contributions: Conceptualization, B.U.D. and A.D.; methodology, Z.S.P. and B.U.D.; software, A.D.; validation, B.U.D. and A.D.; formal analysis, A.D.; investigation, B.U.D.; resources, A.D.; data curation, B.U.D.; writing—original draft preparation, A.D. and B.U.D.; writing—review and editing, Z.S.P. and B.U.D.; visualization, A.D.; supervision, Z.S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Acknowledgments: The authors acknowledge Yüksel Akinay for the analysis of SEM images and Naci Murat for statistical analysis. The article must contain the following statement fully acknowledging the original conference poster. This article is a revised and expanded version of a poster entitled Effects of Thermal Cycle and UV Aging On Mechanical Properties And Repair Bond Strength of CAD/CAM Resin Nano-Ceramic, which was presented at RDD Kış Sempozyumu ve Ana Bilim Dalları Toplantısı, Adana, Türkiye, 17–18 December 2022, where it was awarded second prize for the best research project.

Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Characteristics of Occlusal Force and Masticatory Performance in Female Patients Who Selected Implant Treatment for a Missing Mandibular Second Molar: A Retrospective Study

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Abstract: Background: In this study, we aimed to investigate the association between a patient's selection of implant treatment for a missing mandibular second molar and the magnitude of occlusal force, masticatory ability, mandibular morphology, and age before treatment intervention. Materials and Methods: We retrospectively assessed occlusal force, masticatory performance, and mandibular morphology in female patients who either selected or declined implant treatment for a missing unilateral mandibular second molar. Results: Thirty-three women (mean age of 56.1 ± 9.7 years) were divided into an implant treatment (IT) group and a no-treatment (NT) group. The IT group showed significantly higher occlusal force ($p = 0.021 < 0.05$), while masticatory performance and gonial angle demonstrated no significant difference. Conclusion: The IT group had significantly higher occlusal force, and age had no significant effect on it. Notably, masticatory performance in the IT group increased significantly with age ($p = 0.047 < 0.05$).

Keywords: dental implant; second molar; occlusal force; masticatory performance; single implant

1. Introduction

Implant-prosthetic treatment for missing teeth has been established as a treatment capable of restoring high functionality and esthetics, and many studies have reported its high long-term predictability [1–3]. Additionally, alveolar bone resorption due to periodontitis and apical periodontitis often causes tooth extraction. Through the use of horizontal or vertical bone augmentation, maxillary sinus augmentation using guided bone regeneration, and short implants, the potential for implant treatment is expanding, even in the molar area. This region is susceptible to bone volume deficiency due to issues related to the anatomical morphology of the mandibular canal and maxillary sinus. Additionally, a systematic study revealed that the implant insertion process has been made simpler by alveolar ridge preservation, a technique that was recently developed to retain the alveolar crest in extraction sockets [4–7].

Particularly, implant-prosthetic treatment intervention for missing molar free-end cases is more effective than conventional removable partial dentures in terms of favorable restoration of the occlusal support area, the acquisition of occlusal force that is equivalent to that of natural teeth, a reduction in the burden on the remaining teeth, and lower caries

risk. Moreover, numerous studies along with systematic reviews have demonstrated that it results in high patient satisfaction and improves oral-related quality of life (QOL) [8,9].

Conversely, many studies have reported complications caused by implant treatment, including biological complications, mainly due to bacterial infection, such as peri-implant inflammation and the failure of osseointegration, as well as mechanical complications, including the fracture of prosthetic devices due to excessive occlusal force, the loosening or fracture of screws, and the fracture of implant bodies [2,10–13]. Particularly, implant treatment for mandibular molars reportedly has a higher risk of causing biological and mechanical complications compared to those for other areas. The oral vestibule can become narrow due to a lack of keratinized mucosa, and plaque control is difficult as patients wear a prosthetic device that is difficult to clean, with excessive occlusal force applied at the same time [14,15]. Specifically, because it is a single crown, implant treatment for a single missing mandibular second molar is vulnerable to the fracture of the implant body and superstructure, screw loosening, and loss of osseointegration due to occlusal pressures and bruxism, among other factors.

Because of these risks, prosthetic treatment in the molar area, especially for the second molar, is avoided in some cases. Prosthetic treatment up to the first molar area has reportedly yielded satisfactory outcomes in terms of functionality and oral-related QOL [16–19]. Therefore, although implant treatment is now recognized as an option for prosthetic treatment for missing teeth, the second molar is the most distal area, and there is no established theory regarding whether prosthetic intervention should be provided to this region, which has a high risk of developing complications. Particularly for implant treatment for missing second molars, while it is difficult for dentists to judge whether they should provide the treatment intervention, they often encounter patients who desire implant treatment due to the chief complaint of masticatory difficulty.

In this study, we aimed to clarify the clinical characteristics of patients who request implant treatment for a missing second molar as a first step toward developing evidence-based guidelines for such cases. Specifically, we evaluated objective factors—maximal occlusal force, masticatory performance, mandibular morphology, and age—in female patients prior to treatment, and we compared these parameters between those who selected to receive implant placement and those who did not.

2. Materials and Methods

Measurements were obtained from patients with missing second molars. Patients were informed about the treatment options—partial denture, implant, and no prosthesis—as well as the advantages and disadvantages of each. Those who chose either an implant or no prosthesis were then compared. Additionally, patients were informed that they could withdraw from this study at any time without affecting their future treatment.

This study was retrospective and targeted female patients aged 40 years and older who had a missing unilateral mandibular second molar with an opposing maxillary second molar and visited the Showa University Dental Hospital Implant Center between April 2021 and April 2023. The study group (IT group) consisted of patients who experienced masticatory difficulty and had selected implant treatment. The control group (NT group) consisted of patients who did not select prosthetic intervention for a missing mandibular second molar (Figure 1). All patients in both groups were selected after their doctors had fully explained the advantages and disadvantages of implant treatment interventions for second molars.

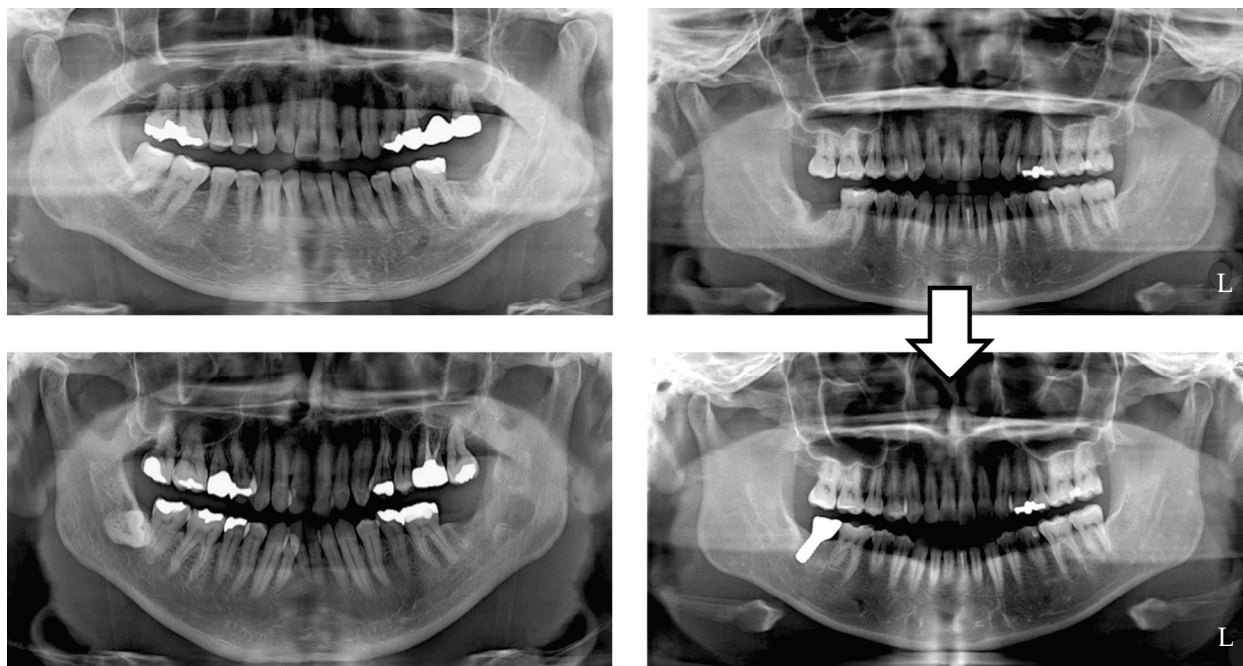


Figure 1. (Left) A representative patient in the NT group. (Right) A representative patient in the IT group (NT: no treatment; IT: implant treatment).

The exclusion criteria were as follows:

1. Patients who had untreated, missing teeth in areas other than the one planned for implant treatment.
2. Patients who were undergoing implant treatment in other areas.
3. Patients with at least one erupted third molar on the left or right side of the upper or lower jaw.
4. Patients who had lost a mandibular second molar within the past 3 months.
5. Patients with insufficient bone volume, in whom the distance to the mandibular canal of the second molar where an implant was placed was ≤ 10 mm.
6. Patients wearing a removable prosthetic device.
7. Patients with temporomandibular disorders.
8. Patients with mobile teeth due to periodontitis.
9. Patients who had difficulty communicating due to mental illness.
10. Patients with bone metabolic disease (such as osteoporosis and rheumatoid arthritis).
11. Patients who had previously received radiation therapy for the jawbone.
12. Patients taking bone resorption inhibitors.

The items examined were occlusal force, masticatory performance, and gonial angle. These were measured as described below, with all measurements in the IT group taken before implant placement surgery. Furthermore, the NT group underwent maintenance every 3 to 6 months to check the extension of the opposing teeth, changes in occlusion, and plaque control to prevent periodontal disease.

(1) Maximal occlusal force (Figure 2)

A film designed for an occlusal force measuring system (Dental Prescale II®; GC Corp., Tokyo, Japan) was placed over the entire dentition, and the participants were instructed to clench their teeth with maximum force in the intercuspal position for 3 s. Two measurements were taken, with an interval of 1 min. The maximal occlusal force was analyzed using a reading scanner (GT-X830; Seiko Epson Corp., Nagano, Japan) and analysis software (Bite Force Analyzer; GC Corp., Tokyo, Japan). These measurements

were conducted by the same examiner, and the maximal occlusal force was taken as the mean value of the two.

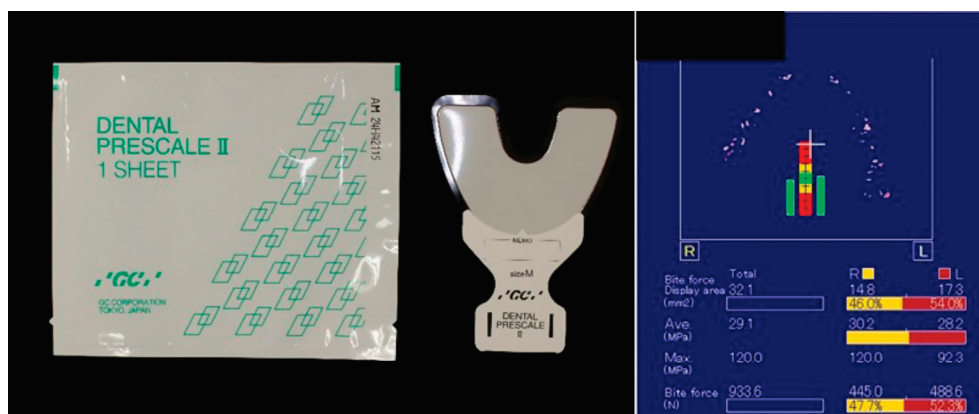


Figure 2. Measurement of maximal occlusal force.

(2) Masticatory performance (Figure 3)

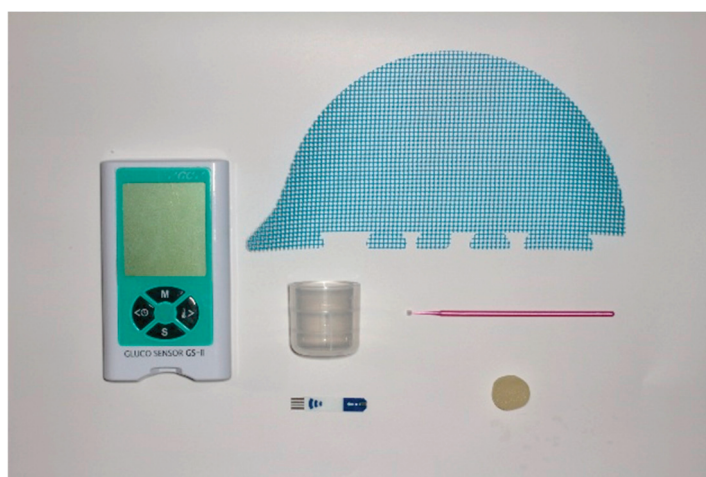


Figure 3. The glucose-measuring device used to determine masticatory performance.

The participants were instructed to place a glucose-containing gummy jelly (Glucol-umn; GC Corp., Tokyo, Japan) in their mouths and masticate for 20 s without swallowing saliva or the jelly. After 20 s, the participants held 10 mL of water in their mouths and spat out the masticated jelly and water together into a cup equipped with a filter mesh. The amount of glucose eluted from the filtrate in the cup was measured using a glucose-measuring device (Gluco Sensor GS-II; GC Corp., Tokyo, Japan), and masticatory performance was calculated [20–22].

(3) Gonial angle (Figure 4)

The gonial angle was considered an indicator of patients' occlusal forcefulness, and it was measured according to the method described by Anderson et al. [23]. The images were acquired using a dental CBCT (KaVo 3D Exam; KaVo Dental Systems, Biberach, Germany). The imaging parameters were set to 120 kVp, 5 mA, an acquisition time of 8.9 s, an axial slice thickness of 0.25 mm, and an isotropic voxel size of 16 × 16 cm. Prior to any measurement, the cone-beam computed tomography (CBCT) volume was adjusted such that the Frankfort plane (eye–ear plane) and interorbital line were parallel to the horizontal plane, and any right–left rotations were corrected. The gonial angle, as conventionally described, is that between the intersection of the ramus and the mandibular lines. All the images were

recorded in Digital Imaging and Communications in Medicine (DICOM) format, and the DICOM data were analyzed using simulation software (Invivo5: Anatomage, Santa Clara, CA, USA).

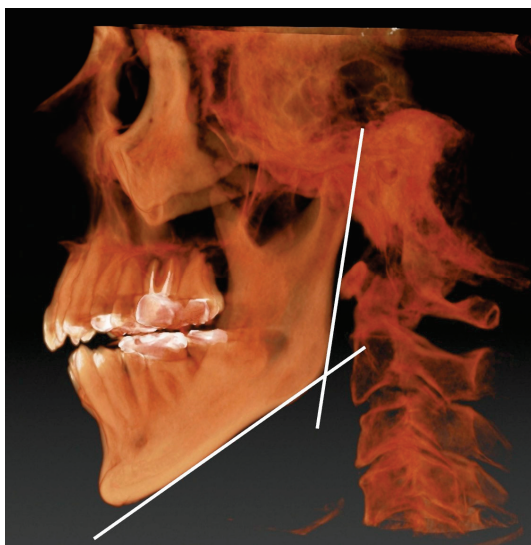


Figure 4. Measurement of the gonial angle. The angle formed at the intersection of the ramus and mandibular line on a 3D image was measured.

The gonial angle was taken as the mean value of the left and right sides.

One radiologist with over 10 years of experience conducted the gonial angle measurements based on the CBCT.

Statistical Analysis

Each item was compared between the IT and NT groups, and the effect of age was examined. A statistical analysis was conducted using the Mann–Whitney U-test and Spearman’s rank correlation coefficient ($p < 0.05$).

The Ethics Committee of Showa University approved this study protocol (approval number DH2018-009; approval date: 14 August 2018).

3. Results

The participants included 33 females (mean age: 55.1 ± 7.1 years), with 21 in the IT group (mean age: 54.5 ± 7.1 years) and 12 in the NT group (mean age: 56.0 ± 7.8 years). The measurement results of the items examined are shown in Table 1.

Table 1. Measurement results.

	IT Group	NT Group	<i>p</i> -Value
Age	54.5 ± 7.1 years	56.0 ± 7.8 years	0.78
Maximal occlusal force	739.2 ± 374.9 N	584.1 ± 293.4 N	$0.021 < 0.05$
Masticatory performance	235.8 ± 127.1 mg/dL	215.5 ± 76.5 mg/dL	0.51
Gonial angle	$125.0 \pm 5.1^\circ$	$121.9 \pm 9.8^\circ$	0.314

NT: no-treatment; IT: implant treatment.

1. Maximal occlusal force

The mean occlusal force was 739.2 ± 374.9 N in the IT group and 584.1 ± 293.4 N in the NT group, with the IT group showing significantly higher occlusal force ($p = 0.021 < 0.05$).

The effect of age was small in both the IT and NT groups, with no significant difference between the groups (IT group: $r = -0.16$; $p = 0.315$; NT group: $r = 0.65$; $p = 0.117$) (Table 2).

Table 2. Relationship with age.

	<i>r</i>	<i>p</i>
Maximal occlusal force		
IT group	−0.16	0.316
NT group	0.65	0.117
Masticatory performance		
IT group	0.53	0.047 < 0.05
NT group	0.13	
Gonial angle		
IT group	0.16	0.248
NT group	0.65	0.159

NT: no-treatment; IT: implant treatment.

2. Masticatory performance

The mean masticatory performance was 235.8 ± 127.1 mg/dL in the IT group and 215.5 ± 76.5 mg/dL in the NT group, with the IT group showing higher values. However, there was no significant difference between the groups ($p = 0.51$). Additionally, masticatory performance in the IT group showed a positive correlative relationship with age ($r = 0.53$; $p = 0.047 < 0.05$) (Table 2).

3. Gonial angle

The mean gonial angle was $125.0 \pm 5.1^\circ$ in the IT group and $121.9 \pm 9.8^\circ$ in the NT group, with no significant difference between them ($p = 0.314$). There was no statistically significant difference in correlation with age for either the IT group or the NT group (Table 2).

4. Discussion

The concept of providing prosthetic intervention for missing molars has been examined in several studies. Regarding removable prosthetic intervention, Walter et al. investigated the periodontal health of patients who had lost their molars by conducting a 10-year comparative analysis between patients wearing partial removable denture prostheses and those receiving no intervention. The latter showed favorable outcomes in all parameters relating to remaining teeth (clinical attachment loss, bleeding on probing, and plaque index) [24]. Furthermore, as regards providing implant-prosthetic intervention for a missing second molar, it has been reported in some studies that implant treatment is more effective than other prosthetic treatments. Contrastingly, others have opined that implant-prosthetic intervention in this region is unnecessary because of its higher risk of developing complications compared to that in other areas, and this thereby remains a controversial topic [25,26].

In clinical practice, treatment using the all-on-four concept, which does not involve prosthetic treatment for the second molar area, can also achieve favorable long-term outcomes [27–29]. Malo et al. reported high cumulative survival and success rates of 93% and 91.7%, respectively, after a long-term follow-up of 10 years and up to 18 years. Based on the all-on-four concept, which is characterized by prosthetic appliances up to the first molar without a second molar, they examined the prognosis of fixed full-arch prosthetic treatment for the mandible. Additionally, they concluded that the treatment method was very predictive, with low marginal bone loss values after 10 and 15 years following treatment of 1.72 mm and 2.32 mm, respectively [27]. According to Uesugi et al.'s longitudinal study of

Japanese patients with 3–17-year survival rates, the cumulative survival rate for the maxilla was 94.4% at the patient level and 97.4% at the implant level, while that for the mandible was 96.7% at the patient level and 98.9% at the implant level [28]. The results indicated that the treatment of the mandible was a successful therapeutic strategy for Japanese patients. Additionally, there was increased enjoyment and quality of life associated with oral health in the edentulous individuals who received full dentures supported by all-on-four implants.

Patients whose rehabilitation was based on the all-on-four concept had excellent oral health-related quality of life and satisfaction, according to a systematic review of the concept that used the oral health impact profile (OHIP) and visual analog scale (VAS) assessment [29]. Therefore, it has been demonstrated that even with prosthetic appliances up to the first molar without a second molar, good long-term prognosis may be achieved, and patient satisfaction and quality of life are high when it comes to full-arch prosthetic treatment with implants.

Regarding treatment planning for older adults with periodontal disease, Curtis et al. noted that the use of removable partial dentures can pose problems, including increased plaque accumulation and caries risk in proximal plates and rests. Moreover, they emphasized the importance of keratinized mucosa and crown contours of prosthetic devices in risk assessment when selecting implant treatment. They further cited difficulty in plaque removal due to keratinized tissue with a size of <2 mm and difficulty in oral hygiene access as factors [30]. Furthermore, patients undergoing implant treatment require professional maintenance, and the incidence rate of peri-implantitis has been reported to differ significantly based on the presence or absence of hospital visits for maintenance purposes [31–33].

In a study by Horibe et al., which used a device similar to that employed in this study, the occlusal force in 391 males and females aged 65 years and older was measured to be 546.5 ± 418.6 N [34]. Additionally, Shiga et al. investigated the effect of aging on the occlusal force of patients with natural dentition by comparing three age groups—a young group (20–39 years), a middle group (40–59 years), and an old group (60 years and over)—and they reported a significant decrease in occlusal force among those aged 60 years and older in both gender [35]. Moreover, a similar study conducted by Sano et al. reported that both sexes showed significant decreases in occlusal force with aging, with a stronger tendency in females, and that the young group aged 21–49 years had a maximal occlusal force of 495.5 ± 144.3 N [20]. In this study, the IT group demonstrated a significantly high occlusal force of 739.2 ± 374.9 N, with no effect of age, which was different from the results of the studies by Horibe et al., Shiga et al., and Sano et al. These results suggest that the patients who selected implant treatment for their second molars had high occlusal forces at any age.

Regarding masticatory function, Shiga et al. examined the relationship of masticatory performance with gender difference and age using a similar device to that in this study. Masticatory performance was significantly higher in males than in females, and there was no effect of aging in either gender [21]. Additionally, Sano et al. examined the association among gender difference, occlusal force, and masticatory performance, reporting that occlusal force and masticatory performance were significantly lower in females (208.8 mg/dL in the young group and 197.4 mg/dL in the older group), and they were positively correlated with each other in both genders [20]. Furthermore, Nimura et al. examined the association among occlusal force, masticatory function, and physical constitution (height, weight, and body mass index (BMI)), showing that occlusal force and masticatory performance were significantly lower in females (452.9 N and 194.4 mg/dL, respectively) and that although occlusal force was associated with physical constitution (especially weight), there was no association with masticatory performance [36]. Additionally, Yokoi

et al. conducted a functional assessment of patients with a missing second molar in either the upper or lower jaw using a similar device to that in this study, and they analyzed the differences between the implant treatment intervention group and the untreated group. They concluded that there was no difference in masticatory performance or occlusal force between the two groups [37]. In this study, the IT group showed a masticatory performance of 235.8 ± 127.1 mg/dL, which was significantly higher than those reported in other studies, and it showed a significant improvement with age. The results suggest that the patients who selected implant treatment for their second molars also exhibited high masticatory ability, which was not undermined by age. This could be attributed to the small sample of patients that had small numbers of missing teeth, good oral hygiene, and affluent socio-economic status.

Regarding the gonial angle, Miwa et al. examined the relationship between this and the maximum occlusal force in young men and women (average age of 24.6 years) using panoramic radiographs. They reported that the maximum occlusal force in men was significantly higher than that in women, and though there was no gender difference in the gonial angle in a multiple regression analysis, a strong negative correlation with the maximum occlusal force was observed [38]. Similarly, Bakan et al. measured the gonial angle using CBCT and examined differences between age and gender, and they reported that there were no differences regarding each of these three factors [39]. In this study, although the IT group had a significantly higher maximum occlusal force, there was no difference in the gonial angle, and age had no effect, consistently with the findings of Bakan et al. The discrepancy with the results from Miwa et al. was possibly due to the difference in measurement methods, as this study targeted relatively older people in their 40s to 60s, with a narrow age range within the patient groups. Recently, Akter et al. investigated the relationship between gonial angle, which is considered the indicator of the magnitude of occlusal force, and late implant loss in order to determine whether occlusal overload is a risk factor for late implant loss. The results of the multivariate analysis reported a significant correlation between a decrease in the gonial angle and late implant loss [40]. Therefore, practitioners should be cautious when implant therapy is planned for a missing mandibular second molar, which is subject to a single crown and high occlusal forces, for patients with smaller gonial angles.

Greenstein et al. created guidelines for the management of missing second molars, stating the following: (1) The decision to replace a missing second molar with implant treatment is optional and should be judged based on patient-specific criteria. (2) Because the over-eruption of an unopposed second molar tends to occur early after an extraction, it is prudent to perform implant treatment as early as possible if it is planned. (3) The bone volume should be thoroughly examined using CT images if implant treatment is considered. (4) Routinely replacing a second molar due to reduced masticatory efficiency is unnecessary. Generally, patients function well with first-molar occlusion. Continued monitoring is recommended for asymptomatic patients who are not manifesting any occlusal alterations. (5) Position changes in opposing teeth can be avoided through preventive and conservative measures (e.g., the bonding of the second molar to the first molar and fabrication of a retainer). (6) Because opposing teeth with periodontal disease tend to over-erupt more than healthy molars, increased monitoring is needed to prevent this [41]. In addition, Esposito et al. compared the prognoses of short implants (4.0 mm long) and normal implants (8.5 mm long or longer) in an RCT investigating the efficacy of short implants for posterior jaws and reported comparable survival rates, albeit for a shorter time period. The results showed that the survival rates were comparable between short implants and normal implants [42]. In this respect, an individual with a missing second molar can be a good candidate for implant treatment even with limited bone height.

The findings in this study suggest that patients who report masticatory discomfort or difficulty due to a missing mandibular second molar and who actively seek treatment are often characterized by possessing a relatively large occlusal force and superior masticatory performance compared to those who do not pursue such treatment. This observation implies that individuals with higher functional demands—those who naturally generate stronger occlusal loads and exhibit more efficient chewing—may be more sensitive to the absence of posterior occlusal support, even in areas that are sometimes considered less critical, such as the second molar region.

It is therefore speculated that these patients perceive the functional deficit caused by the missing second molar more acutely, potentially due to their habitual reliance on a full complement of posterior teeth to maintain their high chewing efficiency. As a result, they may experience a more pronounced decline in oral function when a second molar is lost, leading to a stronger motivation to seek implant-based restoration. This inclination suggests that, despite general trends showing lower prosthetic demand for second molars, there exists a subset of patients with high occlusal capabilities who regard the second molar as essential for maintaining optimal function and comfort in daily mastication.

Based on the lack of an effect of age, we believe that sufficient informed consent is needed from patients indicated for implant treatment, covering issues such as the presence of keratinized mucosa, the ability to control plaque, the selection of superstructure materials, periodic occlusal adjustment, the fabrication of a mouthpiece as necessary, and bone volume. Additionally, for patients who have not received implant treatment for missing mandibular second molars, regular maintenance is essential for monitoring the extension of opposing teeth, changes in occlusion, and plaque control to prevent periodontal disease, as shown in the guidelines of Greenstein et al. [41].

Based on previous reports of gender differences in occlusal force and masticatory performance, only females were targeted in this study [20,21,43]. It is reported that females generally show lower occlusal force and masticatory performance compared to males, with a tendency towards a larger gonial angle. We also used bone volume factors as exclusion criteria to ensure that the evaluations were based on the same criteria. The final sample size of this study was smaller than originally anticipated, primarily due to difficulties encountered during patient recruitment. Despite thorough preparation and outreach efforts, several factors contributed to the lower-than-expected enrolment. These included a limited pool of eligible participants based on the inclusion and exclusion criteria, a relatively short recruitment period, and, in some cases, patient hesitancy to participate in clinical research. Consequently, the reduced sample size may limit the statistical power of this study, and the results should be interpreted with caution, particularly in terms of their generalizability and robustness. Future studies with a larger sample size will be necessary to confirm and expand upon the findings presented here. In addition, patients' socio-economic status was not included in the analysis as one of the determinants of treatment selection because we could not retrospectively identify any variety in this among the participants. Nevertheless, this can be a potential determinant of treatment selection, and so it should be scrutinized and included as a parameter in any future prospective studies. Previous studies have suggested that occlusal force and masticatory performance are associated with physical constitution (including height, weight, and BMI), the period after tooth extraction, and the presence or absence of bruxism, angle classification, and masseter muscle thickness [44], which we believe require further investigation. As this study included patients with systemic diseases, such as controlled diabetes and cardiovascular disease, other than mental illness and bone metabolic disease, the impacts of these and their associated medications were not examined.

In this study, we focused exclusively on females, limiting generalizability across gender. In addition, bruxism, BMI, and muscle thickness, which are factors known to influence bite force, were not recorded and may have impacted the outcomes.

Additionally, since this study involved Japanese participants, it is possible that their dietary habits, culture, and preferences could have affected their decision to choose implant treatment for a single missing second molar, yielding different findings from research completed in the U.S. and Europe. The necessity of implant treatment for a missing mandibular second molar requires a sufficiently detailed study, including a survey using food questionnaires to assess the pre- and post-operative differences caused by implant treatment, because the same effects on patient satisfaction and quality of life can be taken into consideration.

In our future research, we would like to increase the number of patients and include males to investigate the effects of gender and age. Additionally, we will aim to investigate long-term prognoses with and without treatment intervention and examine the adequacy of implant treatment for a missing mandibular second molar.

5. Conclusions

Based on the results of this study, the following conclusions were drawn about the characteristics of the patients who selected implant treatment for a missing mandibular second molar: The IT group had a significantly higher occlusal force, with no effect of age. There was no statistically significant difference in masticatory performance between the IT and NT groups; however, the IT group showed a significant improvement with age. The effect of the gonial angle was small.

Author Contributions: Conceptualization, T.A. and M.M.; data curation, T.Y. and K.B.; formal analysis, T.A. and M.M.; investigation, T.A. and T.Y.; methodology, T.A., M.M. and D.S.; project administration, M.M. and K.Y.; supervision, M.M.; visualization, T.A. and M.M.; writing—original draft preparation, T.A. and M.M.; writing—review and editing, M.M., K.Y. and D.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The Ethics Committee of Showa University approved this study protocol (approval number: DH2018-009; approval date: 14 August 2018).

Informed Consent Statement: All participants verbally provided informed consent. The study information was disseminated to participants both verbally and in written form.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the corresponding author on request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Antibacterial Characteristics of Nanoclay-Infused Cavit Temporary Filling Material: In Vitro Study

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Abstract: In pediatric endodontic procedures, final crown placement is often delayed, requiring the use of temporary filling materials to seal the access cavity. Given the importance of antibacterial properties in temporary restorations and the known antimicrobial effects of nanoclay particles, this study aimed to evaluate the antibacterial efficacy of a nanoclay-infused temporary dressing against cariogenic and residual intracanal bacteria. A commercial temporary material (CAVISOL, Tehran, Iran) was blended with nanoclay (SOUTHERN, Gonzalez, TX, USA; average size 95 nm), using eugenol as a wetting agent. The antibacterial effects on *Streptococcus mutans*, *Enterococcus faecalis*, and *Escherichia coli* were assessed using disc diffusion, well diffusion, and microtiter plate assays after 24 h of incubation at 37 °C (six material groups, three bacterial strains, three replications for each antibacterial test). Statistical analyses were performed using Shapiro–Wilk and ANOVA tests ($p < 0.05$). The results showed that formulations containing 60% and 80% nanoclay significantly inhibited the growth of all tested bacteria, outperforming pure Cavit ($p < 0.05$). The most substantial inhibition was observed in *E. coli*, while *S. mutans* exhibited the least susceptibility ($p < 0.05$). These findings suggest that incorporating nanoclay into temporary fillings may enhance efficacy to increase the success rate of pediatric endodontic treatments, although further physicochemical and clinical evaluations are warranted.

Keywords: antibacterial; nanoclay; nanoparticles; temporary dental filling

1. Introduction

Success in treatments like pulpotomy and pulpectomy is closely related to the complete elimination of microorganisms from the root canals through proper biomechanical cleaning. Effective disinfection and sealing of the access cavity with a restorative material are key to treatment success [1]. Physical cleaning significantly reduces bacterial count, but due to the anatomical complexity of root canals, especially in deciduous teeth, complete microorganism elimination may not be achievable in treatments like pulpotomy and pulpectomy [2]. To maintain the aseptic chain, coronal sealing during treatment sessions is

crucial, preventing saliva and microorganisms from infiltrating the cleaned canals. Studies indicate a direct relationship between coronal sealing and treatment success. Therefore, temporary filling materials used for access cavities should have antibacterial properties and maintain this feature over time [3]. Currently, in many dental procedures such as root canal treatment, final crown restoration does not occur in the first session, necessitating the temporary filling of the access cavity. A temporary filling material is recognized as a restorative that remains inside the access cavity until the final restoration is completed. However, microleakage of filling materials may lead to bacterial contamination in the canal and periapical complications in the post-treatment period. Studies indicate that materials based on zinc oxide have superior antibacterial properties compared to materials based on resin or calcium hydroxide [4,5]. The most widely used zinc oxide-containing material among multi-session temporary filling materials is Cavit [6]. Between treatment sessions, Cavit provides specific advantages such as resisting bacterial contamination, preventing saliva leakage, and preventing the fracture of thin tooth walls [7]. According to surveys, the use of this material is convenient both for filling cavities and for easy removal before final restoration [8]. However, studies by Brännström and colleagues have shown that Cavit lacks proper sealing ability, leading to bacterial contamination in the cavity [9]. Torabinejad and colleagues demonstrated that this bacterial infiltration can reach the apical area within 48 h [10]. Cavit temporary fillings can demonstrate an antimicrobial effect against coronal leakage for a maximum of 7 days [1]. Therefore, adding antibacterial materials to temporary fillings allows the maintenance of a bacteria-free environment, even in cases of partial bacterial infiltration from the coronal access cavity, and can contribute to strengthening the coronal seal, preventing the proliferation of remaining microorganisms in inaccessible areas [11]. Studies on oral bacteria, such as *Streptococcus mutans*, *Escherichia coli*, and *Enterococcus faecalis*, have identified them as common causes of dental caries and periodontal diseases [12,13]. We chose *Escherichia coli* as a model Gram-negative organism to demonstrate the material spectrum. We will clarify this rationale and consider testing more clinically relevant species such as *Lactobacillus* or *Actinomyces* in future studies. Moreover, there is also a significant association between acute dental caries and chronic diseases affecting the growth of children [14]. Given the escalating bacterial resistance, there is a crucial need to identify viable alternatives to conventional antibacterial methods. In the field of dentistry, the emergence of nanotechnology has garnered significant interest, as antibacterial agents derived from nanomaterials offer advantages such as cost effectiveness, stable structures, exceptional antibacterial properties, and a wide-ranging antibacterial spectrum [15]. Nanotechnology involves imaging, modifying, and modeling functions on a nanometer scale. Nanoparticles, which are smaller than 100 nanometers, exhibit unique properties such as high surface activity, chemical reactivity, and biological activity [16].

Nanoclays are nanoparticles that are used as antibacterial agents in dental materials [17]. Montmorillonites (MMT) are a type of nanoclay that has gained significant research interest in recent years, with layered structures that are used extensively in polymers to enhance their mechanical properties [18–20]. Incorporating only a small amount of nanoclay into polymers improves their mechanical performance [21]. The use of montmorillonite nanoparticles is an effective strategy for formulating new dental materials with clinical applicability [22,23]. Even adding 2% weight of quaternary ammonium-modified montmorillonite nanoparticles increases the antibacterial properties of temporary restorative materials [24]. Studies have reported that nanoclay is non-toxic to the human body and exhibits the highest antimicrobial property without side effects at low concentrations [25]. While the antibacterial property of temporary restorations is crucial for treatment success, studies have shown that commercially available temporary restorations often lack this property [11].

This study is aiming to assess the hypothesis that incorporating nanoclay into Cavit will enhance its antibacterial properties against common oral microorganisms, including *Streptococcus mutans*, *Escherichia coli*, and *Enterococcus faecalis*.

2. Materials and Methods

This in vitro experimental study was conducted in a controlled environment, ensuring aseptic conditions.

2.1. Materials and Bacteria

All tests were conducted on standard strains of *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* PTCC 1683, and *Escherichia coli* ATCC 25922, obtained from the microbiology department of Zanzan University of Medical Sciences. The temporary filling material used in this study was CAVISOL, obtained from Golchai Iran Pharmaceutical Company (Tehran, Iran). The nanoclay, designated as 5A, was acquired from the Southern Company, Atlanta, GA, USA. The average particle size was 59 nanometers, with an interlayer spacing of 2.7 nanometers.

2.2. Experimental Groups

To prepare the experimental groups, the following materials were mixed in the specified proportions. Eugenol drops were added to achieve a paste-like consistency and ensure a homogeneous mixture of the temporary filling material. The study consisted of 6 different groups:

1. Cavit1 (C1): negative control = 100% by weight CAVISOL temporary filling material + 3 drops eugenol.
2. Cavit2 (C2): 80% by weight CAVISOL temporary filling material + 20% by weight nanoclay + 3 drops eugenol.
3. Cavit3 (C3): 60% by weight CAVISOL temporary filling material + 40% by weight nanoclay + 3 drops eugenol.
4. Cavit4 (C4): 40% by weight CAVISOL temporary filling material + 60% by weight nanoclay + 3 drops eugenol.
5. Cavit5 (C5): 20% by weight CAVISOL temporary filling material + 80% by weight nanoclay + 3 drops eugenol.
6. Cavit6 (C6): positive control = 100% by weight nanoclay + 3 drops eugenol.

2.3. Tests

In this study, the following antibacterial tests were conducted:

1. Zone of Inhibition Testing: the zone of inhibition was evaluated using two methods, disk diffusion and the well diffusion assay.
2. The microtiter dish assay.

2.4. Implementation Method

2.4.1. Preparation of Experimental Groups

The temporary filling material, Cavisol (Tehran, Iran, Golchai), was physically mixed with nanoclay powder (Closite 5A, Gonzalez, TX, USA, Southern) with particle sizes averaging 59 nanometers using a digital balance (Ohaus Pro Scout SP601, Parsippany, NJ USA) to ensure precise weight measurement. The mixture was prepared with weight ratios of 0.8 to 0.2, 0.6 to 0.4, 0.4 to 0.6, and 0.2 to 0.8, respectively.

2.4.2. Preparation of Culture Media

In this study, MUELLER-HINTON AGAR, BLOOD AGAR, and BROTH media from Merck Germany were used. These powders were mixed with distilled water according to the manufacturer's instructions, poured into Erlen Meyer flasks, and then boiled. The flasks containing the culture media were then placed in an autoclave under standard conditions to be sterilized. After the autoclaving process was completed, the flasks containing the culture media were removed from the autoclave and, next to the flame, AGAR was poured into plates, and BROTH was poured into test tubes. After solidification of the mentioned media, plates containing culture media were placed in an incubator for 24 h to detect any potential contamination during their preparation.

2.4.3. Preparation of Bacterial Suspension

Standard bacterial strains, lyophilized in ampoules, were prepared by following aseptic standards and microbiological methods, washed, and then used to prepare a suspension. These suspensions were introduced into Muller–Hinton agar culture media and incubated to promote the bacterial growth of *Enterococcus faecalis* and *Escherichia coli*. For *Streptococcus mutans* bacteria, BLOOD AGAR culture media was used. To ensure uniform colonies of bacteria, the McFarland 0.5 standard was employed using a BROTH culture.

2.4.4. Disc Diffusion Test

Antibiotic discs with a diameter of 6 mm were prepared and immersed in the experimental groups (Cavit 1–6). A yellow sampler was dipped into a microbial suspension with a 0.5 McFarland concentration, and 200 microliters of the microbial solution was poured onto a 10 cm agar plate. Bacteria were cultured on the surface of the plate using the spread plate method. Discs soaked in the prepared compositions (Cavit 1–6) were placed on the plate. A disc without immersion in any mixture was used as a negative control. After 24 h of incubation at 37 degrees Celsius, the plates were examined to evaluate the growth inhibition halo diameter, measured in millimeters using a caliper. Moreover, the plate containing *Streptococcus mutans* was placed in a CO₂ incubator to create anaerobic growth conditions. This procedure was performed for all three bacteria, and the testing process was repeated three times for each bacterium.

For each method, we used 6 material groups $\times$ 3 bacterial strains $\times$ 3 replicates = 54 samples; the total sample size was 162.

This approach is consistent with previous in vitro antimicrobial studies, where triplicate testing per group is considered standard and sufficient for statistical analyses such as one-way ANOVA or Kruskal–Wallis tests [26,27].

2.4.5. Well Diffusion Test

In this method, initially, under sterile conditions next to the flame and within the laminar flow hood, 200 microliters of the 0.5 McFarland bacterial suspension was spread on the surface of the plate containing Muller–Hinton agar (blood agar for *Streptococcus mutans*) using the spread plate method. Subsequently, five wells were created with a diameter of 5 mm, spaced at 2 cm intervals on the plate's surface. Then, 0.15 g of each of the compounds from groups 1–6 was placed in its own well. In this test, group 6 served as the positive control, and group 1 was the negative control. All plates were incubated at 37 degrees Celsius for 24 h. After this incubation period, bacterial cultures were examined for the presence or absence of growth inhibition zones, and the diameter of these zones was measured in millimeters using a caliper. This test was repeated three times [28].

2.4.6. Biofilm Assessment Test (Microtiter Dish Assay)

Aqueous extracts from temporary filling materials were prepared using groups 1–6, each with distinct weight percentages, and their impact on the formation of biofilm by *Enterococcus faecalis*, *Streptococcus mutans*, and *Escherichia coli* was assessed [29].

This test was conducted in multiple stages:

- Preparation of PBS Solution:

To prepare one liter of PBS solution, 8 g of NaCl, 0.2 g of KCl, 0.2 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ were dissolved completely in 800 milliliters of distilled water. The pH was adjusted to 7.4 using sodium hydroxide (NaOH) solution with a pH meter, and the final volume reached one liter.

- Preparation of Extracts from Groups 1–6:

A 0.2 g sample of each composition was placed inside labeled glass vials for groups 1–6, and 1 milliliter of PBS solution was added to each vial. The vials were shaken slightly and incubated for 10 h in a shaking incubator at 37 degrees Celsius and 81 rpm. This process was repeated for each group. The extracts were then transferred to labeled microtubes. In each well of a 24-well plate, 150 microliters of the extract was added using a blue sampler. Next, 150 microliters of PBS was added to two wells and they functioned as the control groups. The following procedure was performed for the three bacteria using three separate plates.

- The cultivation of seven bacterial strains in broth culture medium and the preparation of 0.5 McFarland concentration.
- Treatment with extracts from temporary filling materials and nanoclay: in each well containing the extract, 50 microliters of the bacterial suspension with a concentration of 0.5 McFarland was dispensed using a yellow sampler.
- Incubation: incubation was performed for 24 h, with the plates containing *Escherichia coli* and *Streptococcus mutans* placed in an incubator at 37 degrees Celsius, and the plates containing *Enterococcus faecalis* placed in a CO₂ incubator at 37 degrees Celsius.
- Fluorescent biofilm staining: After incubation, the contents of the plates were carefully aspirated using a sterile pipette. To detach non-adherent bacteria, each well was washed three times with 200 microliters of sterile PBS. Subsequently, in each well, 200 microliters of 4% crystal violet was added. After 12 min, the excess dye was poured off, and the plates were washed under a gentle stream of distilled water.
- Recording biofilm absorption using the ELISA plate reader (Tecan EL-Reader, Männedorf, Switzerland): After washing the crystal violet, 200 microliters of 33% acetic acid solution was added to each well for 15 min. At this stage, the colors that had penetrated the biofilm were released, and then they were read using the ELISA instrument at a wavelength of 600 nanometers. The more bacterial biofilm remained, the more color it absorbed. Therefore, after adding acetic acid, it produced more color, ultimately registering a higher absorption in the ELISA instrument.

3. Results

3.1. Well Diffusion Test

The findings are depicted in Figure 1, with the individual results for each bacterium provided in Table 1. A Picture of the inhibition zones is provided in Figure 2.

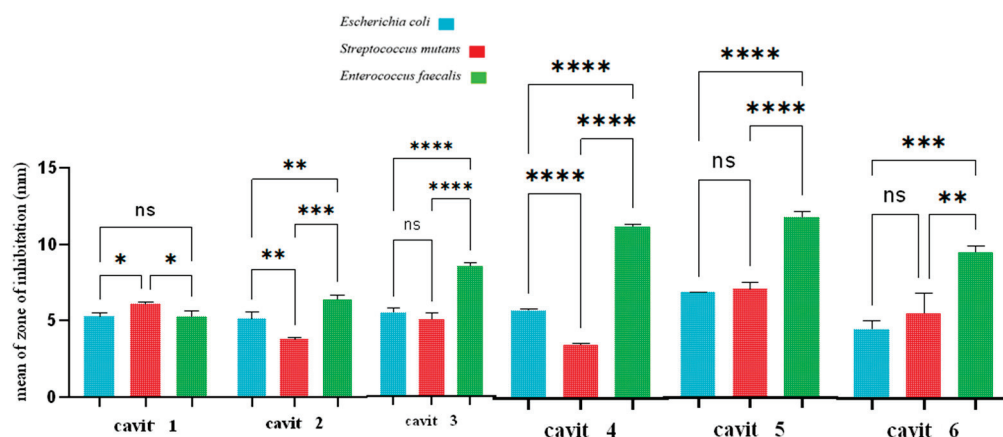


Figure 1. Comparison of inhibition zones in well diffusion test. ns: not significant, *: p -value < 0.05, **: p -value < 0.01, ***: p -value < 0.001, ****: p -value < 0.0001.

Table 1. Hierarchy of bacterium inhibition zones in well diffusion test, from most to least in diameter (left to right). Cavity groups are listed based on inhibition zones, from largest to smallest.

Bacterial Species	Cavity Groups					
<i>E. coli</i>	Cavit3>	Cavit5>	Cavit2>	Cavit4>	Cavit1>	Cavit6
<i>S. mutans</i>	Cavit5>	Cavit3=	Cavit1	Cavit6>	Cavit2>	Cavit4
<i>E. faecalis</i>	Cavit5>	Cavit3>	Cavit4>	Cavit6>	Cavit2>	Cavit1

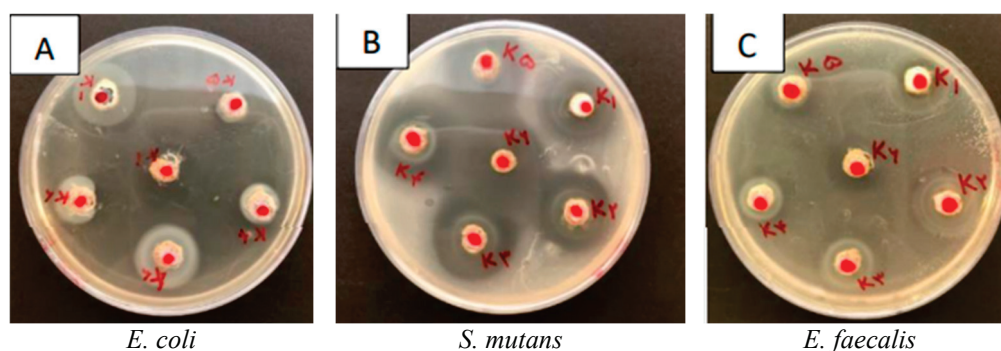


Figure 2. Inhibition zones of each bacterium in well diffusion test. (A) *E. coli*, (B) *S. mutans* (C) *E. faecalis*.

3.2. Disk Diffusion Test

The findings are depicted in Figure 3, with the individual results for each bacterium provided in Table 2.

Table 2. Hierarchy of bacterial species inhibition zones during disk diffusion test. Cavity groups are listed based on inhibition zones, from largest to smallest.

Bacterial Species	Cavity Groups					
<i>E. coli</i>	Cavit6>	Cavit5>	Cavit4>	Cavit3>	Cavit2>	Cavit1
<i>S. mutans</i>	Cavit5>	Cavit4>	Cavit3>	Cavit2>	Cavit6>	Cavit1
<i>E. faecalis</i>	Cavit6>	Cavit5>	Cavit4>	Cavit2>	Cavit3>	Cavit1

In disk and well diffusion assays, an increased diameter of the inhibition zone reflects higher inhibitory efficacy against the tested microorganisms (Tables 1 and 2).

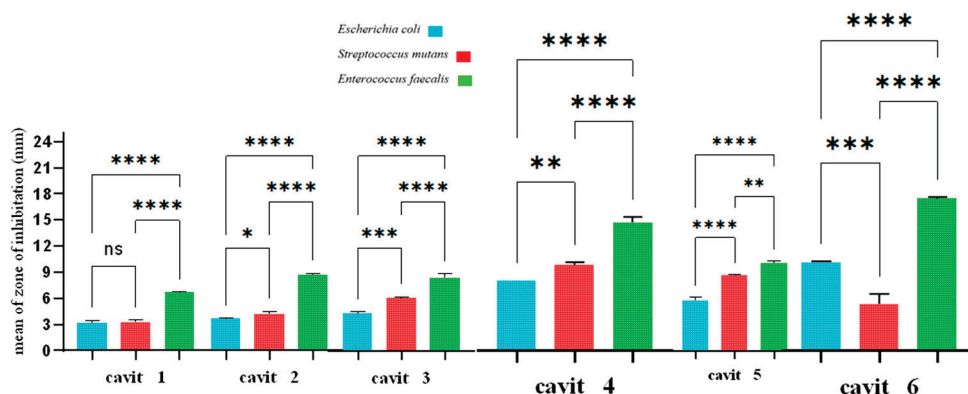


Figure 3. Comparison of inhibition zones in disk diffusion test. ns: not significant, *: p -value < 0.05, **: p -value < 0.01, ***: p -value < 0.001, ****: p -value < 0.0001.

For *E. coli*, Cavit-3 in the well diffusion test and Cavit-6 in the disk diffusion test showed significantly greater inhibition than the other groups. For *S. mutans*, Cavit-5 demonstrated markedly superior inhibition compared to the other groups in both the well diffusion test and the disk diffusion test. For *E. faecalis*, Cavit-5 in the well diffusion test and Cavit-6 in the disk diffusion test achieved a markedly enhanced inhibitory performance over the other groups (Figures 1 and 3).

The findings from both the disk and well diffusion assays demonstrate that the antibacterial activity of the Cavit–nanoclay formulations differs depending on the bacterial species tested. The results suggest that *E. coli* is more susceptible to formulations with higher nanoclay content, whereas *S. mutans* responds best to Cavit-5, and *E. faecalis* shows moderate sensitivity across different formulations. This variability highlights the need to consider bacterial species-specific responses when designing antimicrobial dental materials, to ensure maximum efficacy against targeted pathogens.

3.3. Microtiter Dish

The findings are depicted in Figure 4, with the individual results for each bacterium provided in Table 3.

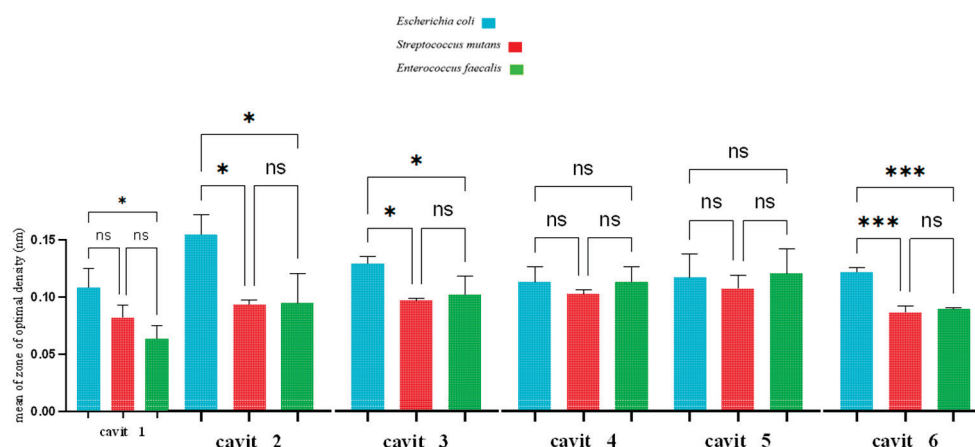


Figure 4. Comparison of inhibition zones in microtiter dish assay test. ns: not significant, *: p -value < 0.05, **: p -value < 0.001.

In this test, smaller values denote higher inhibitory efficacy against the tested microorganisms (Table 3).

Table 3. Antibacterial effect on each bacterial species during microtiter dish assay. Cavity groups are listed based on inhibition zones, from largest to smallest.

Bacterial Species		Cavity Groups				
<i>E. coli</i>	Cavit4>	Cavit5>	Cavit6>	Cavit3>	Cavit2>	Cavit1
<i>S. mutans</i>	Cavit6>	Cavit2>	Cavit3>	Cavit4>	Cavit5>	Cavit1
<i>E. faecalis</i>	Cavit6>	Cavit1>	Cavit2>	Cavit3>	Cavit4>	Cavit5

For *E. coli*, Cavit-4 showed significantly greater inhibition than Cavit-3 and Cavit-2 ($p < 0.001$). For *S. mutans*, Cavit-6 produced the largest inhibition zone, with no significant differences among the other groups. For *E. faecalis*, no statistically significant differences were observed among the tested groups.

Overall, these results indicate that the antibacterial effect of the materials varies by bacterial species, with *E. coli* being more sensitive to higher nanoclay concentrations, while *S. mutans* responded most strongly to Cavit-6, and *E. faecalis* showed comparable resistance across formulations.

4. Discussion

We demonstrated that incorporating nanoclay Cloisite 5A particles into Cavit can augment its antibacterial characteristics. The following sections will elaborate on the material weight percentage that exhibits the most significant antibacterial impact on each bacterium.

E. faecalis: In the well diffusion method, the Cavit5 group (20 wt% Cavit + 80 wt% nanoclay + 3 drops eugenol) demonstrated the highest antibacterial efficacy, and in the disk diffusion method, the Cavit6 group (100 wt% nanoclay + 3 drops eugenol) did the same. Moreover, all groups showed statistically significant differences in inhibiting bacterial growth. This finding aligns with the research conducted by Alzaidy et al., where they observed an augmentation in the antibacterial characteristics of root canal sealers against *Enterococcus faecalis* upon the addition of silver nanoparticles [30].

E. coli: In the well diffusion method, the Cavit3 group (60 wt% Cavit + 40 wt% nanoclay + 3 drops eugenol) exhibited the largest zone of *Escherichia coli* growth inhibition, and in the disk diffusion method, the Cavit6 group (100 wt% nanoclay + 3 drops eugenol) did the same, although its difference was not statistically significant (p -value = 0.05).

S. mutans: The Cavit5 group (20 wt% Cavit + 80 wt% nanoclay + 3 drops eugenol) showed the highest antibacterial properties for *Streptococcus mutans* in both the disk diffusion and well diffusion methods. The study titled “Nanoclay-reinforced polymethyl-methacrylate and its mechanical properties” demonstrates that even modest nanoclay additions (1–2 wt%) can significantly reduce the tensile strength of PMMA resin. Therefore, incorporating nanoclay at levels as high as 80% is not considered clinically feasible and should be regarded as applicable only in the context of in vitro microbiological testing [31].

Consistent with the current study, Yin et al. showcased the enhanced antibacterial properties of dental materials through the addition of silver nanoparticles [27]. Similarly, Fareed et al. found that incorporating 2% weight of nanoclay improves antibacterial and physical properties, supporting our results [32].

However, Barzegar et al.’s study, using 20A Cloisite nanoclay, contradicts our findings. They could not statistically demonstrate enhanced antibacterial and mechanical properties in polymethyl methacrylate materials combined with nanoclay [31]. The difference likely arises from the nanoclay type (5A Cloisite) and variations in nanoparticle size. Particle size and morphology significantly influence antibacterial effects [33]. Organically modified nanoclays show higher antibacterial activity than unmodified forms due to increased surface reactivity [34]. Smaller particles and better dispersion typically enhance efficacy [35].

Changes in nanoparticle size and surface-to-volume ratio impact distribution and biocompatibility, as extensively studied and confirmed [36]. Our study consistently observed an increase in the diameter of the inhibition zones during the well diffusion and disk diffusion tests with a rise in the percentage of nanoclay. This rise can be attributed to the antimicrobial and bacteriostatic effects of nanoclay. Additionally, Song et al. demonstrated the applicability of nanoparticles due to their small size and high surface-to-mass ratio, which are highly suitable for enhancing antimicrobial effects [37]. The antibacterial mechanism of nanoclay involves interacting with the cell wall and peptidoglycan membrane, resulting in cell lysis, disrupting bacterial proteins and protein synthesis, and interacting with bacterial DNA to hinder DNA replication. As the percentage of nanoclay rises, the antibacterial mechanism intensifies, enhancing antibacterial properties [38].

Among the three examined bacteria, *Escherichia coli* exhibited an increase in antibacterial properties proportional to the increase in the percentage of nanoclay. This implies that the antibacterial properties of the experimental groups, from Cavit1 to Cavit5, consistently increased in *Escherichia coli*. The groups containing 60% and 80% nanoclay showed excellent antibacterial results against *Escherichia coli*. These findings align with the results of the study by Ghorbanpour et al. [38]. The lower sensitivity to the other two bacteria, *Enterococcus faecalis* and *Streptococcus mutans*, can be attributed to their Gram-positive nature. Studies have shown that Gram-positive bacteria, with their thick cell walls, exhibit greater resistance to nanoparticles compared to Gram-negative bacteria such as *Escherichia coli* [39]. Nevertheless, pairwise comparison of the bacteria indicated that among the three bacteria, *Enterococcus faecalis* showed the largest growth inhibition zone. Regarding this contradiction between the results, it should be noted that the type of numerical comparison used for the diameter of the growth inhibition zones may not be accurate, as the growth of each bacterium in a culture medium with another bacterium differs. Therefore, the consistent enhancement of antibacterial properties with an increase in the weight percentage of nanoclay serves as a more reliable criterion for comparing bacterial growth inhibition. This consistent pattern was observed in *Escherichia coli* with both the well diffusion and disk diffusion methods. The results of the microtiter dish assay lacked consistency and did not exhibit a clear pattern in terms of antibacterial properties. One possible explanation is the dissolving of the mixtures (Cavit1 to Cavit6) in PBS. These materials contained eugenol, which is hydrophobic [40]. Thosar et al. investigated the solubility of various materials, including eugenol, and found that eugenol exhibited the least solubility among the materials studied [41]. Although Cavit contains eugenol, its antimicrobial activity is known to be limited and inconsistent, particularly against resistant species such as *E. faecalis*. Previous studies have shown that eugenol exhibits weak antibacterial properties when used in zinc oxide-based pastes [42]. In the present study, significant inhibition zones were only observed with increased nanoclay concentrations, supporting the conclusion that the observed antibacterial enhancement is primarily due to nanoclay rather than eugenol. Considering that PBS is hydrophilic, the dissolution process may have been disrupted. In this study, the well diffusion method proved to be the most promising and reliable approach among the antibacterial tests for evaluating dental materials in combination with nanoparticles. This in vitro study investigated the antibacterial effects of nanoclay in combination with Cavit temporary filling material, acknowledging the different conditions in the oral cavity. Factors like oral temperature, pH, and saliva flow can influence antimicrobial efficacy. Recent reports using talc-mediated nanoclay composites show nearly complete bacterial kill rates with acceptable cell viability (~84%) in vitro, suggesting that antibacterial efficacy can be enhanced without excessive cytotoxicity if phase and surface chemistry are optimized [43]. Future studies should broaden the evaluation of nanoclay's

effects on various oral bacteria, assess its cytotoxic effects on human cells, and thoroughly examine the mechanical properties of this new material.

5. Conclusions

This study demonstrates that the incorporation of nanoclay particles significantly enhances the antibacterial properties of Cavit temporary filling material. Among the tested concentrations, higher nanoclay loadings demonstrated superior antibacterial efficacy *in vitro*; however, further investigation is required to identify the optimal concentration that balances antimicrobial activity, mechanical performance, and cytocompatibility. Although broad clinical application cannot yet be inferred, these findings underscore the necessity for comprehensive *in vivo* studies and rigorous biocompatibility assessments. Establishing a safe and effective nanoclay concentration may enable its integration as a targeted antimicrobial additive in restorative dentistry. Future studies should also address the long-term stability and potential cytotoxicity of nanoclay-based formulations prior to clinical translation.

Author Contributions: Conceptualization, B.N.S. and A.R.; methodology, S.M.N.; software, S.N. and A.N.; validation, S.D., B.N.S., and I.L.; formal analysis, S.M.N.; investigation, S.N.; resources, B.N.S.; data curation, S.N. and A.N.; writing—original draft preparation, S.N.; writing—review and editing, S.D., I.L., and A.R.; visualization, A.R.; supervision, B.N.S. and I.L.; project administration, S.M.N.; funding acquisition, B.N.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The corresponding author can provide data upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Comparative Evaluation of Shear Bond Strength Between Monolithic Zirconia and Three Different Core Build-Up Materials

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Abstract: This in vitro study aimed to evaluate and compare the shear bond strength (SBS) between monolithic zirconia (MZ) and different core build-up materials. Sixty cylindrical MZ specimens were fabricated and divided into three groups ($n = 20$) based on the type of core build-up material: nanohybrid composite resin (NHCR), glass ionomer cement (GIC), and zirconia-reinforced glass ionomer cement (zirconomer). All specimens were subjected to airborne-particle abrasion with aluminum oxide and bonded using a self-adhesive dual-cure resin cement. After 24 h of storage in distilled water at 37 °C, SBS testing was performed using a universal testing machine. Failure modes were examined under a stereomicroscope and classified as adhesive, cohesive, or mixed. The NHCR group exhibited the highest SBS values (48.32 ± 12.49 MPa), followed by the Zirconomer group (14.19 ± 3.66 MPa), and the GIC group (10.37 ± 4.21 MPa). The SBS of NHCR was significantly higher than that of both Zirconomer and GIC ($p < 0.001$). Although no significant difference was found between Zirconomer and GIC, Zirconomer demonstrated higher mean bond strength. Within the limitations of this study, NHCR showed the highest bond strength to monolithic zirconia. Zirconomer performed better than conventional GIC; however, further investigations involving different surface treatments and long-term clinical conditions are recommended to enhance its bonding efficacy.

Keywords: zirconomer; core build-up; nanohybrid composite; monolithic zirconia; shear bond strength

1. Introduction

Despite the increasing awareness of the importance of oral and dental health, the demand for endodontic treatment remains consistently prevalent [1]. The long-term prognosis of endodontically treated teeth is influenced by several key factors, such as the quality of the coronal seal, the number of remaining cavity walls, the presence of a ferrule, post placement, and the application of indirect restorations [1,2]. After endodontic treatment, core restorations play a crucial role in restoring the natural anatomical form of the tooth and reinforcing its strength to withstand functional and parafunctional stresses [2].

Core build-up is defined as a restorative procedure designed to enable the reconstruction of a significant portion of the coronal structure in teeth with extensive loss of hard tissue [3,4]. It is typically indicated when more than 50% of the crown structure is missing [5]. Since stress distribution differs between intact and restored teeth, compressive, tensile, and shear forces may simultaneously act on both the restoration and the

tooth–restoration interface. Therefore, the mechanical strength of core build-up materials is a critical determinant for material selection [6].

An ideal core build-up material should demonstrate adequate compressive and flexural strength, ensure biocompatibility with surrounding tissues, and establish a durable bond with tooth structure, pins, posts, and luting cements. For this purpose, a variety of materials have been employed, including cast metal cores, amalgam, composite resins, glass ionomer cements (GICs), porcelain, and compomers [7,8]. Among these, composite resins are widely preferred due to their superior mechanical properties [2,9].

GICs have been widely used in core restorations for many years due to their advantageous properties such as chemical adhesion to hard dental tissues, reduced postoperative sensitivity, and fluoride release [10]. To overcome the mechanical limitations of conventional GICs, Zirconomer was recently introduced through the incorporation of zirconia particles, thereby combining the adhesive bonding capacity and fluoride release capability of GICs with improved mechanical strength and occlusal resistance, making it a promising alternative in restorative dentistry [3,11]. The powder component of Zirconomer contains fluoroaluminosilicate glass, zirconium oxide, and pigments, whereas the liquid component consists primarily of polyacrylic acid and tartaric acid [7,12].

For teeth with extensive structural loss, indirect full or partial coverage restorations are recommended to provide cuspal protection following direct restorations. The bond strength between the core build-up and the crown is a decisive factor in the long-term success of all-ceramic restorations [13,14]. In high-strength all-ceramic materials such as zirconia crowns, bonding quality directly affects mechanical stability and marginal integrity, making both micromechanical retention and chemical interaction with resin cements essential for clinical success. In the literature, numerous studies have investigated the effects of various surface treatments (e.g., sandblasting, silica coating, plasma treatment) and adhesive resin systems applied before cementation to improve the bond strength of zirconia crowns [15,16]. However, while most studies primarily focused on the zirconia substrate, there is limited evidence on the bonding behavior of zirconia crowns to underlying core build-up materials, and comparative data on materials such as GIC, composite resins, or modified formulations remain scarce [2,3,7,8]. Understanding the influence of core material selection is therefore critical for the long-term success of restorations in endodontically treated teeth.

The aim of this *in vitro* study was to evaluate and compare the shear bond strength between monolithic zirconia and three core build-up materials: NHCR, GIC, and Zirconomer. In addition, the distribution of failure modes (adhesive, cohesive, and mixed) at the bonding interfaces was analyzed. The null hypothesis stated that there would be no statistically significant difference in shear bond strength among the tested core build-up materials.

2. Materials and Methods

In this experimental *in vitro* study, 60 monolithic zirconia specimens were bonded to three different core build-up materials using a self-adhesive dual-cure resin cement, and their shear bond strength was evaluated. According to a power analysis ($\alpha = 0.05$, power = 0.8), 60 square-shaped zirconia specimens and 60 disc-shaped core build-up specimens were fabricated and randomly allocated to three groups ($n = 20$ per group).

Monolithic zirconia blocks (inCoris TZI C disc 22 A1, Sirona Dental Systems, Bensheim, Germany; Batch No. 2016290053-1055) were cut from CAD/CAM blocks and sectioned using a precision micro-cutting machine (Struers Secotom-60, Struers A/S, Ballerup, Denmark). During the cutting process, the shrinkage expected after sintering was considered, and the specimens were prepared in larger dimensions to achieve the final size of $10 \times 10 \times 2$ mm. Accordingly, the zirconia blocks were cut to approximately

12.5 × 12.5 × 2.5 mm prior to sintering. After sintering, the zirconia blocks shrank by approximately 20% and reached the targeted final dimensions. Sintering was performed at 1510 °C for 8 h.

Afterwards, only one surface of each zirconia specimen was subjected to sandblasting. Sandblasting was performed using 30 µm aluminum oxide (Al₂O₃) particles (CoJet, 3M Deutschland GmbH, Neuss, Germany) at 2 bar pressure, from a 10 mm distance, for 15 s. Sandblasting was chosen as the sole surface treatment method because it is considered essential for achieving sufficient bond strength in high-strength ceramics [15] and is also a common and clinically relevant approach widely adopted for conditioning zirconia surfaces [16]. The specimens were then divided into three groups according to the core build-up material to be tested (*n* = 20).

The disc-shaped core build-up specimens (*n* = 20 for each core build-up material) were prepared using a mold with dimensions of 10 mm × 4 mm. The core build-up specimens were prepared to the same size as the zirconia discs to standardize the bonding area for shear bond strength testing and to simulate the clinical situation where zirconia restorations fully cover the core surface. Three different core build-up materials, GIC (Ketac Molar, 3M ESPE, Seefeld, Germany, Batch No. 1114316), NHCR (RubyCompNano, Inci Dental, Istanbul, Turkey, Batch No. 5068), and Zirconomer (zirconia-reinforced glass ionomer, SHOFU, Kyoto, Japan, Batch No. 05220583), were placed into the mold using manual instruments.

Zirconomer specimens were prepared according to the manufacturer's instructions by mixing at a 2:1 powder-to-liquid ratio on a glass slab with an agate spatula, then placed into the mold and left to self-cure at room temperature. Similarly, the Ketac Molar material used for the GIC group was mixed at a 2.5:1 powder-to-liquid ratio according to the manufacturer's instructions, placed into the mold, and chemically self-cured. The NHCR (RubyCompNano) specimens were light-cured for 20 s according to the manufacturer's instructions using a LED curing unit (Radii Plus, SDI, Bayswater, Australia; intensity: 1200–1500 mW/cm²; wavelength: 440–480 nm). The composition and manufacturer information of all materials are presented in Table 1.

Table 1. Composition and manufacturer information of the materials used in this study.

Material	Type	Manufacturer (Country)	Main Composition *
RubyCompNano (NHCR)	Nanohybrid composite resin	Inci Dental, Turkey	Bis-GMA, UDMA, silica/zirconia nano-fillers
Ketac Molar (GIC)	Conventional glass ionomer cement	3M ESPE, Germany	Fluoroaluminosilicate glass, polyacrylic acid, tartaric acid
Zirconomer	Zirconia-reinforced GIC	Shofu, Japan	Fluoroaluminosilicate glass + zirconia fillers, polyacrylic acid, pigments
RubySE Cem	Self-adhesive dual-cure resin cement	Inci Dental, Turkey	10-MDP monomer, resin matrix, inorganic fillers

* The main composition data were obtained from the manufacturers' product information sheets.

Each specimen was examined for surface irregularities, and the final dimensions were confirmed using a precision caliper. The sandblasted surface of the monolithic zirconia specimens was covered with a self-etching and self-adhesive dual-cure resin cement containing the 10-MDP monomer (RubySE Cem, Inci Dental, Turkey, Batch No. RSC002), which is known to enhance chemical bonding to zirconia surfaces [8,16]. The core build-up materials were subsequently placed onto the zirconia specimens (Figure 1a). A constant

load of 5 kg was applied to ensure uniform cement thickness, and excess cement was carefully removed. All specimens were light-cured for 20 s from four directions.

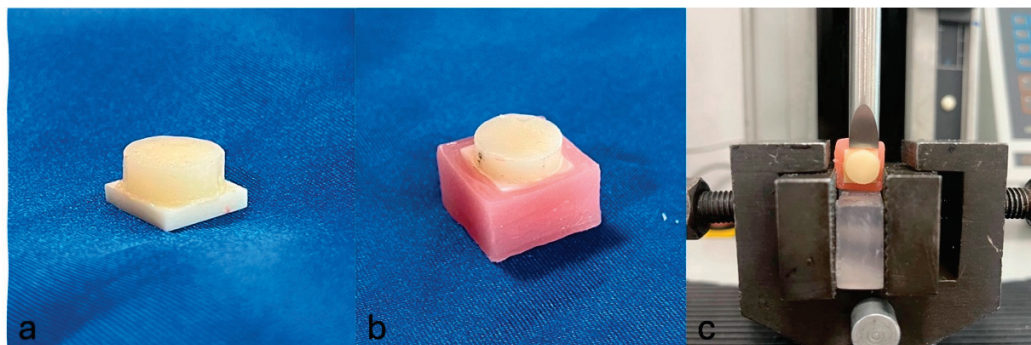


Figure 1. (a) Bonding of the core build-up material to the monolithic zirconia specimen, (b) specimen embedded in acrylic resin, and (c) evaluation of the specimen during shear bond strength testing using a universal testing machine.

After cementation, the specimens were stored in distilled water at 37 °C for 24 h to simulate the moist and stable temperature conditions of the oral environment *in vitro* and to ensure complete polymerization and stabilization of the mechanical properties of both the core build-up materials and the resin cement. Subsequently, a mold was prepared using type C silicone (Zhermack, Italy) for embedding the specimens in acrylic resin (Ivoclar Vivadent, Liechtenstein). The cemented and water-stored specimens were embedded in acrylic blocks so that half of the thickness of the monolithic zirconia remained exposed above the acrylic surface (Figure 1b).

For the evaluation of bond strength, all specimens were subjected to a shear bond strength test. The test was performed using a universal testing machine (Lloyd Instruments, Model LRX, Bognor Regis, UK) operating at a crosshead speed of 1 mm/min until failure occurred at the bonding interface (Figure 1c). The maximum load (N) sustained by each specimen was recorded. This value was then divided by the bonding area (mm²) to calculate the shear bond strength in MPa. All statistical analyses were performed using the shear bond strength values expressed in MPa.

In addition, all specimens were examined for failure modes. Each specimen was evaluated and classified according to semi-quantitative criteria as one of three failure types: adhesive (score 0, >75% of the failure surface exposed), cohesive (score 2, >75% covered with residual core material or fracture within the core material), or mixed (score 1, neither criterion fully met). The evaluations were carried out using a stereomicroscope (LEICA MZ12, Leica Microsystems, Wetzlar, Germany) at 10× magnification. All specimens were evaluated and classified by a single experienced examiner (E1) who had previously participated in similar studies. Prior to the main evaluations, E1 underwent a calibration session using a set of representative stereomicroscopic images. A predefined scoring guide was used, including explicit decision rules and a visual aid to estimate the 75% threshold for class assignment (adhesive, cohesive, mixed). Standardized imaging conditions (magnification, illumination, and working distance) were established and maintained for all observations.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics v26.0 (IBM Corp., Armonk, NY, USA). The normality of the data distribution was assessed using the Shapiro–Wilk test, while Levene’s test was used to evaluate the homogeneity of variances among the groups. Depending on the distribution and variance characteristics of the data,

appropriate parametric or non-parametric tests were applied for group comparisons. The Kruskal–Wallis test was used to compare the shear bond strength values, and when a significant difference was found, pairwise comparisons were performed using the Bonferroni-corrected Dunn’s post hoc test. The distribution of failure modes was analyzed using Pearson’s Chi-square test. A significance threshold of $p < 0.05$ was adopted for all statistical evaluations.

3. Results

The descriptive statistics of the shear bond strength values for the tested core materials are summarized in Table 2. The highest mean bond strength was observed in the NHCR group (48.32 ± 12.49 MPa), followed by the zirconomer (14.19 ± 3.66 MPa) and GIC (10.37 ± 4.21 MPa) groups. The NHCR demonstrated significantly higher bond strength compared with both GIC and zirconomer groups ($p < 0.001$). Although the difference between the GIC and zirconomer groups was not statistically significant ($p = 1.000$), the zirconomer group exhibited higher bond strength values than GIC. These findings suggest that the NHCR exhibit superior bonding performance to zirconia surfaces compared to the other tested core materials.

Table 2. Descriptive statistics of shear bond strength (MPa) for each core material group.

Group	Mean $\pm$ SD (MPa)	Median (MPa)	Min (MPa)	Max (MPa)
Zirconomer	14.19 ± 3.66^a	13.58	7.23	20.48
GIC	10.37 ± 4.21^a	10.22	2.6	17.04
NHCR	48.32 ± 12.49^b	48.76	23.54	71.01

Different superscript letters (^a, ^b) indicate statistically significant differences between groups (Dunn’s test, $p < 0.05$). Identical letters indicate no significant difference. SD: Standard deviation; Min: Minimum; Max: Maximum; MPa: Megapascal.

The distribution of failure modes among the core materials is summarized as follows. In the Zirconomer group, failures were predominantly mixed (65%), followed by cohesive (30%) and adhesive (5%). The GIC group showed no adhesive failures, with 40% cohesive and 60% mixed failures. In the NHCR group, 15% of the failures were adhesive, 40% cohesive, and 45% mixed. Representative stereomicroscopic images of the typical failure patterns for each group are shown in Figures 2–4. Statistical comparison of the distributions using the Chi-square test revealed no significant differences between groups ($\chi^2 = 4.6283$; $df = 4$; $p = 0.327$).

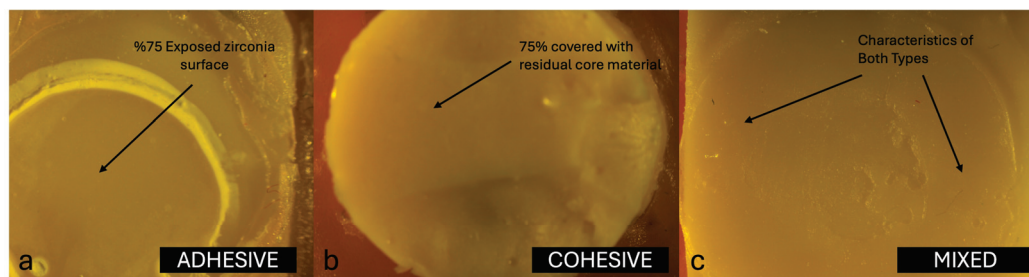


Figure 2. (a) Adhesive failure, (b) Cohesive failure, and (c) Mixed failure pattern of zirconomer group at $10\times$ magnification of a stereomicroscope.

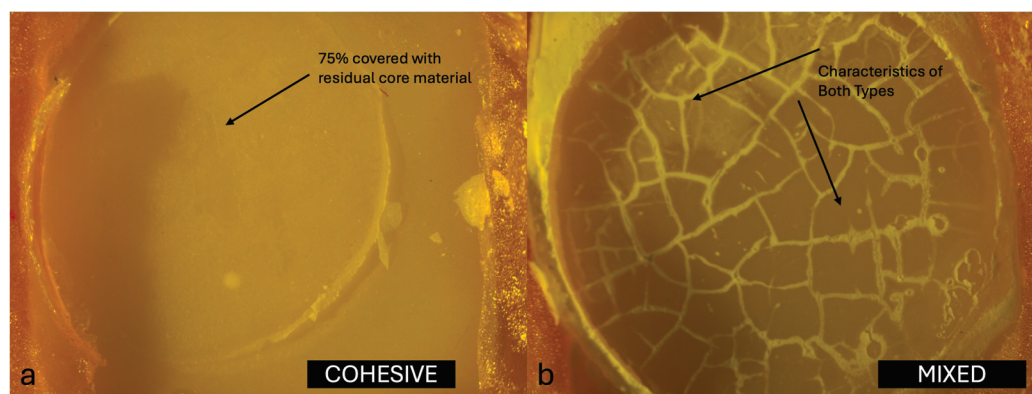


Figure 3. (a) Cohesive failure and (b) Mixed failure pattern of GIC group at 10× magnification of a stereomicroscope.

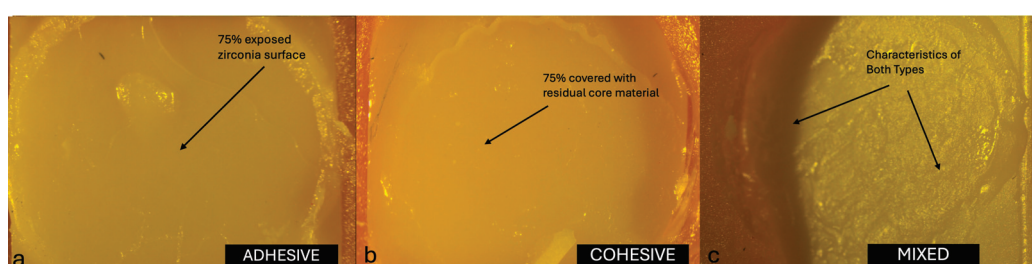


Figure 4. (a) Adhesive failure, (b) Cohesive failure, and (c) Mixed failure pattern of NHCR group at 10× magnification of a stereomicroscope.

4. Discussion

In this *in vitro* study, the shear bond strength between monolithic zirconia and three core build-up materials (NHCR, GIC, and Zirconomer) was evaluated. NHCR showed the highest bond strength, while Zirconomer presented higher mean values than GIC, although the difference was not statistically significant. Accordingly, the null hypothesis stating that “there is no statistically significant difference in shear bond strength between monolithic zirconia and different core build-up materials” was rejected.

The superior properties of composite resins have been demonstrated in numerous studies [17–21], and their advantages over amalgam as core build-up materials have been frequently emphasized [17,18,21]. Agrawal and Mala [21] showed that nano-filled resin composites could serve as an effective alternative to amalgam core materials in terms of their mechanical properties. Tavakolizadeh et al. [13] evaluated the shear bond strength of zirconia bonded to various core build-up materials (non-precious gold alloy, zirconia ceramic, natural dentin, and composite resin) and found that composite resin achieved the highest values (11.58 ± 1.74 MPa), confirming its status as the most favorable material. Among different composite types, NHCRs exhibit superior mechanical properties compared with microhybrid and microfill resins [3,19], which is consistent with our findings, where NHCR demonstrated the highest shear bond strength (48.32 ± 12.49 MPa).

Capp and Warren [18] reported that composite and acrylic resins can serve as acceptable alternatives to core build-up materials, whereas glass ionomer cements (GICs) are considered the weakest option in terms of mechanical performance. This observation underscores the inherent limitations of GICs and highlights the clinical rationale for favoring resin-based materials in situations where high strength and durability are required. Similarly, Goldman et al. demonstrated that glass ionomer materials exhibit substantially lower fracture toughness compared with composite resins. Since fracture toughness is a critical property that directly influences a material’s ability to withstand

functional stresses and resist crack propagation under masticatory forces, this finding further accentuates the structural deficiencies of GICs.

In the present study, the lowest shear bond strength values were also observed in the GIC group (10.37 ± 4.21 MPa), in full agreement with previous reports [4,18,22–24]. These results suggest that GICs possess a limited capacity to establish a durable adhesive interface with monolithic zirconia, and their relatively poor mechanical characteristics may have further contributed to their inferior bonding performance. Taken together, the evidence indicates that conventional GICs are not ideally suited for use as core build-up materials in conjunction with high-strength ceramics such as zirconia, and their clinical application may be more appropriately restricted to temporary or low-stress restorative contexts.

An ideal core build-up material is expected to possess adequate mechanical properties and effectively transmit functional forces. Zirconomer (Zr), developed to overcome the disadvantages of conventional GIC, has been introduced as an alternative material [7]. In a study conducted by Thomas et al. [22], Zirconomer was shown to have higher flexural strength compared with traditional GICs. Similarly, in our study, although the difference between the Zirconomer and GIC groups was not statistically significant, the shear bond strength of Zirconomer (14.19 ± 3.66 MPa) was higher than that of conventional GIC (10.37 ± 4.21 MPa). However, Zirconomer exhibited significantly lower shear bond strength values compared with the NHCR group, and this difference was statistically significant.

The differences in bond strength may be partly explained by the distinct compositions of the materials; the incorporation of zirconia fillers in Zirconomer could account for its improved performance over conventional GIC, while the resin matrix and nano-fillers in NHCR likely contributed to its superior bonding properties [4]. Recent international studies have shown that the incorporation of additives such as chitosan, glass fibers, and graphene oxide into conventional glass ionomer cements (GICs) significantly enhances their mechanical performance compared with unmodified formulations. These findings indicate that bio-reinforcement strategies may overcome the structural limitations of GICs and highlight the critical role of material composition in determining the bonding behavior and durability of restorative systems [23,24].

Prabakaran et al. [3] reported that NHCR exhibits superior mechanical properties compared with Zirconomer when used as core build-up materials. This has been further supported by Aditi et al. [7], who found that composite resins bonded to zirconia had significantly higher strength (7.25 MPa) than Zirconomer (0.74 MPa) ($p \leq 0.001$). Similarly, Nakade et al. [20] showed that composite resins had higher flexural strength than Zirconomer, and these findings are consistent with the present study. Abraham et al. [25] also compared the shear bond strength (SBS) of GIC, Zirconomer, and Luxacore core build-up materials to zirconia, reporting values of 9.51, 13.94, and 17.48 MPa, respectively. Based on these results, they recommended the combined use of chemical and mechanical surface treatments to improve the bond strength between MZ and Zirconomer.

The failure at the interface between monolithic zirconia (MZ) and core build-up materials is generally classified as adhesive, cohesive, or mixed, depending on the exact location at which the separation occurs during testing. Adhesive failures are characterized by debonding strictly at the interface, cohesive failures occur within the material itself, and mixed failures present features of both types. In a study by Aditi et al. [7], detailed analysis of the failure modes revealed 100% adhesive failures in the Zirconomer group, indicating a weaker interfacial bond between this material and zirconia. In contrast, the composite resin group demonstrated a more balanced distribution of failure types, with 43.8% cohesive, 31.2% mixed, and 25.0% adhesive failures, suggesting improved bonding performance and a stronger internal integrity within the material. Similarly, Giti and

Zarkari [8] reported 100% cohesive failures in their investigation, in which composite resin was bonded to zirconia discs, further emphasizing the relatively high internal strength of composite resins when compared with glass ionomer-based materials. These findings collectively highlight how the type of core build-up material can significantly influence the nature of failure at the zirconia interface, underlining the importance of material selection in restorative applications.

In this study, the failure modes observed in the Zirconomer group were 5% adhesive, 30% cohesive, and 65% mixed. In the GIC group, no adhesive failures were recorded; instead, 40% were cohesive and 60% were mixed. In the NHCR group, 15% of the failures were adhesive, 40% were cohesive, and 45% were mixed. The distribution of failure modes provides insight into both the internal strength of the core build-up materials and their bonding capacity with the zirconia surface. The high percentage of mixed (65%) and cohesive (30%) failures in the Zirconomer group suggests that the material formed only a limited bond with zirconia and may also lack sufficient structural strength. Similarly, the complete absence of adhesive failure in the GIC group, combined with a predominance of cohesive and mixed failures, indicates that this material also exhibited poor adhesion to the zirconia surface and had relatively low mechanical properties. In contrast, the more balanced distribution of failure types in the NHCR group (15% adhesive, 40% cohesive, 45% mixed) reflects both a strong internal structure and a more stable bond to zirconia. These findings support the clinical advantage of NHCR as core build-up materials [26].

Since this study focused on monolithic zirconia as the restorative material, its clinical performance is particularly relevant. Beyond the *in vitro* context, long-term clinical data are essential to validate the performance of zirconia restorations. In this regard, Lolos et al. [27] retrospectively evaluated 1143 zirconium oxide restorations over a 5-year period and reported an overall survival rate of 96.3%, with monolithic restorations achieving a 100% survival rate compared to 95.8% for layered restorations, where failures were mainly related to veneer chipping. These findings support the clinical reliability of monolithic zirconia, further reinforcing its relevance in prosthetic applications and aligning with the high bond strength values observed in our study. Moreover, Beketova et al. [28] demonstrated that the incorporation of zirconia nanoparticles into contemporary dental luting cements enhanced their physicochemical properties and shear bond strength to monolithic zirconia, highlighting how material innovations can further optimize the long-term clinical success of zirconia-based restorations.

This study has several limitations. Firstly, it was conducted under *in vitro* conditions, which limits the simulation of complex biological and mechanical factors encountered in the clinical setting (e.g., fluctuating moisture, temperature, pH, masticatory forces, and biofilm formation). In addition, thermocycling and artificial aging protocols were intentionally not included, as the primary aim was to establish a baseline comparison of shear bond strength between zirconia and different core build-up materials. Nevertheless, such protocols are essential for mimicking the clinical environment, and future studies should incorporate them to provide more clinically relevant outcomes. In this study, sandblasting was selected as the sole surface treatment together with a single self-adhesive dual-cure resin cement (RubySE Cem), which contains the 10-MDP monomer. This choice was made to ensure methodological standardization and reliable bonding to zirconia. Nevertheless, other surface conditioning methods (e.g., tribochemical silica coating, laser irradiation, plasma treatment) and alternative resin systems with different adhesive compositions or protocols may also influence bonding outcomes and therefore warrant further investigation. Since only one commercial brand was used for each core build-up material, the findings are specific to these products and cannot be generalized to all similar materials. Furthermore, this study evaluated only the shear bond strength; other loading types (e.g., microtensile,

flexural) and long-term durability or aging tests were not included. Therefore, to ensure the clinical applicability of these findings, more advanced and long-term clinical studies are needed.

5. Conclusions

Within the limitations of this study, the findings emphasize the importance of selecting appropriate core build-up materials to ensure the long-term success of zirconia-based restorations. In particular, nanohybrid composite resins demonstrated superior bonding potential, while zirconia-reinforced glass ionomer cements showed promise as alternatives to conventional GICs. Clinically, these results underline how material choice can directly influence the durability of zirconia restorations. Future research should focus on optimizing the bonding performance of modified glass ionomer systems and validating these outcomes through long-term clinical conditions.

Author Contributions: Conceptualization, A.Ş., B.A. and F.G.; methodology, A.Ş., F.G. and M.A.K.; investigation, O.A.H., S.K. and M.A.K.; resources, A.Ş.; data curation, A.Ş., B.A. and F.G.; writing—original draft preparation, A.Ş. and S.K.; writing—review and editing, A.Ş., B.A. and F.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors gratefully acknowledge Mustafa Yeşil of the Research Laboratory, Faculty of Dentistry, Ankara University, for his valuable assistance and support.

Conflicts of Interest: The authors declare no conflicts of interest.

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Systematic Review

Effects of Implant Silver Coatings on Bone Formation in Animal Models: A Systematic Review and Meta-Analysis

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Abstract: Background/Objective: Clinical statistics show that bacterial infection is a major driver of implant failure. To enhance antibacterial performance, some metallic elements, such as silver (Ag), zinc (Zn), and copper (Cu), are commonly used to modify the titanium surface. Despite the promising antibacterial performance of Ag, concerns persist regarding dose-dependent cytotoxicity, systemic accumulation, and potential effects on local bone metabolism. This review aimed to investigate the effects of incorporating or coating titanium (Ti) implant surfaces with Ag on bone formation around implants. Methods: A search was undertaken using three main databases (PubMed, Web of Science, and Scopus). The search was limited to studies published within the last 20 years that involved animal experiments using endosseous implants coated with or incorporating Ag. Meta-analyses were performed for bone-to-implant contact (BIC), bone formation (BA), and bone volume (BV/TV) around the implant in control and test groups. The compared groups were subjected to similar implant surface treatments aside from the presence of silver in the test group. Results: Sixteen studies met the inclusion criteria in this study and were included. The analysis of BIC values revealed a statistically significant overall effect in favor of silver-coated implants ($Z = 2.01$, $p = 0.04$), along with 95% confidence intervals (CIs). The BA analysis found no significant difference between silver-coated and control implants ($Z = 1.09$, $p = 0.28$). The BV/TV analysis also showed no statistically significant overall difference ($Z = 0.35$, $p = 0.73$). Conclusions: In animal models, silver-coated Ti implants improve bone–implant contact without altering peri-implant bone volume metrics.

Keywords: silver-coated titanium implants; bone formation; peri-implantitis; antibacterial coatings; animal models; meta-analysis

1. Introduction

Titanium (Ti) and its alloys are regularly used in implantable medical and dental devices because of their favorable biocompatibility, mechanical strength, and corrosion resistance [1,2]. Ti implants now represent the preferred modality for the restoration of edentulous spaces in dental practice [3,4]. Osseointegration around titanium (Ti) implants is a coordinated cascade of immune, angiogenic, and osteogenic events shaped by surgical protocol, implant surface chemistry/topography, primary stability, functional loading, and host factors [5,6]. However, unlike many other surgical procedures performed under strict aseptic conditions, dental implants are immediately exposed to the complex oral environment, which is home to diverse microorganisms [7,8].

Peri-implant infections are among the most significant complications related to dental implants, which involve inflammation of peri-implant tissues and continuing marginal bone loss [3,9]. These problems are more pronounced in individuals whose bone healing

is compromised by diabetes, older age, or osteoporosis [10,11]. Numerous studies have demonstrated a strong association between peri-implant infections and implant failure, with microbial biofilms playing a pivotal role in disease initiation and progression [6,10]. Once an implant-related infection is established, treatment often requires surgical debridement, implant removal, prolonged antibiotic therapy and, eventually, revision surgery, which imposes substantial clinical and financial burdens on both patients and healthcare systems [10,12].

To reduce such risks, several studies have focused on modifying implant surfaces to enhance osseointegration while minimizing bacterial colonization [13,14]. Nanoscale or microscale roughness could improve the adhesion and proliferation of osteoblasts, accelerating bone healing [13,15]. However, rougher surfaces at exposed regions can also promote bacterial attachment, potentially increasing the risk of infection [16,17]. Coating Ti implants with antimicrobial agents is one strategy to address this issue. Such coatings aim to provide an initial antibacterial effect during the early healing phase, when infection risk is highest, while maintaining compatibility with host tissues [18].

Silver (Ag) is one of the most extensively investigated antimicrobial agents for implant coatings [19,20]. It is active against a wide spectrum of pathogens, including many multidrug-resistant isolates, and its bactericidal properties have been attributed to multiple mechanisms: bacterial membrane disruption, reactive oxygen species generation, and inhibition of essential enzymatic systems [21,22]. Silver nanoparticles (Ag NPs) have enhanced antimicrobial activity compared with bulk Ag, mainly due to their large specific surface area and continuous release of Ag⁺ ions [20,23].

In vitro and in vivo reports have revealed the ability of Ag-coated Ti surfaces to reduce bacterial adhesion and biofilm formation without significantly impairing osteoblast function when used at controlled concentrations [24,25]. For example, Ti implants modified with Ag NPs have shown significant antibacterial effects against *Staphylococcus aureus* while maintaining favorable cell viability and alkaline phosphatase activity in osteoblast cultures [26]. Preclinical models have also reported reduced peri-implant infection with Ag-coated Ti implants and bone–implant contact ratios comparable to those of uncoated controls [27,28].

Several methods have been investigated to incorporate Ag into Ti implant surfaces, including plasma-immersion ion implantation, electrochemical deposition, and sol–gel techniques. Each method influences the release kinetics of Ag ions, which is critical for balancing antimicrobial efficacy with cytocompatibility [29,30]. Controlled, low-level Ag release is particularly desirable to minimize cytotoxic effects on host cells, as high local concentrations can impair osteoblast proliferation and differentiation [31,32].

The clinical use of Ag-coated Ti implants in orthopedic applications is more established, with Ag-coated prostheses having been successfully used in tumor surgery to prevent deep infections [33,34]. In dentistry, however, the clinical data remain limited. Early-stage studies have suggested that Ag coatings can reduce early colonization by pathogenic bacteria, but the long-term effects on bone remodeling and implant survival remain unclear [35]. Despite the promising antibacterial performance of Ag, concerns persist regarding its potential cytotoxicity, systemic accumulation, and the possibility of altering local bone metabolism. Some studies have reported delayed bone healing or reduced bone–implant contact at higher Ag concentrations, highlighting the need for precise control over release profiles [36,37]. The cytotoxicity of AgNPs is dose- and time-dependent [38]; however, at appropriately low concentrations, AgNPs can enhance cell proliferation and activity [39].

Given these considerations, this review investigated the effects of incorporating or coating Ti implant surfaces with Ag on bone formation around implants and whether antibacterial benefits can be achieved without compromising osseointegration.

2. Materials and Methods

2.1. PICO Question

This review was performed in accordance with the PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses, INPLASY registration number: INPLASY202590035) [40]. Before initiating the systematic literature search, the PICO framework was defined as follows: the Population (*p*) was defined as animal models; the Intervention (*I*) was Ti implants modified or coated with silver; the Control (*C*) was defined as Ti implants without silver modifications or coatings; and the Outcome (*O*) was bone formation.

2.2. Search Strategies

The literature search was undertaken in May 2025 using three main databases: PubMed, Web of Science, and Scopus. Only studies published within the last 20 years were considered. The search strategies in all the databases included terms related to four primary aspects: bone, implant, silver, and animal studies. The search terms used for the electronic search were (“bone formation” OR “osseointegration” OR “bone growth” OR “bone development” OR “bone remodeling” OR “bone regeneration”) AND (“implants” OR “titanium implants” OR “dental implants” OR “biocompatible coated materials” OR “endosseous dental implantation” OR “bone-implant interface”) AND (“antibacterial” OR “antimicrobial” OR “infection” OR “failure” OR “Coatings” OR “silver” OR “silver nanoparticles” OR “silver coating” OR “Ag”) AND (“in vivo” OR “animals” OR “animal models” OR “animal experimentation”).

2.3. Inclusion and Exclusion Criteria

The eligibility criteria were (1) studies conducted on animals that evaluated the use of silver coatings with endosseous implants. (2) The silver should be incorporated into the substrate or applied as a surface coating for these implants, either prior to or at the time of insertion. (3) Measurements of bone–implant contact (BIC), bone area (BA), or bone volume (BV/TV) should be reported for the control and test groups. (4) The compared groups should have received similar implant surface treatments aside from the presence of silver in the test group. (5) Only articles published in English were included. Clinical studies or in vitro investigations were excluded. Studies that did not report BIC, BA, or BV/TV values and the number of implants used in the evaluated groups were excluded.

2.4. Study Selection

All studies identified in the initial search were screened for eligibility according to the prespecified inclusion criteria. Full-text retrieval was performed for studies that fulfilled these criteria and those whose titles/abstracts lacked sufficient detail to determine their eligibility.

2.5. Data Extraction

When available, the following variables were collected via a standardized extraction form: study title, publication year, coating method, form of silver, animal species, number of animals and implants, duration between implant placement and animal euthanasia, as well as the mean values and standard deviations for BIC, BA, and BV/TV percentages around the implants.

2.6. Quality Assessment

The methodological quality was evaluated using the ARRIVE 2.0 (Animal Research: Reporting of In Vivo Experiments) guidelines [41]. Each of the 21 items was categorized as

follows: “reported” (two points) if all subitems were fully addressed, “not reported” (no points) if none were addressed, and “unclear” (one point) if some details were missing. Based on these scores, the quality coefficient was calculated for each study, which was categorized as follows: Poor (<0.5), Average (0.5–0.8), or Excellent (0.8–1).

2.7. Risk of Bias in Individual Studies

The risk of bias was estimated using the SYRCLE (Systematic Review Centre for Laboratory Animal Experimentation) RoB tool [42].

2.8. Data Analysis

The continuous outcomes assessed were BIC, BA, and BV/TV. The weighted mean differences were used to generate forest plots, with the number of implants in each group serving as the statistical unit. Records with insufficiently reported outcomes of interest were excluded from the analysis. Heterogeneity between the studies was quantified using the I^2 statistic, which represents the percentage of total variation due to heterogeneity. When statistical heterogeneity reached significance ($p < 0.05$), a random-effects (inverse-variance) model was used; if not, a fixed-effect model was used. Intervention effects are reported as mean differences (MDs) in percentage, along with 95% confidence intervals (CIs). The meta-analysis was completed using the Review Manager software (version 5.3.3, The Nordic Cochrane Centre, The Cochrane Collaboration).

3. Results

3.1. Literature Search

Figure 1 shows an overview of the study selection process. Following database searching, screening of titles and abstracts, and removal of duplicates, the full-text articles were evaluated for eligibility. In total, 16 animal studies met the eligibility criteria and were included in both the qualitative and quantitative syntheses [28,43–57]. PRISMA checklist is shown in Supplementary Materials (Table S1).

The study characteristics of the included articles are shown in Table 1. The investigations were conducted in a range of animal models including Wistar rats, New Zealand White rabbits, Sprague–Dawley rats, Labrador dogs, beagle dogs, pigs, and Merino sheep. A variety of silver modification strategies were reported, including nanotubular structures, plasma immersion ion implantation (PIII), electrodeposition, atomic layer deposition, wet chemical plating, magnetron sputtering, and hybrid coatings. Silver was delivered in several forms, such as nanoparticles, ions, nanolayers, and silver oxide. The outcome assessment methods predominantly employed micro-computed tomography (micro-CT) and histomorphometric analysis.

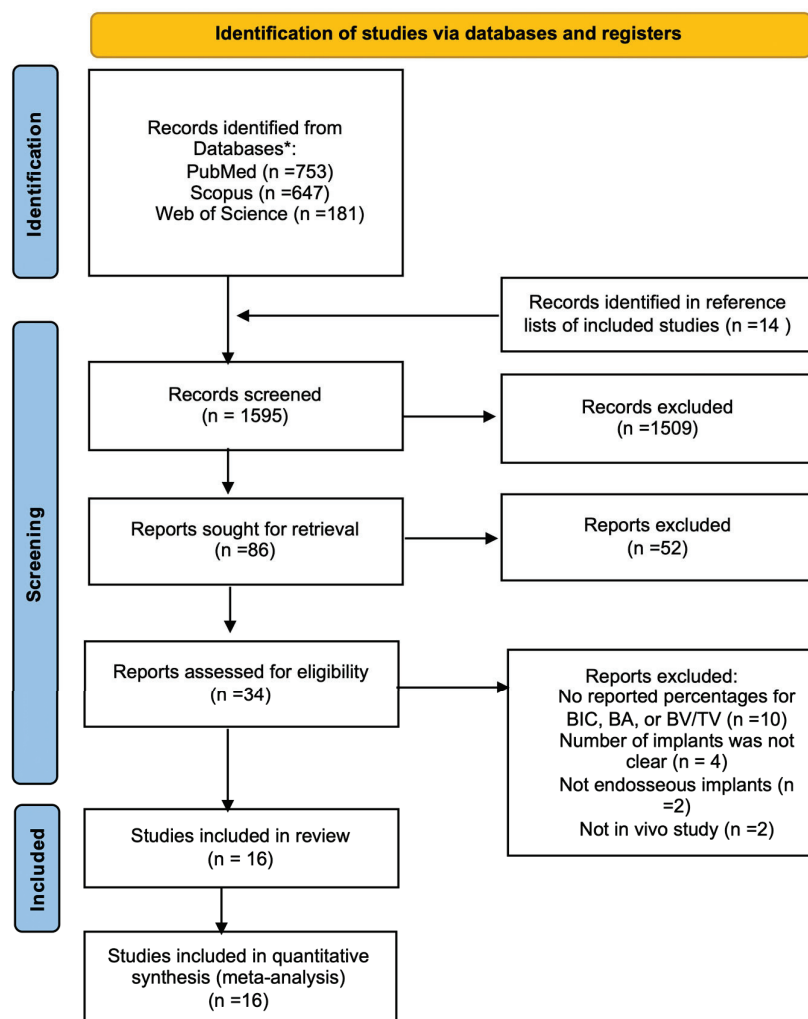


Figure 1. PRISMA flow diagram for the search process to identify studies to include in this review. The “*” represent the three main databases used for the electronic search: PubMed, Web of Science, and Scopus.

Table 1. Overview of characteristics of included studies.

Author	Silver Coating Technique	Silver Form	Animal Model	Bone Formation Outcome(s)	Evaluation Method(s)	Evaluated Groups	Healing Period
Cheng, 2013 [43]	Silver nanoparticle (Ag NPs) incorporated nanostructured Ti coating	Ag NPs	SD rats	BIC and BA	Histological evaluation	Control: Ti oxide nanotubes (TiO ₂ -NT); test group: NT-Ag	8 weeks
Svensson, 2013 [44]	Wet chemical plating process	Nanostructured coating	New Zealand White rabbits	BIC and BA	Histological evaluation	Control: non-coated implants; test group: coated implants	6 and 12 weeks
Qiao, 2015 [45]	Plasma immersion ion implantation of silver (Ag-PIII)	Ag NPs	Labrador dogs	BA and BIC	Micro-CT and histological evaluation	Control: sandblasted and acid-etched (SLA) implants; test groups: implants treated for (1) 30 min with 30 Ag PIII, (2) 60 min with 30 Ag PIII, and (3) 90 min with 30 Ag PIII	3 months
Devlin-Mullin, 2015 [46]	Silver nanolayer deposition via atomic layer deposition	Ag Nanolayer	Wistar rats	BA	Micro-CT analysis	Control: Ti; test group: Ti-Ag	12 weeks
Zhao, 2015 [47]	Plasma immersion ion implantation of silver (Ag-PIII)	Ag NPs	New Zealand White rabbits	BIC, BA, and BV/TV	Micro-CT and histological evaluation	Control: Ti; test groups: (1) Ag-PIII and (2) Mg/Ag-PIII	8 weeks
Godoy-Gallardo, 2016 [48]	Implants with silver electrodeposition	Ag electrodeposition	Beagle dogs	BIC and BV/TV	Micro-CT and histological evaluation	Control: Ti; test group: Ti-Ag	2 months

Table 1. Cont.

Author	Silver Coating Technique	Silver Form	Animal Model	Bone Formation Outcome(s)	Evaluation Method(s)	Evaluated Groups	Healing Period
Cheng, 2016 [49]	Silver (Ag)-loaded nanotubular structures	Ag NPs	Rats	BIC, BA, and BV/TV	Micro-CT and histological evaluation	Control: TiO ₂ -NTs; test groups: (1) NT10-Ag1, (2) NT10-Ag2, and (3) NT40-Ag1	6 weeks
Smeets, 2017 [50]	Silver-doped polysiloxane coating	Ag particles	Pigs	BIC	Histological evaluation	Control: grit-blasted and acid-etched Ti implants coated with polysiloxane; test group: silver-doped polysiloxane	3 months
Liu, 2019 [51]	Ti-Ag alloy nanotubular coatings (TNT)	Ag ions	Sprague–Dawley rats	BA	Micro-CT and histological evaluation	Control: TNT; test groups: (1) Ti1% Ag-NT, (2) Ti2% Ag-NT, and (3) Ti4% Ag-NT	(1, 2, and 4 weeks)
Xie, 2019 [52]	Coating consisting of hydroxyapatite (HA), AgNPs, and chitosan (CS)	Ag NPs	SD rats	BIC	Micro-CT and histological evaluation	Control: Ti implants; test group: HA/Ag/CS-coated implants	12 weeks
Stein, 2020 [53]	Titanium–vanadium implants (Ti6Al4V) coated with a polysiloxane layer with embedded elementary silver particles	Ag particles	Merino sheep	BIC	Histological evaluation	Control: uncoated Ti implants; test group: silver-coated implants	6 months
Chen, 2020 [54]	TiO ₂ nanotubes loaded with silver nanoparticles	Ag NPs	Sprague–Dawley rats	BV/TV	Micro-CT analysis	Control: TiO ₂ nanotubes (TiO ₂ -NTs); test group: AgTiO ₂ -NTs	2 weeks
Nakashima, 2022 [28]	Silver-containing hydroxyapatite coating	Silver oxide	Sprague–Dawley rats	BIC, BA, and BV/TV	Micro-CT and histological evaluation	Control: HA; test group: Ag-HA	4 and 8 weeks
Yao, 2022 [55]	Magnetron sputtering was used to form uniform Ag and strontium titanate (SrTiO ₃) coatings on Ti	Ag ions	New Zealand White rabbits	BV/TV	Micro-CT analysis	Control: AH-Ti; test groups: (1) AH-Ti/Ag and (2) AH-Ti/Ag/Sr	1 month
Li, 2023 [56]	Ti implants immersed in AgNP solution	Ag NPs	Sprague–Dawley rats	BIC, BA, and BV/TV	Micro-CT and histological evaluation	Control: Ti; test group: AgNP	4 weeks
Pinera-Avellaneda, 2023 [57]	Ti implants with an Ag-doped calcium–Ti surface layer	Ag ions	New Zealand albino rabbits	BV/TV	Micro-CT analysis	Control: Ti; test group: Ti-Ag	2 and 4 weeks

3.2. Quality Assessment

Quality was assessed using the ARRIVE 2.0 guidelines and the results are presented in Figure 2 and Table 2. The calculated coefficients ranged between 0.71 and 0.93. Eight studies achieved a rating of Excellent [28,44,45,48,50,52,53,56], while the remaining eight were graded as Average. No study was categorized as Poor.

Table 2. Quality coefficients of included studies.

Authors	Year	Animal Model	Coefficient	Quality
Cheng et al. [43]	2013	Rat	0.761	Average
Svensson et al. [44]	2013	Rabbit	0.809	Excellent
Qiao et al. [45]	2015	Dog	0.880	Excellent
Devlin-Mullin et al. [46]	2015	Rat	0.785	Average
Zhao et al. [47]	2015	Rabbit	0.714	Average
Godoy-Gallardo et al. [48]	2016	Dog	0.904	Excellent
Cheng et al. [49]	2016	Rat	0.738	Average
Smeets et al. [50]	2017	Pig	0.904	Excellent
Liu et al. [51]	2019	Rat	0.761	Average

Table 2. Cont.

Authors	Year	Animal Model	Coefficient	Quality
Xie et al. [52]	2019	Rat	0.904	Excellent
Stein et al. [53]	2020	Sheep	0.857	Excellent
Chen et al. [54]	2020	Rat	0.714	Average
Nakashima et al. [28]	2022	Rat	0.928	Excellent
Yao et al. [55]	2022	Rabbit	0.761	Average
Li et al. [56]	2023	Rat	0.857	Excellent
Pinera-Avellaneda et al. [57]	2023	Rabbit	0.761	Average

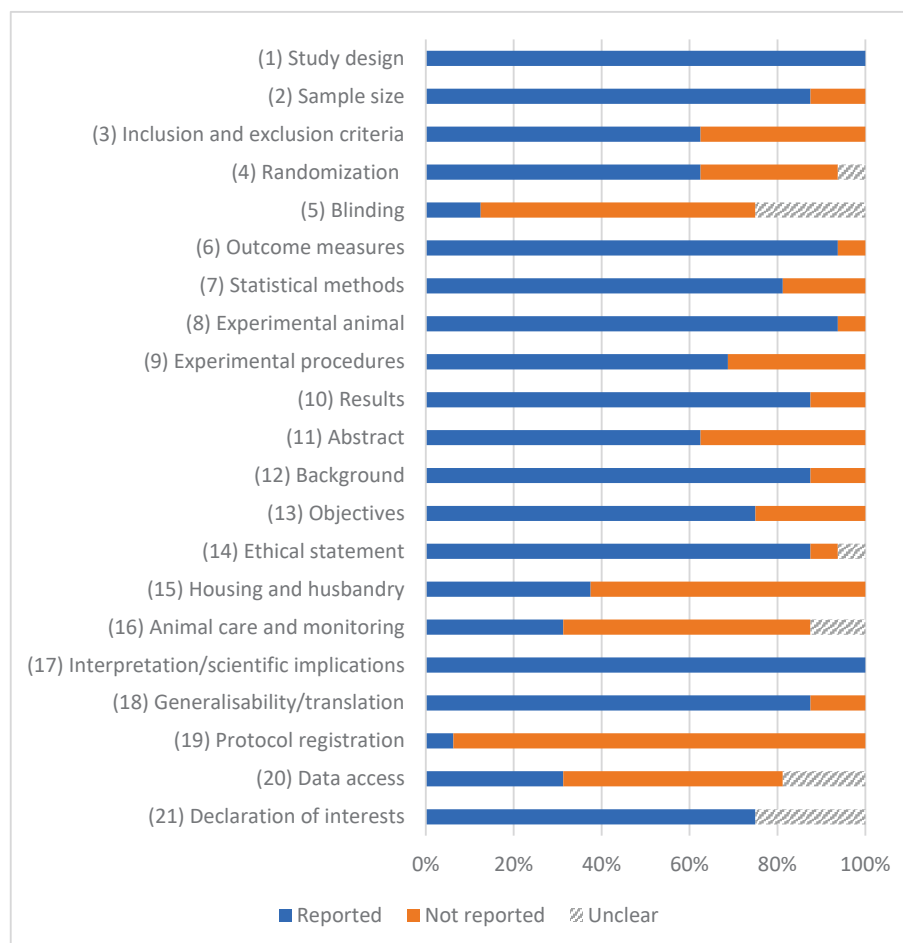


Figure 2. ARRIVE 2.0-based quality evaluation of included studies. Values are presented as percentages.

3.3. Risk of Bias

The assessment of the risk of bias based on the SYRCLE guide is displayed in Figure 3. In most studies, the risk of bias was judged to be unclear for both allocation concealment and outcome assessor blinding. Domains related to random sequence generation and selective outcome reporting showed lower levels of concern. Overall, variability in methodological care was observed among the included studies.

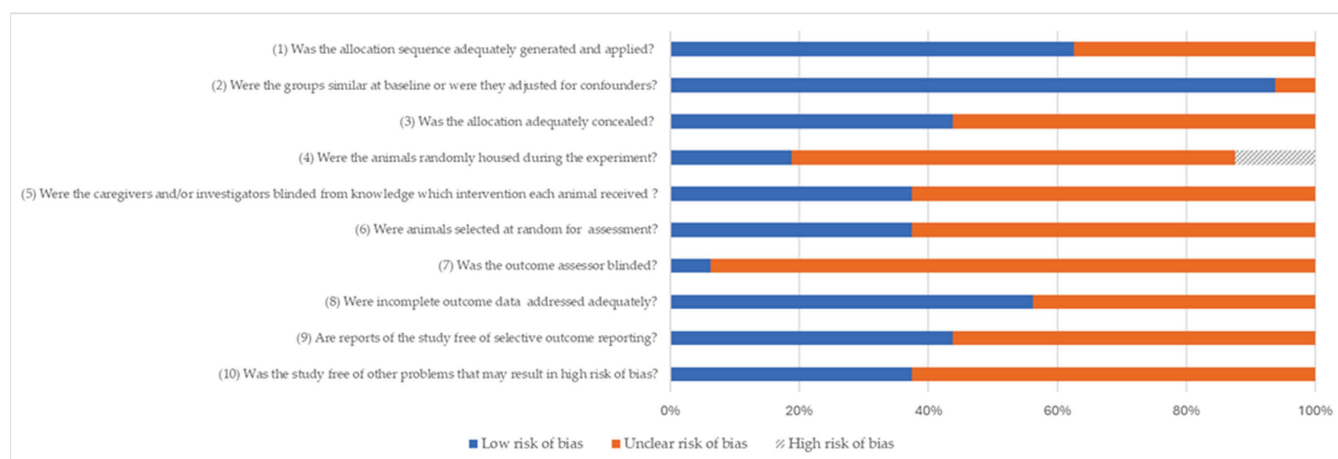


Figure 3. Risk of bias distribution according to Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) tool. Values are presented as percentages.

3.4. Meta-Analysis of Bone Formation Outcomes

3.4.1. BIC

The pooled analysis of BIC values (Figure 4) revealed a statistically significant overall effect in favor of silver-coated implants ($Z = 2.01$, $p = 0.04$). Substantial heterogeneity was observed ($\text{Tau}^2 = 190.60$; $\text{Chi}^2 = 555.54$, $\text{df} = 16$ ($p < 0.001$); $I^2 = 96\%$).

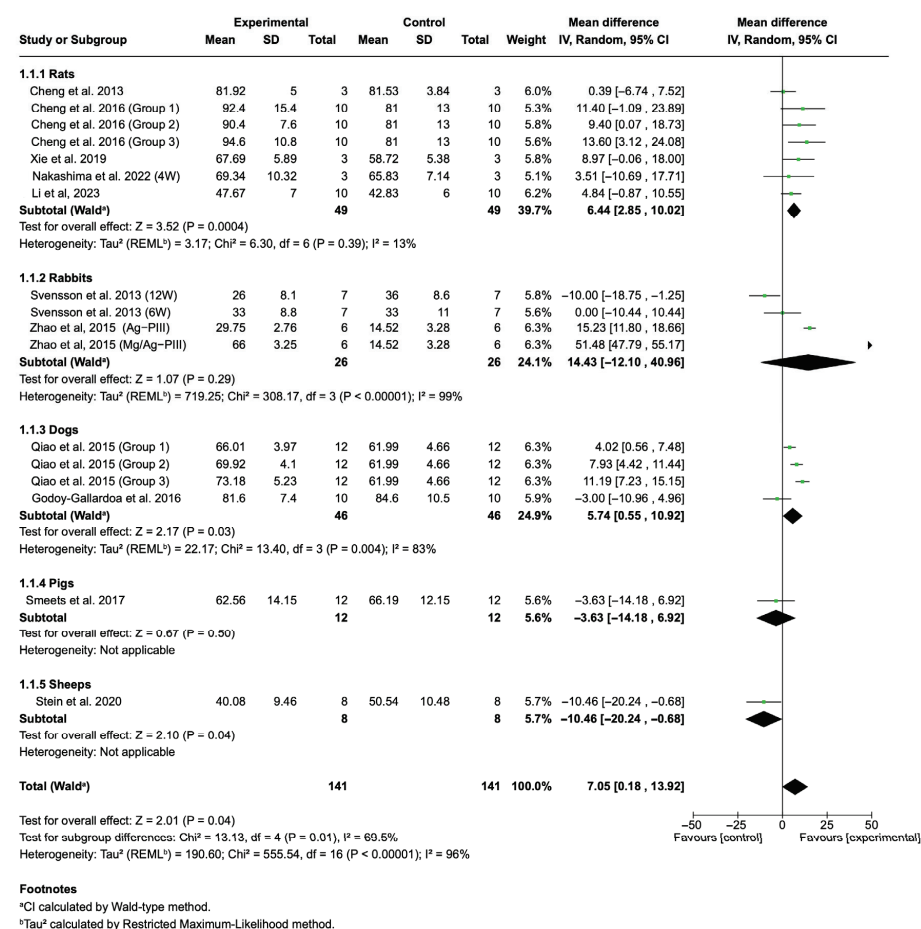
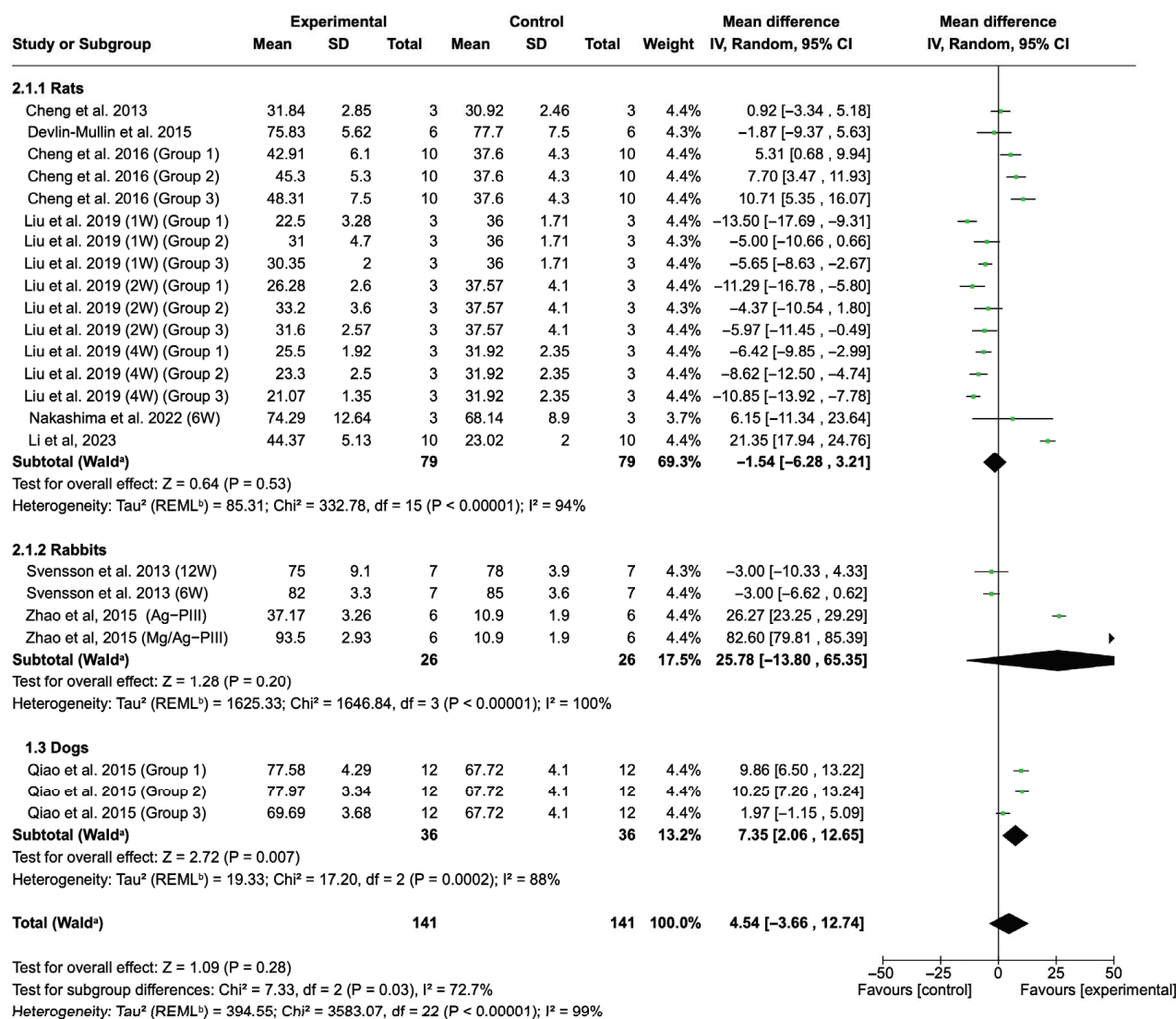


Figure 4. Forest plot summarizing the BIC values from the included studies on Ti implants with silver coatings [28,43–45,47,48,50,52,53,56]. The pooled analysis revealed a statistically significant overall effect in favor of silver-coated implants ($Z = 2.01$, $p = 0.04$).

3.4.2. BA

The pooled analysis of BA (Figure 5) demonstrated no significant difference between silver-coated and control implants ($Z = 1.09$, $p = 0.28$). Considerable heterogeneity was observed ($\text{Tau}^2 = 394.55$; $\text{Chi}^2 = 3583.07$, $\text{df} = 22$ ($p < 0.001$); $I^2 = 99\%$).



Footnotes

^aCI calculated by Wald-type method.

^b Tau^2 calculated by Restricted Maximum-Likelihood method.

Figure 5. Forest plot summarizing the BA values from the included studies on Ti implants with silver coatings [28,43–47,49,51,56]. The pooled analysis demonstrated no significant difference between silver-coated and control implants ($Z = 1.09$, $p = 0.28$).

3.4.3. BV/TV

The analysis of BV/TV (Figure 6) also showed no statistically significant overall difference ($Z = 0.35$, $p = 0.73$). High heterogeneity was detected across studies ($\text{Tau}^2 = 112.23$; $\text{Chi}^2 = 2432.44$, $\text{df} = 23$ ($p < 0.001$); $I^2 = 98\%$).

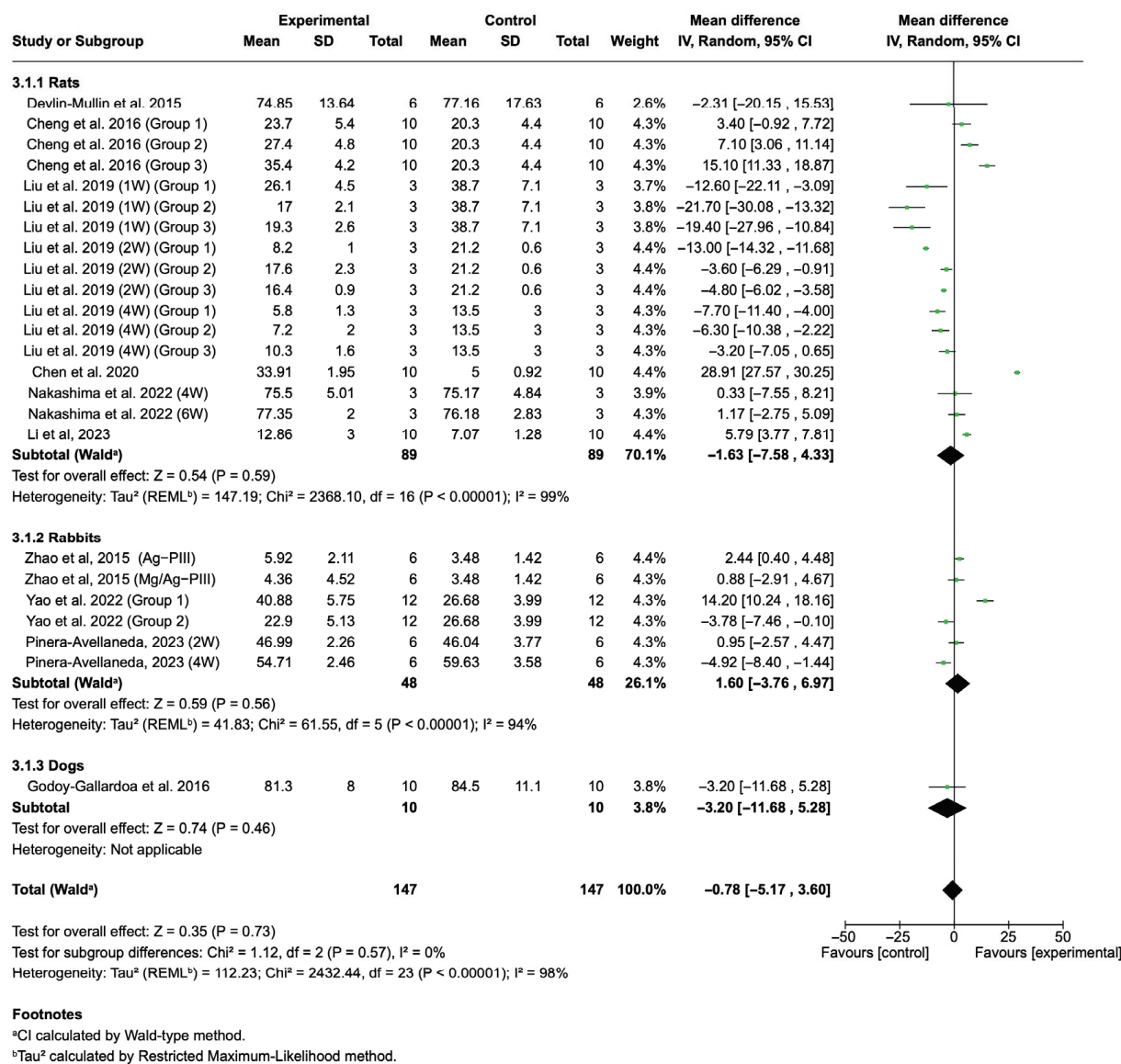


Figure 6. Forest plot summarizing the BV/TV values from the included studies on Ti implants with silver coatings [28,46–49,51,54–57]. The analysis of BV/TV showed no statistically significant overall difference ($Z = 0.35$, $p = 0.73$).

4. Discussion

This review evaluated the effect of Ag-coated Ti implants on bone formation around implant in animal models. Sixteen studies were included, and the pooled findings revealed that Ag coatings significantly improved BIC, while no significant differences were found for BA or BV/TV. These results indicate that Ag modifications may facilitate bone integration but do not consistently enhance overall peri-implant bone remodeling.

The integration of Ag into dental implant surfaces has been primarily driven by its antimicrobial potential, as peri-implant infections are among the most prevalent reasons for implant loss. Peri-implantitis is initiated by bacterial colonization and biofilm formation on implant surfaces, leading to a cascade of host immune responses, chronic inflammation, and progressive marginal bone loss [58,59]. Therefore, antibacterial surface modifications are being increasingly explored to prevent early microbial colonization and reduce the incidence of biological complications. Among these modifications, Ag has emerged as a particularly attractive candidate because of its broad-spectrum antibacterial ability and relatively low tendency to develop resistance [19,60].

At the molecular level, Ag ions exert bactericidal effects by binding to bacterial cell membranes, increasing membrane permeability, denaturing enzymes, and interfering with DNA replication [61]. Nanosilver particles also generate reactive oxygen species, further enhancing their antimicrobial efficacy. This potent antibacterial activity has been observed both *in vitro* and *in vivo*, with Ag-coated implants showing significantly reduced bacterial adhesion compared with unmodified Ti implants [35,62]. In the orthopedic field, clinical studies have confirmed reduced infection rates with Ag-coated prostheses [63,64] while dental research has focused on whether similar benefits can be achieved in oral implantology. Beyond antibacterial effects, Ti surfaces modified with Ag can promote early macrophage polarization from a pro-inflammatory M1 state to a pro-healing M2 phenotype, thereby moderating inflammation and supporting osteogenesis [54]. In parallel, sustained Ag release is associated with pro-angiogenic signaling, enhanced endothelial tube formation, and an increased microvessel density, collectively strengthening the angiogenesis–osteogenesis coupling required for durable osseointegration [24].

The significant improvement in BIC found in this review suggests that Ag coatings may support early osseointegration, either directly by enhancing osteoblast behavior or indirectly by suppressing bacterial competition at the interface (Figure 4). Several *in vivo* studies have confirmed this effect. For instance, Cheng et al. [49] demonstrated that Ag NP-modified nanotubular implants promoted greater BIC in rat models, while Xie et al. [52] observed improved osseointegration with Ag–hydroxyapatite hybrid coatings. At the cellular level, Ag has been reported to stimulate osteoblast adhesion and proliferation at low concentrations [35,65]. Furthermore, recent evidence has suggested that Ag coatings may also influence immune modulation, promoting a favorable M2 macrophage phenotype that enhances osteogenesis and angiogenesis [30,66]. This dual antibacterial–immunomodulatory function may explain why Ag coatings improved BIC across diverse experimental conditions.

In contrast, no significant differences were observed in BA or BV/TV, which is a volumetric indicator of peri-implant bone formation (Figures 4 and 5). This discrepancy between interfacial contact and bone volume suggests that the effect of Ag may be localized to the implant–bone interface rather than extending to overall bone remodeling. Several factors may account for this variability. First, the dose-dependent effects of Ag are critical: while low concentrations are osteoconductive, higher concentrations may impair osteoblast proliferation, collagen synthesis, and matrix mineralization [31,38]. Zhao et al. [35] demonstrated that excessive nanosilver exposure reduced osteoblast activity, while Greulich et al. [67] showed cytotoxicity to bone marrow stromal cells at elevated Ag doses. Second, discrepancies in coating techniques (e.g., plasma immersion ion implantation, electrodeposition, magnetron sputtering, and atomic layer deposition) lead to differences in Ag release profiles, which strongly influence biological outcomes [35,50]. Third, most studies used short- or medium-term follow-up, which may not capture the long-term remodeling processes critical for implant survival.

These findings position Ag coatings as dual-function surfaces in implant dentistry: their primary role remains antibacterial, protecting against peri-implant infections, while also appearing to maintain or even enhance interfacial bone contact. More importantly, none of the included studies in this review reported detrimental effects on bone formation, addressing the concerns about the potential cytotoxicity of Ag. This is reassuring for clinical translation, as infection control and bone preservation are interdependent requirements for long-term implant success. Notably, peri-implantitis and marginal bone loss often coexist, particularly in patients at high risk such as those with diabetes, poor oral hygiene, or osteoporosis [10,11,68]. For these populations, Ag-coated implants could offer a valuable adjunctive strategy to reduce complications.

When comparing Ag with other antimicrobial coatings, such as zinc, copper, or chitosan, the evidence suggests that while they all provide antibacterial activity, Ag remains the most extensively studied and exhibits one of the broadest spectrum of antimicrobial efficacy [35,66]. However, other materials may provide additional osteogenic benefits [69]. Zinc (Zn) coatings on titanium implants exhibit favorable bactericidal effects attributable to Zn incorporation [70], and in vivo studies suggest bacteria show limited resistance to Zn²⁺ [71]. Zn has also been associated with enhanced osteoblastic activity, supporting its use in bone-related applications [72,73]. Copper (Cu) can provide broad-spectrum antimicrobial activity [74]. Furthermore, Cu can be integrated into implant alloys such as Ti-6Al-4V to strengthen antibacterial performance and to enhance angiogenesis [75,76]. However, unlike Ag, elevated Copper ions (Cu²⁺) release has been linked to cytotoxicity, which necessitate careful control of release kinetics and coating thickness [35,77]. Comparative research is needed to determine whether Ag provides superior clinical benefits or whether combinations of antimicrobial and osteoinductive coatings may represent the optimal approach.

The limitations of the available evidence must be acknowledged. Large discrepancies were observed in all the pooled outcomes, reflecting the differences in animal species, sample sizes, healing times, and methodological approaches (Table 1). The risk of bias was often unclear due to inadequate reporting of allocation concealment and blinding (Figure 3). Moreover, peri-implantitis and marginal bone loss are long-term complications, but the included studies largely reported short-term results (≤ 6 months). Thus, it remains uncertain whether the antibacterial advantages of Ag coatings translate into sustained improvements in peri-implant bone stability and implant survival.

Future research should prioritize long-term preclinical studies using standardized protocols and large animal models to better approximate clinical conditions. Optimization of Ag concentration, particle size, and release kinetics is necessary to balance antibacterial efficacy with cytocompatibility. Moreover, comparative clinical trials are needed to assess Ag's effectiveness relative to other antibacterial coatings and to confirm whether the observed improvements in BIC can be translated into reduced risks of peri-implant disease and bone loss in patients.

5. Conclusions

This review demonstrated that Ag-coated Ti implants are associated with a significant improvement in BIC, whereas no consistent effects were observed for BA or BV/TV. These findings suggest that Ag coatings, while primarily applied for their antibacterial properties, may also support interfacial bone healing without compromising peri-implant bone formation. Further preclinical and clinical investigations are warranted to clarify the dose-response relationship, optimize coating strategies, and determine whether the antibacterial advantages of Ag coatings translate into improved peri-implant bone preservation and implant survival in dental practice.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/jfb16100369/s1>, Table S1: PRISMA checklist. Reference [78] is cited in the Supplementary Materials.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: The researcher would like to thank the Deanship of Graduate Studies and Scientific Research at Qassim University for providing financial support (QU-APC-2025).

Conflicts of Interest: The author declares no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BIC	bone–implant contact
BA	bone area
BV/TV	bone volume

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Article

Influence of Different Biomaterials Extracted from Autologous Blood on the Cell Migration of Stem Cells from Dental Pulp

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Abstract: Background: This study aims to evaluate the effect of different types of platelet concentrates (autologous blood biomaterials) on the migration potential of human dental pulp stem (hDPSCs). Materials and Methods: Our team created a model of human dental pulp stem cells (hDPSCs). Various types of AB biomaterials were produced from blood samples from volunteers using the protocols presented: A-PRF+, Gel A-PRF+, and Solid PRF. The scratch wound healing assay was used to examine the closure of the experimental wounds on day 1 and day 14. The wound areas were quantified using Image J software. Statistical analysis was performed with the Kruskal–Wallis and Mann–Whitney U tests, as the data did not follow a normal distribution, which was confirmed by the Shapiro–Wilk test ($p < 0.05$). Results: The results demonstrate significantly faster closure of the experimental wounds on day 14 of the studied biomaterials AB: A-PRF+, Gel A-PRF+, and Solid PRF compared to the control group of cells. Gel A-PRF+ exhibited the most pronounced stimulatory effect on cell migration ($p = 0.0036$ vs. control), followed by Solid PRF and A-PRF+. Conclusions: The results indicate that autologous blood platelet concentrates stimulate the migration of hDPSCs in vitro. Gel A-PRF+ demonstrated the strongest effect, underscoring its potential clinical relevance for applications in tissue engineering.

Keywords: dental pulp stem cells; platelet concentrates; A-PRF; Solid PRF; scratch wound healing assay; dental pulp regeneration; tissue engineering

1. Introduction

Regenerative endodontics has progressed from descriptive biology to clinically oriented strategies that leverage human dental pulp stem cells (hDPSCs) to promote reparative dentinogenesis and preserve pulp vitality. Recent studies demonstrate that early recruitment and directional migration of pulp progenitors to the exposure site are prerequisite steps that precede matrix deposition and dentin bridge formation, providing a mechanistic basis for vital pulp therapy. Emerging translational approaches—combining bioactive scaffolds, controlled growth-factor release, and minimally invasive protocols—aim to enhance the pulp–dentin complex’s innate healing potential and shorten recovery timelines [1].

From a patient perspective, vital pulp therapy offers tangible clinical benefits: it reduces treatment invasiveness, preserves sensibility and immune surveillance within the tooth, maintains structural integrity, and may lower the risk of long-term fractures relative

to full pulpectomy. These advantages translate to fewer appointments, shorter chair time in selected cases, and improved long-term tooth survival, reduced need for root-canal therapy, particularly when indications are carefully selected and hemostasis and asepsis are rigorously controlled [2].

In the context of endodontic healing, the term “migration to damaged tissues” is used to denote the early recruitment of hDPSCs to the site of pulp exposure. This process is a necessary prerequisite for initiating subsequent reparative dentinogenesis, expressed by the formation of a hard-tissue dentin bridge, and for maintaining the vitality of the pulp’s soft-tissue component [1,2].

Accordingly, the ultimate therapeutic objectives of regenerative endodontics are twofold: (i) to induce and stabilize a hard-tissue response in the form of reparative dentin, establishing a biological barrier, and (ii) to preserve pulp vitality long-term with functional restoration and maintenance of the pulp–dentin complex. These parameters serve as clinically relevant endpoints for evaluating the effectiveness of biological and bioactive approaches, including the application of PRF biomaterials [1,2].

Seo BM and co-authors prove that human mesenchymal stem cells isolated from pulp, periodontal ligament, and bone marrow regenerate these tissues [3]. Human dental pulp stem cells (hDPSCs) have high proliferative capacity and multilineage differentiation potential. They have the ability to migrate and close tissue defects. This is essential for the regeneration of injuries in pulp tissues. Shi and Huang found that hDPSCs are promising for differentiation into odontoblast-like cells [4,5].

From human dental pulp mesenchymal stem cells in vitro conditions, a cell model can be created for conducting in vitro studies. This model is used to investigate the potential of various biological materials for repairing damage to the dental pulp. One such method is the scratch wound healing assay, which is used to study cell migration in two dimensions. The leading indicator for assessing wound healing is the rate at which the gap closes. It quantitatively reflects the dynamics of the collective movement of stem cells. The rate is determined by measuring the reduction in the cell-free area over time. By analyzing the results, the migration potential of biomaterials under different experimental conditions is compared [6].

Calcium–silicate cements, including MTA and Biodentine, constitute the benchmark for direct pulp capping because they ensure durable sealing, exhibit high biocompatibility, and consistently induce reparative dentin. In contrast, autologous PRF is positioned as a complementary bioactive adjunct that delivers a fibrin scaffold with sustained growth-factor release. Current evidence does not establish PRF as a full substitute for calcium–silicate cements; combined protocols remain a reasonable hypothesis pending rigorous clinical validation [7–9].

Platelet-rich fibrin (PRF) biomaterials are a second-generation platelet concentrate developed as a biologically more active material than platelet-rich plasma (PRP). Unlike PRP, the PRF manufacturing protocol does not use anticoagulants or exogenous thrombin, allowing for slow and natural fibrin polymerization and the formation of a three-dimensional network close to the physiological one. The PRF structure retains platelets and growth factors, which are released gradually, thus creating an autologous matrix that promotes cell migration, proliferation, and tissue regeneration [10–12]. Among the main factors identified in PRF are platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- β 1), as well as interleukins (IL-1 β , IL-6, IL-4) and tumor necrosis factor α (TNF- α), which together regulate the inflammatory response, angiogenesis, and stimulate cell migration [12]. PRF serves as a reservoir of bioactive molecules that stimulate wound healing and bone regeneration processes. Recent studies demonstrate the specific effect of PRF

on pulp stem cell migration [13]. Margono et al. (2023) report that culturing these cells in the presence of Advanced PRF+ (A-PRF+) significantly increases their migration rate, confirming the therapeutic potential of PRF in tissue engineering [14].

Various protocols have been established for the preparation of autologous blood biomaterials, including A-PRF+, Gel A-PRF, and Solid PRF. However, despite their increasing use in regenerative dentistry, the comparative biological effects and clinical outcomes of these PRF variants in vital pulp therapy remain insufficiently investigated, and no comprehensive data are currently available in the scientific literature [15].

This investigation aims to evaluate the effect of different types of autologous blood biomaterials on the migration potential of hDPSCs using a scratch wound healing assay. The null hypothesis is that there is no difference in the stimulation of cell migration between the different types of biomaterials from autologous blood and the control conditions.

2. Materials and Methods

In accordance with the Helsinki Declaration II, a set of ethical principles for medical research involving human subjects, all subjects provided informed written consent for participation. The Medical University of Sofia Research Ethics Committee, a body responsible for ensuring the ethical conduct of research involving human participants, approved this project (approval code: 57 from 12 July 2024), a map for scientific developments and projects anticipating scientific research involving human participation. Ethical approval was obtained for the use of extracted human teeth, for venipuncture blood sampling, and for the conduct of clinical investigations.

The study was conducted on human dental pulp stem cell cultures. The cultures were prepared as follows:

2.1. Isolation and Cultivation of hDPSC

Human dental pulp samples were obtained from extracted third molars of healthy donors aged 18–25 years. The teeth were removed for orthodontic reasons under aseptic conditions. Third molars were impacted or partially erupted but not carious; teeth with prior endodontic treatment, restorations, cracks, or caries were excluded. After extraction, each tooth was cleaned of soft deposits and placed individually in separate sterile containers filled with DMEM (Dulbecco's Modified Eagle Medium, high glucose; Sigma-Aldrich (St. Louis, MO, USA), Cat. No. D5648) and immediately transported to the laboratory under sterile conditions to ensure the preservation of cell viability. In the laboratory, each tooth was processed separately to avoid cross-contamination. The crown was carefully separated, and the dental pulp was extirpated with sterile endodontic instruments. The tissue fragments were incubated for 60 min at 37 °C in a solution containing Collagenase type I (Sigma-Aldrich, Cat. No. C0130) and Dispase II (Sigma-Aldrich, Cat. No. D4693), with periodic stirring. After centrifugation at 2700 rpm for 3 min, the cell pellet was resuspended in DMEM (Dulbecco's Modified Eagle Medium, high glucose; Sigma-Aldrich, Cat. No. D5648).

The cells were cultured in DMEM, high glucose, enriched with 10% FBS (Fetal Bovine Serum, heat-inactivated; Sigma-Aldrich, Cat. No. F7524), 1% Antibiotic-Antimycotic solution (100×; Penicillin/Streptomycin/Amphotericin B; Sigma-Aldrich, St. Louis, MO, USA) and NaHCO₃ (≥99.7%, Merck (Rahway, NJ, USA)/Sigma-Aldrich, Cat. No. A0384), at 37 °C, 5% CO₂ and saturated humidity. The medium was replaced daily.

Upon reaching approximately 80% confluence, the cells were meticulously separated by treatment with 0.25% Trypsin-EDTA (Sigma-Aldrich, Cat. No. T4049) for 10 min, and then resuspended in PBS (Phosphate-Buffered Saline, pH 7.4; Sigma-Aldrich, Cat. No. P3813) and cultured in new Petri dishes. Human dental pulp stem cell cultures (passage P4) were seeded

in 6-well plates and cultured until a confluent cell layer was reached within 24–48 h, ensuring the highest standards of cell culture.

To prove the human stem cell origin of the isolated hDPSCs, the cell cultures were stained immunofluorescently with the markers CD44 (HCAM, DF1485)—mouse monoclonal IgG1, 200 µg/mL (Santa Cruz Biotechnology, USA), Nestin (D-9)—mouse monoclonal IgG2a, 200 µg/mL (Santa Cruz Biotechnology, USA), CK19 (ab52625)—Cytokeratin 19, rabbit monoclonal, 0.838 mg/mL (Abcam, UK) and DSPP (LFMb-21)—mouse monoclonal IgG2b, 200 µg/mL (Santa Cruz Biotechnology, USA) and the nuclei were stained with DAPI (D9542)—nuclear fluorescent dye (Sigma-Aldrich, USA). The images were captured at $\times 60$ magnification using IN Cell Analyzer 6000 system (GE Healthcare, Pittsburgh, PA, USA).

2.2. Preparation of Biomaterials from Autologous Blood (AB Biomaterial)

2.2.1. Donor Inclusion and Exclusion Criteria

All participants provided written informed consent prior to blood collection. The inclusion criteria comprised clinically healthy ten male donors aged 25–35 years, non-smokers, with no history of systemic, infectious, or autoimmune diseases. The participants were not professional athletes and were not taking any medications known to affect platelet function (anticoagulants, corticosteroids, or nonsteroidal anti-inflammatory drugs).

The exclusion criteria included ongoing therapy with anticoagulant or anti-inflammatory drugs, the presence of acute infections, or hematological disorders.

Thirty milliliters of blood were collected from each participant via venipuncture (three 10 mL tubes). From these samples, three different protocols for AB biomaterials were used to prepare samples for each participant. The blood samples were centrifuged in two different centrifuges.

2.2.2. Type of Centrifuges Used to Obtain Biomaterials from PRF

1. Centrifuge with fixed angle of the slots: DUO Quattro PRF centrifuge (Process for PRF, Nice, France) with the corresponding tubes provided according to the specifications of the same manufacturer with a red cap A-PRF (A-P by Choukroun, Process for PRF, Nice, France) and a green cap S-PRF (S Process for PRF, Nice, France) and are anticoagulant-free. The tubes differ in the internal treatment of their walls and are free from anticoagulants. The AB biomaterials A-PRF+ and Gel A-PRF+ were obtained according to the protocols of Choukroun J [10].
2. Centrifuge with horizontal arrangement of the slots during operation: Bio-PRF Horizontal Centrifuge with the corresponding tubes provided according to the specifications of the same manufacturer. Bio-PRF Red Glass Tubes, 100% glass (Bio-PRF, Jupiter, FL, USA). The test tubes differ in the internal treatment of their walls and are free of anticoagulants. The AB biomaterial Solid PRF was obtained according to Miron's protocols [15].

A comparison of the technical characteristics of the two centrifuge types is presented in Table 1

Table 1. Centrifuge Configuration for PRF: Comparative Summary of Fixed-Angle (Choukroun) and Horizontal (Miron) Systems.

Parameter	Fixed-Angle (“Vertical”, Choukroun)	Horizontal (Swing-Out, Miron)
Rotor orientation	Tubes fixed ~25–45°; separation along an inclined column	Buckets swing to true horizontal; separation across a short vertical column
RCF field	Steeper radial RCF gradient along tube length	More uniform in-tube RCF distribution
Cell distribution	Sharper phase demarcation; higher g/longer spins may drive cells toward RBC layer	Broader interface; often higher platelet/leukocyte recovery at matched RCF/time
Typical PRF protocols *	A-PRF/A-PRF+: lower g, longer time (≈few hundred g for ~8–14 min) → looser fibrin, better cell preservation	Solid/liquid PRF variants: multiple validated protocols across a range of g and durations
Tubes/consumables	Prefer glass/plain, no anticoagulant	Same; tube material/coatings can modulate fibrin polymerization
Temperature/braking	Room temperature; gentle braking; start timing after target speed	Same

* The protocol is different for different centrifuges.

Three groups of samples of tested AB biomaterials were created as follows:

1. Group 1. AB biomaterial Gel A-PRF+
2. Group 2. AB biomaterial Solid PRF
3. Group 3. AB biomaterial A-PRF+

2.2.3. Protocols for AB Biomaterial: Gel A-PRF+; Solid PRF and A-PRF+

Protocol for Autologous Blood Biomaterial A-PRF+

Ten milliliters of venous blood, meticulously collected in red-capped A-P by Choukroun glass tubes, was used. The blood was then subjected to a precise centrifugation process in a DUO Quattro PRF centrifuge at 1300 rpm for 14 min. After this, the tubes were opened for 4–5 min, and the fibrin clot was separated from the erythrocyte mass with utmost care and precision, without cutting. The clot was then placed on a grid in a PRF box and compressed for 5–6 min until an A-PRF+ membrane was obtained, ensuring the highest quality of the biomaterial.

The amount of separated transudate collected with the processing box was used to prepare Gel A-PRF+.

Protocol for Autologous Blood Biomaterial Gel A-PRF+

Ten milliliters of venous blood, collected in green-capped S-PRF glass tubes, were used. The blood was centrifuged in a DUO Quattro PRF centrifuge at 1300 rpm for 14 min. After centrifugation, the upper liquid fraction, rich in platelets and fibrinogen, was separated with a sterile needle No. 18 and transferred to a sterile tray, where it was mixed with the transudate separated in the box to obtain A-PRF+. After 10–15 min, gelation occurs, and the resulting gel-like material is compressed six times with a hand tool to reduce the liquid and obtain a Gel-A-PRF+ membrane.

Protocol for Autologous Blood Biomaterial Solid PRF

Ten milliliters of venous blood, collected in Bio-PRF Red Glass Tubes, were used. The blood was centrifuged in a Bio-PRF Horizontal Centrifuge at 700 rpm for 8 min. After centrifugation, the tubes were opened for 4–5 min, after which the fibrin clot was separated from the erythrocyte mass without cutting. The clot was placed on a grid in a Bio-PRF box and compressed for 5–6 min until a Solid PRF membrane was formed.

Each membrane obtained according to the above protocols was weighed on an analytical scale KERN ABT 120-5DM (KERN & Sohn GmbH, Balingen, Germany) to determine the volume of the test materials. They were then placed in a test tube containing 10 mL of DMEM medium and incubated at 4 °C. The membranes remained in DMEM, and samples of the conditioned medium were collected on days 1 and 14.

2.3. Test Method: Scratch Wound Healing Assay

The scratch assay is a standardized surrogate of cell motility, modeling early recruitment relevant to initial closure of a pulp exposure; we acknowledge its limitations (cannot fully separate migration from proliferation) and cite its accepted use in dental tissue-engineering research. On the day before the experiment, the cell cultures were placed in a medium containing 1% FBS to minimize the effect of cell division. The cell cultures were placed in wells. A linear defect (“wound”) scratch zone was created in each well using a sterile tip of a 200 µL pipette. The wells were then washed three times with PBS (Phosphate-Buffered Saline, pH 7.4) to remove any detached cells.

1.8 mL of each type of AB biomaterial from was added to the prepared stem cell cultures for the first and second study periods. After the removal of 1.8 mL from the basal media for the first period of the experiment, an additional 1.8 mL of DMEM was added to the tubes containing the tested PRF membranes in order to restore the initial volume. After placing the conditioned medium in the wells, 0.2 mL of FBS was added to a final concentration of 10% to provide essential nutrients and growth factors for the cells.

The scratch areas were photographed with an inverted phase-contrast microscope Leica DMI3000 B (Leica Microsystems, Wetzlar, Germany, objective 5×/0.12). immediately after the wound was created (0 h). The cultures were maintained at 37 °C, 5% CO₂, and 50% humidity, and were documented again at 24 h and 48 h. The second experiment (14-day incubation of membrane samples) was performed on new cell cultures of pulp stem cells in 6-well plates with cells from passage P4 using the same Protocol (Figure 1).

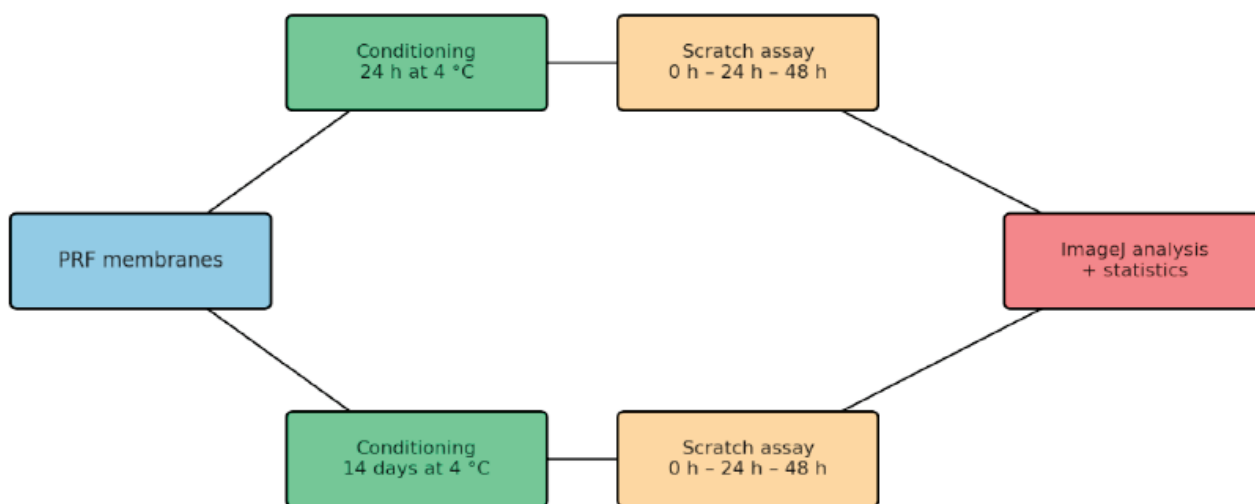


Figure 1. Schematic diagram of the experimental design. The PRF membranes were incubated in DMEM culture medium at 4 °C for two time periods: 24 h to observe the immediate effects, and 14 days to assess the long-term impact of the biomaterials on wound healing.

The obtained images were analyzed using Wound Healing Size plugin for ImageJ (ImageJ v1.x/ Fiji distribution; Version 1.54p, National Institutes of Health, Bethesda, MD, USA) developed by Suarez-Arnedo et al. The plugin automatically distinguishes the wound area from the surrounding cell monolayer using contrast enhancement, variance filtering, and morphological reconstruction (“hole filling”) to isolate the open area. The

analysis was carried out by measuring the wound area at each time point, which allowed for the accurate calculation of the wound closure rate, providing reliable and valid results for the study [16].

Advanced statistical methods were applied using IBM SPSS (Statistics 26, IBM, NY, USA), ensuring the robustness of the data analysis. Graphical representations were then created with Excel 2010, providing a clear and comprehensive visualization of the results. The normality of data distribution was assessed using the Shapiro–Wilk test, which indicated that the data did not follow a normal distribution. Therefore, non-parametric statistical tests were applied: the Kruskal–Wallis test for overall group comparisons and the Mann–Whitney U test with Bonferroni correction for pairwise analyses. A p -value of <0.05 was considered statistically significant. Effect sizes (r) and 95% CIs are reported.

3. Results

To confirm the phenotype of hDPSCs, immunofluorescence staining was performed (Figure 2), which showed positive expression of the mesenchymal marker CD44, confirming the mesenchymal origin of hDPSCs. The expression of Nestin and DSPP is consistent with their neurogenic and odontogenic potential, while CK19 highlights the possibility of epithelial-like differentiation and heterogeneity of the cell population.

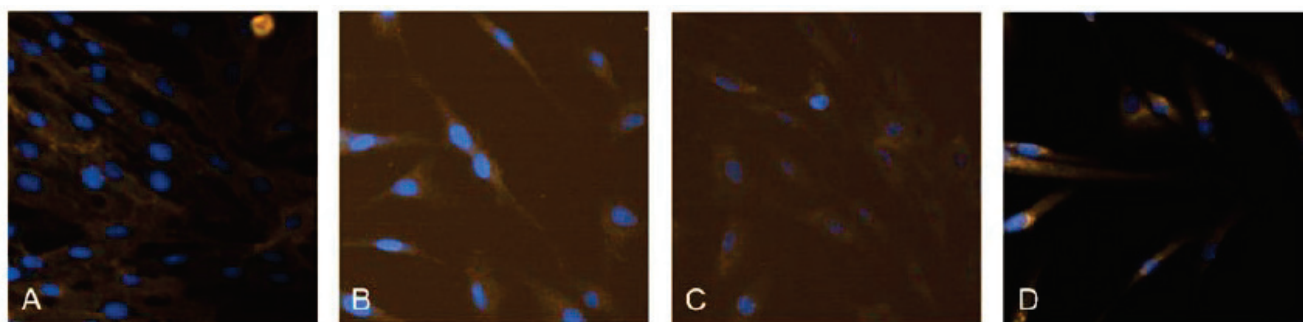


Figure 2. Immunofluorescence characterization of hDPSCs. Images with positive staining for (A) CD44, (B) Nestin, (C) CK19, and (D) DSPP. Nuclei are stained with DAPI. All images were captured at $60\times$ magnification.

The expression of the markers confirms the mesenchymal origin and multipotency of hDPSCs.

3.1. Results from the First Day of the Study

The results from the first day of the study are presented in Figures 3 and 4 and Table 2.

Image J software (Wound Healing Size plugin for Image J) was used to calculate the wound area in pixels, wound width in pixels, and wound area as a percentage. From these data, we calculated the average values, as shown in Figure 2, which illustrates the dynamics of wound closure in the different PRF groups compared to the control group. When tracking Day 1, it is evident that the quantitative assessment of the images reveals that Gel A-PRF+ yields the most pronounced reduction in wound percentage, area in pixels, and width in pixels, as early as 24 h and especially at 48 h. Solid PRF and A-PRF+, on the other hand, did not reach statistical significance compared to the control group, which highlights the specific effectiveness of Gel A-PRF+.

p -values and corrected p -values are indicated. At 24 h, a significant difference was found between the Gel A-PRF+ and A-PRF+ groups, and at 48 h, between the Control group and the Gel-A-PRF+ group. This shows that the effect of the studied biomaterials on cell migration varies over time.

According to the results of the present study, we observed a substantial effect size for the tested materials both relative to the control cells and among the materials themselves. The results obtained are visualized in Figure 4.

Median wound area for hDPSCs at 24 h and 48 h (Day 1)

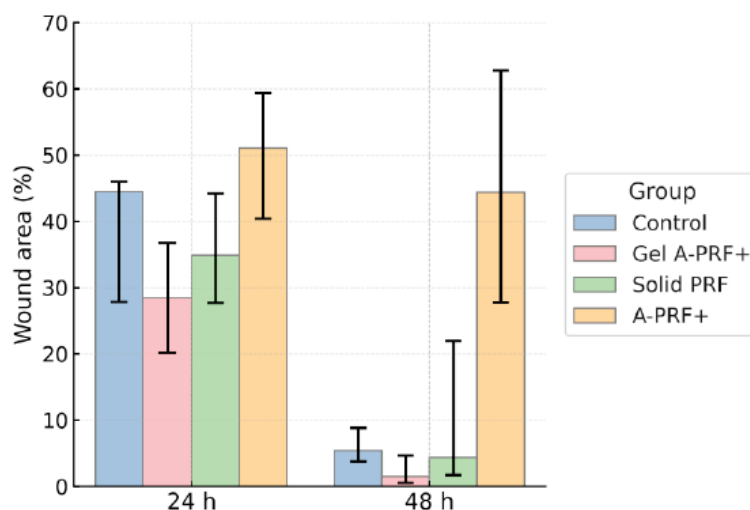


Figure 3. Scratch healing test—day 1. Quantitative analysis of wound area reduction (%) in hDPSCs at 24 h and 48 h after exposure to Control, Gel A-PRF+, Solid PRF, and A-PRF+. The chart illustrates the mean values showing the dynamics of wound closure on Day 1, with Gel A-PRF+ exhibiting the most pronounced effect.

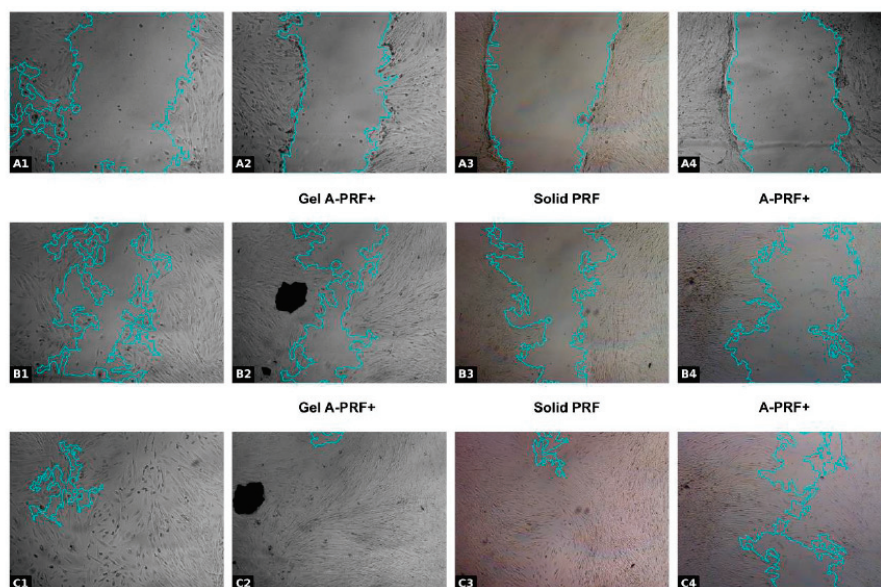


Figure 4. Representative images of wound healing assay at different time points (Day 1: 0 h, 24 h, 48 h) in dental pulp stem cells exposed to various platelet concentrates. Panels: (A1–A4) Control group at 0 h; (B1) Control group, (B2) Gel-A-PRF+ group; (B3) Solid PRF group; (B4) A-PRF+ group at 24 h; (C1) Control group; (C2) Gel-A-PRF+ group; (C3) Solid PRF group; (C4) A-PRF+ group at 48 h; Scale bar = 500 μ m (applies to all panels).

Table 2. Post hoc Mann–Whitney U test with Bonferroni correction for multiple comparisons regarding the percentage of wound area reduction on the first day of the study at 24 and 48 h.

Days of Investigation Comparison Between Group	24 h/Day 1			48 h/Day 1		
	<i>p</i> -Value Mann–Whitney	<i>p</i> -Bonferroni	Effect Size (<i>r</i>)	<i>p</i> -Value Mann–Whitney	<i>p</i> -Bonferroni	Effect Size (<i>r</i>)
Gel-A-PRF+ vs. Control	0.0181	0.1084	0.506 ***	0.0006 *	0.0036 *	0.731 **
Solid-PRF vs. Control	0.067	0.4022	0.394	0.8157	1	0.056
A-PRF+ vs. Control	0.067	0.4022	0.394	0.0001 *	0.0004 *	0.843 ***
Gel-A-PRF+ vs. Solid-PRF	0.1028	0.6168	0.372	0.0252	0.1512	0.507 ***
A-PRF+ vs. Gel-A-PRF+	0.0002 *	0.001 *	0.845 **	0.0002 *	0.0010 *	0.845 **
Solid-PRF vs. A-PRF+	0.011	0.0661	0.575 ***	0.0002 *	0.0010 *	0.845 **

A difference of $p < 0.05$ is considered statistically significant. It is marked with *. Guidelines for effect size: ~0.10 small (*), ~0.30 medium (**), ≥ 0.50 large effect (***).

3.2. Results from the Fourteenth Day of the Study

The results from the first day of the study are presented in Figures 5 and 6 and Table 3.

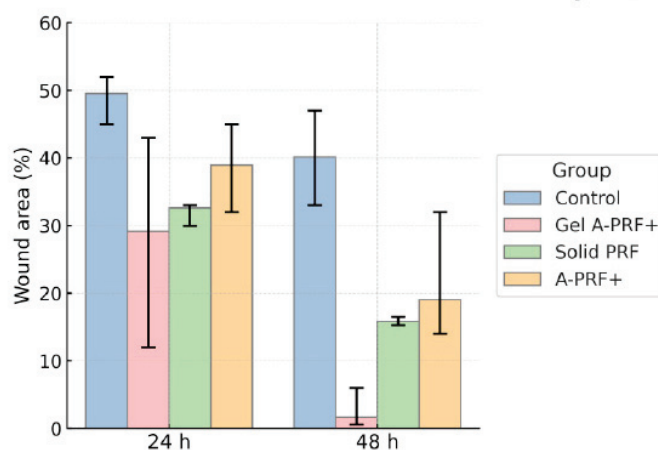
Median wound area for hDPSCs at 24 h and 48 h (Day 14)

Figure 5. Scratch wound healing assay—Day 14. Median wound area (%), min–max) of hDPSCs cultured with autologous platelet concentrates after 14 days of conditioning. Gel A-PRF+ and Solid PRF induced markedly greater wound closure versus control, confirming the time-dependent enhancement of cell migration under prolonged exposure conditions.

Table 3. Results of post hoc analysis with Mann–Whitney U test with Bonferroni correction for multiple comparisons regarding the percentage of wound area reduction for the fourteenth day (day 14) of the 24 and 48 h study.

Days of Investigation Comparison Between Group	24 h/Day 14			48 h/Day 14		
	<i>p</i> -Value Mann–Whitney	<i>p</i> -Bonferroni	Effect Size (<i>r</i>)	<i>p</i> -Value Mann–Whitney	<i>p</i> -Bonferroni	Effect Size (<i>r</i>)
Gel-A-PRF+ vs. Solid-PRF	0.3027	1	0.237	0.0015 *	0.0092 *	0.710 **
Gel-A-PRF+ vs. A-PRF+	0.1028	0.6168	0.372	0.0006 *	0.0033 *	0.778 **
Solid-PRF vs. A-PRF+	0.0042 *	0.0252 *	0.642 *	0.0243	0.1459	0.507 ***
Gel-A-PRF+ vs. Control	0.0006 *	0.0036 *	0.731 **	0.0001 *	0.0004 *	0.843 ***
Solid-PRF vs. Control	0.0001 *	0.0004 *	0.843 ***	0.0001 *	0.0004 *	0.843 ***
A-PRF+ vs. Control	0.0038 *	0.0226 *	0.619 *	0.0038 *	0.0226 *	0.619 *

A difference of $p < 0.05$ is considered statistically significant. It is marked with *. Guidelines for effect size: ~0.10 small (*), ~0.30 medium (**), ≥ 0.50 large effect (***).

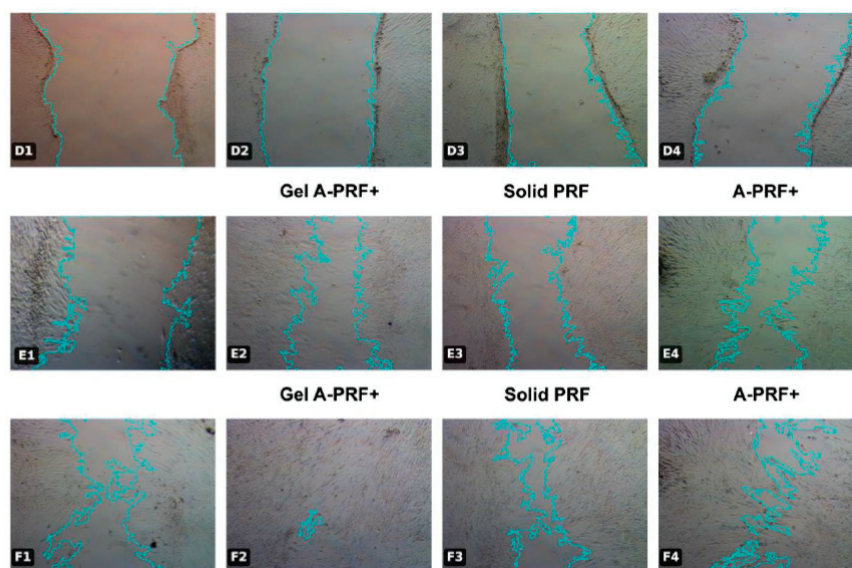


Figure 6. Representative images of wound healing assay at different time points (Day 14: 0 h, 24 h, 48 h) in dental pulp stem cells exposed to various platelet concentrates. Panels: (D1–D4) Control group at 0 h; (E1) Control group, (E2) Gel-A-PRF+ group; (E3) group; (E4) group at 24 h; (F1) Control group; (F2) Gel-A-PRF+ group; (F3) Solid PRF group; (F4) A-PRF+ group at 48 h; Scale bar = 500 μ m (applies to all panels).

A post hoc Mann–Whitney U test, with the Bonferroni correction for multiple comparisons, revealed statistically significant differences in the percentage of wound area reduction between the control group and the three materials studied. The results are presented in Table 3.

The results are illustrated with photographs of the culture media (Figure 6).

4. Discussion

Our results confirm that isolated dental pulp stem cells exhibit characteristic features of mesenchymal human stem cells. The expression of CD44 is consistent with published data, which identify this marker as a reliable indicator of mesenchymal origin [17]. The presence of Nestin and DSPP indicates the potential of hDPSCs for both neurogenic and odontogenic differentiation, which corresponds to their role in regenerative endodontics [18]. CK19 is also expressed, as reported in studies on pulp stem cells [19] and highlighted in a review of oral stem cells [20]. This expression confirms the heterogeneity of the cell population, indicating that these cells have the potential to differentiate into various cell types, contributing to their regenerative capabilities.

Wound healing is a complex physiological process characterized by four main phases: phase (1) hemostasis; phase (2) inflammation; phase (3) proliferation; and phase (4) maturation/remodeling. During hemostasis, activated platelets release growth factors that prepare the environment for the proliferation of mesenchymal stem cells. During the inflammation phase, macrophages release cytokines that direct the migration of mesenchymal stem cells to the damaged site. During the proliferative phase, stem cells migrate to the damaged site, proliferate, and differentiate into various cell types in response to local signals [2].

The scratch wound healing assay is a method for quantitatively assessing cell migration in wound closure in vitro. It is particularly suitable for studying the effect of biological materials such as autologous platelet concentrates, providing a reliable and comprehensive understanding of their impact. It allows tracking the dynamics of wound healing, the time of growth factor release, and their effect on human dental pulp stem cells (hDPSCs) [21].

Le and Nguyen's findings, similar to ours, demonstrate the potential of A-PRF+ in accelerating wound healing in the first 1–2 days, albeit with a focus on stem cells from the apical papilla. In contrast, our study, centered on stem cells from human dental pulp, further underscores this potential [22]. Margono et al. also discovered, in line with our results, that A-PRF enhances hDPSC migration in a concentration-dependent manner, with the most significant effect observed at a 10% concentration, a detail that piques our interest due to the rich content of leukocytes and growth factors [14].

This study compares the effects of three autologous blood-derived biomaterials (A-PRF+, Gel A-PRF+, and Solid PRF) on hDPSCs migration in a standardized scratch model. All PRF variants accelerated “wound” closure versus control, with the strongest effect observed for Gel A-PRF+, Solid PRF showing a more sustained effect over time, and A-PRF+ exhibiting a moderate stimulatory response. These findings support the notion that biological conditioning of the milieu—via growth-factor release and cellular cargo (platelets/leukocytes)—is a key mechanism governing early reparative events in the pulp–dentin complex.

The results of our study found that in the early stages—the first day of the study—the overall closure dynamics remained limited, which can be explained by the fact that the release of biomolecules is in its initial stage.

The time dependence (1 day vs. 14 days) reveals a dynamic release/action profile: short conditioning yields a limited effect, whereas prolonged conditioning results in markedly enhanced migration. This aligns with the recognized biphasic kinetics of PRF—an initial burst followed by sustained, lower-intensity release of bioactive mediators—modulated by fibrin network density and cellular load. The stronger early effect of Gel A-PRF+ may reflect higher initial bioavailability of key mediators (e.g., PDGF, TGF- β , VEGF), whereas Solid PRF likely provides a more stable matrix with prolonged release, favoring durable closure. Although we did not perform biochemical/ultrastructural characterization, differences in fibrin architecture and cellular composition among PRF variants plausibly explain the observed effects.

These results can be explained by the observations of Zwitter et al., who found that PRF matrices release VEGF, PDGF, and TGF- β 1 over a period of 7 to 10 days. Solid forms provide a more sustained release, while liquid forms have a faster but shorter-lasting effect. This explains why the most pronounced effect is observed in our hDPSC cultures precisely in continuously conditioned environments (14 days) [23]. Miron et al. confirm that PRF acts as a three-dimensional fibrin matrix, retaining and gradually releasing growth factors over a period of 10 days [15]. This dynamic creates a favorable microenvironment for cell migration and proliferation, which is fully consistent with our results [24]. Jin et al. show that CGF stimulates the proliferation and migration of pulp stem cells at low concentrations, and Chai et al. demonstrate that i-PRF promotes cell migration even in the presence of induced inflammation [7,25].

It is crucial to understand that different PRF forms exhibit varying effectiveness, largely due to their structural features and centrifugation conditions. A-PRF+ is a softer matrix rich in cells, with a rapid release of growth factors, while Solid PRF is a more compact structure that retains larger amounts of biologically active molecules and ensures their slower, yet more prolonged, release. A-PRF+ contains more leukocytes thanks to the low-speed centrifugation concept (Choukroun & Ghanaati, 2018), which contributes to a moderately stable effect [26]. Miron et al. (2020) confirms that different centrifugation protocols lead to significant differences in cell composition and growth factor release dynamics [15]. This underscores the urgency and significance of protocol optimization, as a balance must be found between a rapid initial effect and long-term biological activity [15].

Chen et al. (2020) conclude that AB PRF biomaterials accelerate the healing of difficult wounds [27]. Our study demonstrates the intriguing potential of A-PRF+ to stimulate the migration of pulp stem cells. In the presence of AB biomaterials: A-PRF+, Gel A-PRF+, and Solid PRF, compared to the control group of cells, we observed accelerated healing of experimental wounds, further highlighting the potential of A-PRF+ in regenerative medicine.

Growing literature and clinical interest position PRF not only as a biological adjunct but also as a potential alternative to calcium–silicate materials in vital pulp therapy. Our in vitro data (enhanced hDPSC migration, most pronounced with Gel A-PRF+) are consistent with this trend, but they cannot be interpreted as evidence of clinical equivalence or superiority. Given the distinct mechanisms of action (biological modulation with PRF versus reliable sealing and hard-tissue induction with MTA/Biodentine), prospective randomized designs—testing stand-alone PRF protocols against standard therapy, as well as combined protocols—are required to determine whether PRF can genuinely replace calcium–silicate cements in selected clinical indications [9].

The present study has several limitations that should be acknowledged. First, the experiments were conducted in vitro, which does not fully replicate the complex biological environment of the dental pulp and surrounding tissues. Second, the sample size was limited, and inter-individual variability among donors may have influenced the results. Third, only the migratory behavior of hDPSCs was analyzed, without further assessment of proliferation, differentiation, or the molecular mechanisms involved in tissue regeneration. Finally, no biochemical or ultrastructural characterization of the PRF biomaterials was performed to correlate the content of growth factors or fibrin architecture with the observed biological effects.

Future research should include in vivo models and more detailed molecular investigations to confirm and expand upon the present findings.

5. Conclusions

In conclusion, the results of the present study show that the effect of PRF on cell migration is dynamic and dependent on the conditioning time. With short-term conditioning (1 day), the effect is more limited, while prolonged conditioning (14 days) significantly accelerates wound closure. Gel A-PRF+ stimulates rapid, early migration. Solid PRF provides a sustained and long-lasting effect, while A-PRF+ maintains a moderate stimulating effect.

Pulp regeneration is a complex and challenging process. The search for the most promising means of preserving its vitality continues. Based on the present in vitro data, PRF—particularly Gel A-PRF+—may be considered a promising stand-alone candidate for vital pulp therapy. However, available evidence is insufficient to assert a substitutive role; rigorous randomized clinical trials with adequate power and predefined endpoints (e.g., clinical success, reparative dentin formation, retreatment rates) are necessary to validate equivalence or superiority.

Author Contributions: Conceptualization, J.N.K.; methodology, J.N.K. and N.D.I.; investigation, R.Z.V. and N.D.I.; resources, J.N.K.; data curation, J.N.K.; writing—original draft preparation, J.N.K. and R.Z.V.; writing—review and editing, J.N.K., R.Z.V., V.P.P. and N.D.I.; visualization, J.N.K., V.P.P. and R.Z.V.; supervision, J.N.K., E.G.D., S.Y. and N.D.I. All authors have read and agreed to the published version of the manuscript.

Funding: The study is financed by the European Union—NextGeneration EU, through the National Recovery Plan of Republic of Bulgaria, Grant no. ZIU-MP-14/09.08. 2024, BG-RRP-2.004-0004-C01 “Strategic Research and Innovation Program for the Development of MU-Sofia”.

Institutional Review Board Statement: The study was conducted in accordance with the Medical University–Sofia Research Ethics Committee (Approval Code 57), with the approval granted on 12 July 2024.

Informed Consent Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AB	Autologous Blood
A-PRF+	Advanced Platelet-Rich Fibrin Plus
CD44	Cluster of Differentiation 44
CGF	Concentrated Growth Factors
CK19	Cytokeratin 19
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DSPP	Dentin Sialophosphoprotein
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
Gel A-PRF+	Gel form of Advanced Platelet-Rich Fibrin Plus
hDPSCs	Human Dental Pulp Stem Cells
IL-1 β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PRF	Platelet-Rich Fibrin
SD	Standard Deviation
Solid PRF	Solid Platelet-Rich Fibrin
TGF- β 1	Transforming Growth Factor Beta 1
TNF- α	Tumor Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor

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Article

Grain Size and Electrochemical Surface Modification Effects on Corrosion, Biological, and Technological Properties of CP Titanium Implants

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Abstract: Commercially pure (CP) titanium is widely used for long-term biomedical implants due to its high biocompatibility and corrosion resistance. However, its relatively low strength limits its use in highly loaded applications. Ultrafine-grained (UFG) titanium obtained through severe plastic deformation offers enhanced mechanical performance while maintaining the stability of CP titanium. This study investigates how electrochemical surface modification by anodization affects the corrosion, biological performance, and technological behavior of UFG titanium. TiO₂ layers with nanotubular and nanoporous morphologies were produced at anodization voltages between 20 and 60 V. Corrosion tests in physiological solution confirmed stable passive behavior with corrosion rates below 4 μm year^{−1}, and surface wettability increased markedly with anodization. Osteoblast-like MG-63 cells exhibited good viability on all anodized surfaces, with improved adhesion and proliferation on samples anodized at 60 V. The porous TiO₂ layers were successfully intercalated with dimethyl sulfoxide and ibuprofen, demonstrating potential for local drug delivery. Implantation simulations on real Nanoimplant[®] prototypes confirmed sufficient mechanical stability of the anodized layer. Overall, the optimized anodization of UFG titanium enhances its biological response while maintaining corrosion resistance, supporting its clinical use in long-term dental and orthopedic implants with integrated drug-release functionality.

Keywords: titanium implants; nanostructure; corrosion; biocompatibility; surface treatment; targeted drug delivery system

1. Introduction

Since approximately 500 BC, when primitive gold application was first used for tooth replacement by the Etruscans, materials for implantology have undergone considerable development [1]. After titanium fixators were first tested in 1978, the material itself became very popular for the construction and manufacturing of long-term and rather permanent applications for hard tissue replacement [2]. The main alloy used is so-called commercially pure titanium (CP Ti). This metal is available in four grades, numbered 1 to 4, according to its purity and interstitial atom content [3]. These grades may differ in corrosion and mechanical properties due to impurities that are likely bound with titanium or otherwise form secondary phases in a titanium matrix. Particles of these phases hinder the dislocation movement on slip planes, which causes precipitation strengthening of the matrix. On the other hand, under certain conditions, they decrease corrosion resistance by the spontaneous initiation of micro-sized corrosion cells in specific environments [3–5].

As the ultimate tensile strength of CP Ti is usually lower than that of austenitic stainless steels (240–550 MPa for grades 1–4, respectively), Ti6Al4V was introduced as CP Ti grade 5, which shows ultimate tensile strength (UTS) above 850 MPa [6]. The microstructure of this alloy is formed by a mixture of HCP α and BCC β phases. Due to higher mechanical properties, CP Ti grade 5 is commonly applied for highly loaded implants, where other titanium alloys fail due to overload or fatigue mechanisms [7]. Later on, some progressive or experimental multicomponent alloys with a two-phase microstructure (e.g., Ti6Al7Nb [8], Ti13Nb13Zr [9], Ti29Nb13Ta5Zr [10], etc.) were introduced and are currently being validated for practical use in the field of implantology. Although these progressive titanium alloys mostly have promising mechanical properties, their corrosion resistance is fundamentally limited by precipitates in the matrix [11]. Particles of secondary phases dispersed in the microstructure degrade corrosion resistance significantly. Furthermore, the elements released into the environment during the active corrosion process of implants may cause secondary body reactions [12].

Another way of improving the mechanical properties of materials is significant grain refinement by intense thermomechanical treatment with limited recrystallization. This was also shown as a promising way for titanium, when CP Ti, after a severe plastic deformation (SPD) process, reaches UTS up to 1240 MPa and yield strength (YS) up to 1200 MPa [13,14]. The corrosion properties of ultrafine grain (UFG) CP Ti are not usually influenced distinctly by the SPD process and remain stable, as there are no secondary phase particles present in the microstructure [15]. Due to the larger area of grain boundaries per unit volume, there exists a massive accumulation of grain boundary surface energy in the microstructure [16]. Consequently, the grain boundaries are more reactive (become less noble) than grain interiors (acting more noble), which makes grain boundaries sensitive to intercrystalline corrosion as a result of the formation of localized submicron corrosion cells [17].

Titanium-based materials are well known for their high potential for hard tissue bonding as discovered during the first tests in 1978 [18]. This is mostly caused by the high stability of physicochemical interactions between titanium dioxide (TiO₂) films, spontaneously formed on oxygen-exposed surfaces, and amino acids contained in different tissues [19] or blood serum [18]. The interaction between the material and the surrounding tissue can be subsequently enhanced by coating of the active surface by bioactive components like hydroxyapatite or calcium phosphate-based minerals, leaving the final surface highly porous and with increased roughness [20]. Other coating processes include composite coatings, titanium nitride coatings, carbon, glass, and ceramic coatings, as well as TiO₂ film coatings [21]. The disadvantage of coating processes is the need for elevated temperature to achieve strong bonding between the coating and the substrate material. Unfortunately, the high temperature may result in microstructure degradation, especially

when applied to UFG titanium, where spontaneous recrystallization occurs, leading to grain coarsening and a decrease in mechanical properties [22]. To minimize this risk, chemical or even electrochemical surface treatments can be applied. Because the titanium-based materials are considered highly chemically resistant, aggressive solutions containing a concentrated mixture of an oxidizer and specific corrosion agents must be applied, leaving the surface covered with wide but shallow unorganized corrosion pits [23]. Less concentrated solutions can be used in the electrochemical process called anodization, where the chemical oxidizer is substituted by an external source of electric current. Highly organized formation of TiO₂ nanopores or even nanotubes can be obtained during this process; furthermore, the geometry of the formation can be actively changed by anodization voltage, and the layer thickness is determined by the passed electric charge, i.e., time of anodization [15]. Chemical treatment of alkalinized titanium surfaces by direct immersion in alkaline solutions leaves the surface significantly active with higher values of wettability [24,25]. On the other hand, the negative effect of defects localized in the anodized TiO₂ layer can be partially reduced by passivation in oxidizing acids [26]. The anodized layer exhibits a large free volume, which can be intentionally saturated by active chemicals and, if applied as an implant, serves as a targeted drug delivery system [27]. Unless this possibility had been studied in the past [28,29], the synergistic combination of ultrafine-grained (UFG) titanium, which provides improved mechanical properties, and its surface modification by anodization to form nanotextured TiO₂ layers capable of hosting bioactive substances, remains insufficiently explored. This study presents a comprehensive evaluation of the anodization process applied to commercially pure titanium with both conventional and ultrafine-grain structures, with an emphasis on electrochemical and biological performance. The aim of this work was to assess how anodization parameters (20, 40, and 60 V) influence surface morphology, corrosion resistance, wettability, and cytocompatibility of UFG titanium and to verify its suitability for application in Nanoimplant[®] prototypes.

Although anodized titanium has been studied extensively, most investigations have focused on materials with conventional grain sizes. The combined effects of the UFG microstructure and electrochemical oxidation on corrosion behavior, surface energy, cell response, and local drug delivery potential have not yet been fully understood. In addition, very few studies address the technological transfer of anodization to real miniature implant geometries with clinically relevant shapes and surface areas.

Based on these gaps, the aim of this study was to verify that anodization of UFG titanium would generate uniform and mechanically stable TiO₂ layers with enhanced biological interaction and an increased ability to intercalate small therapeutic molecules, without compromising corrosion resistance or mechanical integrity.

2. Materials and Methods

2.1. Material and Its Processing Parameters

Investigations were mainly performed on CP titanium grade 4 with ultrafine grain size. This UFG CP Ti was obtained using SPD processing by equal channel angular pressing (ECAP) conducted at 300 °C with 8 passes via the route B_C [14]. Especially for the purpose of corrosion parameter comparison, there was also CP Ti grade 3 with conventional grain size included in the selected tests. The chemical composition and basic mechanical properties of both grades are summarized in Table 1. The mechanical properties of ECAP CP Ti grade 4 were determined by tensile tests; all other values are taken from the ASTM B265 [30].

The microstructure of CP Ti grade 3, revealed by light microscopy (LM) after mechanical polishing, followed by electrochemical polishing (30 V/10 s in Struers A3 solution), is shown in Figure 1. It is obvious that the presented microstructure with equiaxed grains is

typical for material after a hot forming process with spontaneous primary recrystallization. The average grain size is approximately 30 μm .

Table 1. Chemical composition and mechanical properties of tested materials.

Grade	Mechanical Properties			Chemical Composition (max. wt.%)				
	Tensile Strength (MPa)	Yield Strength (MPa)	Elongation (%)	N	C	H	Fe	O
CP Ti grade 3	min. 450	min. 380	min. 18	0.3	0.8	0.015	0.3	0.35
ECAP Ti grade 4	1143	864	15.4	0.3	0.8	0.015	0.5	0.4

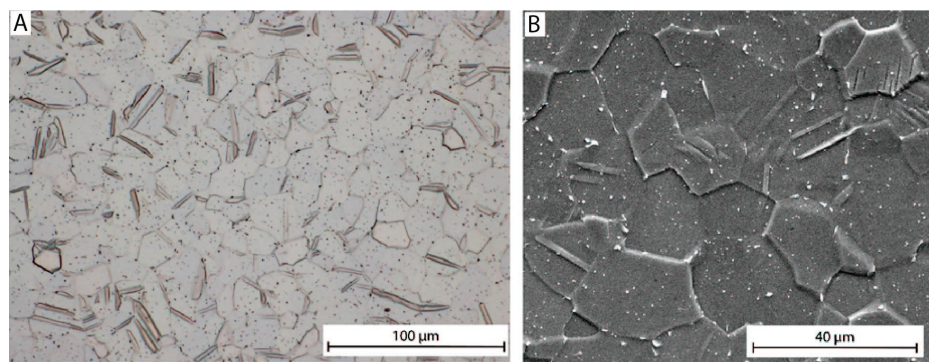


Figure 1. Microstructure of the reference sample (CP-Ti gr. 3) observed by (A) light and (B) scanning electron microscopy image in SE mode.

Figure 2 illustrates a grain size map of CP Ti grade 4 after ECAP processing obtained by the electron back-scattered diffraction (EBSD) technique in a scanning electron microscope (SEM-FEI QUANTA FEG 450+, Brno, Czech Republic). The size of individual grains was evaluated as the equivalent grain diameter, which was defined as the diameter of a circle with the same area. The minimum grain size, which could be determined for the measurement conditions used, was 0.15 μm . Figure 3 shows an IPF orientation map for the LD (longitudinal direction during ECAP). The IPF map reveals a pronounced preferred orientation of grains. The orientation of most grains in the LD is close to $\{10\bar{1}0\}$.

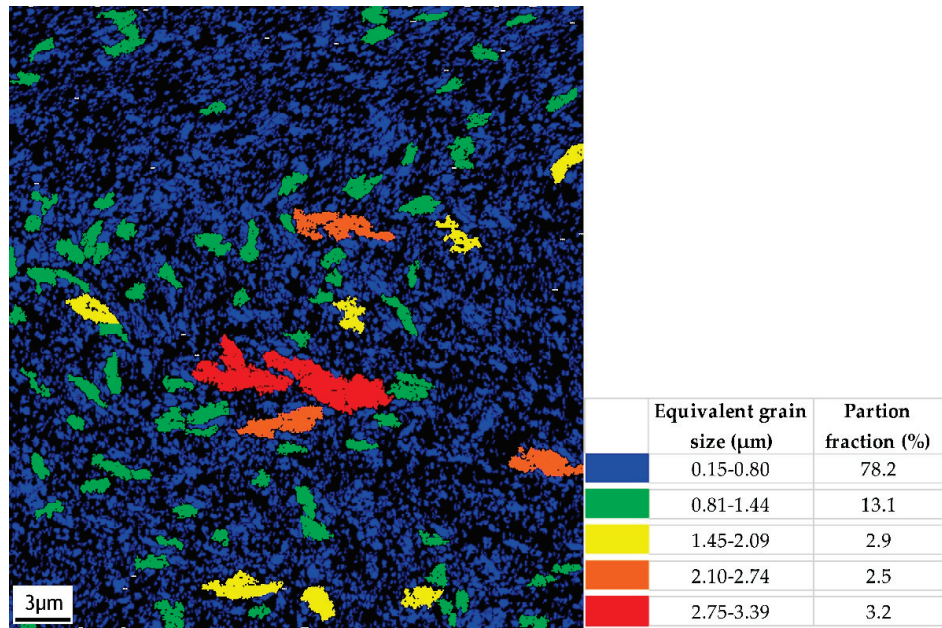


Figure 2. Grain size map, EBSD, sample UFG CP Ti grade 4, cross-section.

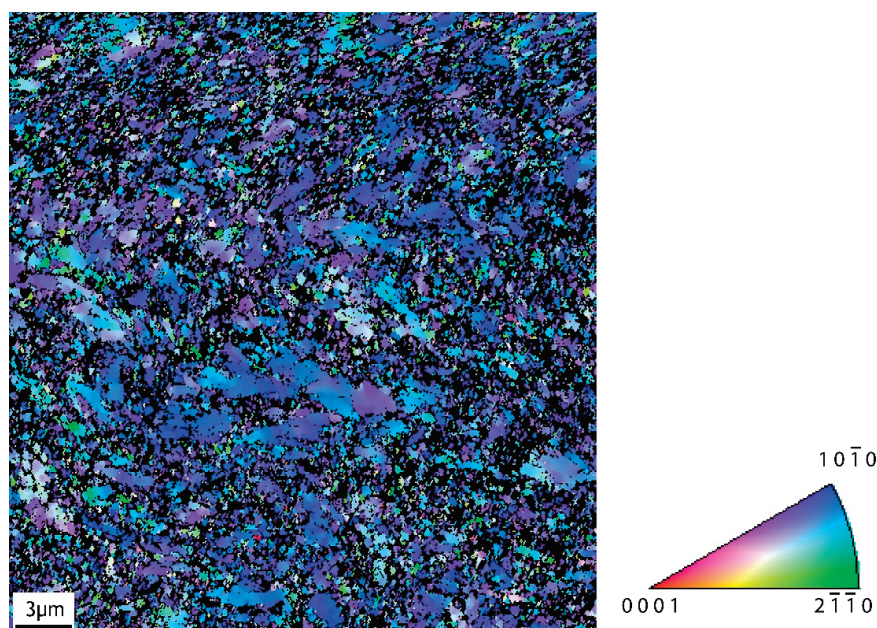


Figure 3. IPF orientation map for the LD, sample UFG CP Ti grade 4, cross-section.

For the purpose of electrochemical oxidation, each material was machined into cylindrical shape, $\phi 6 \times 40$ mm. Samples were partially mounted into bisphenol transparent resin (Specifix by Struers). Orientation of the rods in mounted samples (see Figure 4) allowed mechanical preparation and electrochemical treatment on rods' cross-sections. The investigated sections also remained constant during corrosion tests, where the risk of crevice corrosion under an O-ring spacer was completely eliminated [31].

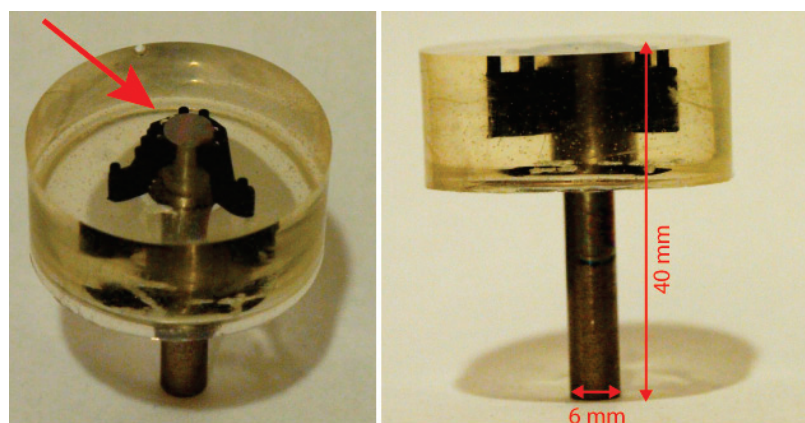


Figure 4. Samples after mounting in resin; an arrow marks the cross-section area for testing.

2.2. Electrochemical Surface Oxidation

After mechanical grinding (SiC papers up to #4000) and polishing with colloidal Al_2O_3 suspension (Struers), the cross-section surfaces of the rods were ultrasonically cleaned and degreased in pure ethanol for 10 min. The samples were then removed from the bath and allowed to dry completely to ensure full evaporation of residual ethanol. For anodization, the samples were fixed in a laboratory holder and partially immersed in an electrolyte composed of 79 wt.% ethylene glycol, 20 wt.% deionized water, and 1 wt.% NH_4F (all reagents from VWR™). A platinized titanium wire, coiled around the sample, was placed in the solution to ensure a homogeneous electric field distribution. The sample and the platinized wire were connected to the positive and negative terminals of a DC power

supply with adjustable voltage using crocodile clips. A schematic representation of the anodization setup is shown in Figure 5.

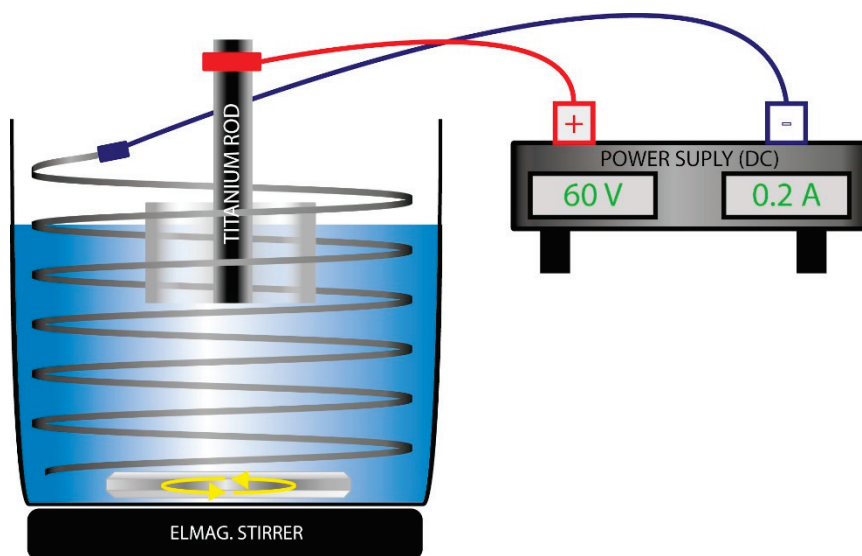


Figure 5. Schema of anodization apparatus.

In general, the mechanism of the TiO_2 porous layer formation on a clean titanium surface (Figure 6A) in fluorine-ion-based electrolytes is said to occur as a result of three simultaneous processes: the field-assisted oxidation of Ti metal to form titanium dioxide (Figure 6B and Equation (1)), the field-assisted dissolution of Ti metal ions in the electrolyte, more precisely the chemical dissolution of TiO_2 due to etching by fluoride ions (Figure 6C and Equation (3)), which is enhanced by the presence of H^+ ions produced by electrochemical water molecules splitting when sufficient external voltage is applied (Equation (2)) [32,33]. This process leaves the surface covered either by nanopores [34] or thin-walled nanotubes [27,35]. As the oxygen bubbles were generated on the anodized surface, to prevent surface/solution contact, electromagnetic stirring was applied at 180 RPM to eliminate them. Anodization time was set to 300 s; according to previous tests and measurements, longer times of anodization led to thicker anodized layers with lower adhesion to the UFG CP Ti substrate.

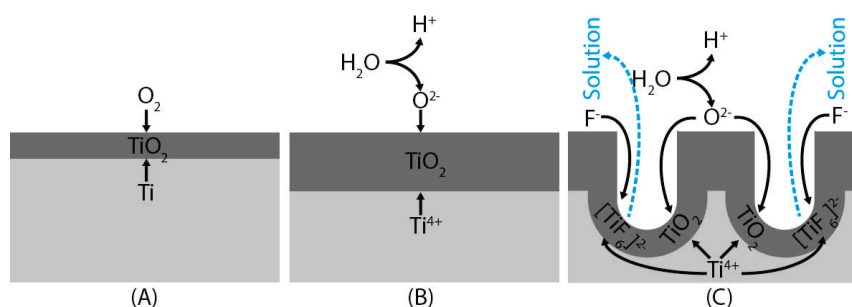
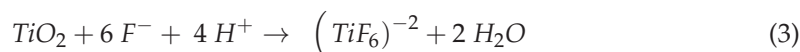


Figure 6. (A) Spontaneous formation of passive oxide layer; (B) accelerated electrochemical oxidation related to electrolysis as a source of oxygen; (C) selective dissolution of oxide layer during accelerated oxidation in fluorine-rich solutions.

Three sets of UFG CP Ti samples were anodized at different voltages for the purpose of the present study. The anodization voltages of 20, 40, and 60 V were selected based on previous optimization studies [36,37], where these values generated TiO₂ layers with distinct nanotubular to nanoporous morphologies and stable adhesion to titanium substrates. Each experimental condition was performed on three independent specimens, and all electrochemical, wettability, and biological tests were conducted in triplicate to ensure reproducibility. A sample of CP Ti grade 3 was not anodized and was used as a reference to the anodized ones. The parameters of processing for each sample are listed in Table 2.

Table 2. Parameters of sample processing.

Sample	Material	Anodization Voltage (V)	Time of Anodization (s)
UFG-20V	UFG CP Ti grade 4	20	300
UFG-40V	UFG CP Ti grade 4	40	300
UFG-60V	UFG CP Ti grade 4	60	300
Reference	CP Ti grade 3	-	-

2.3. Corrosion Tests

Given the substantial corrosion resistance inherent in titanium-based materials, conducting direct immersion corrosion trials would prove inefficient in terms of time consumption. Consequently, the potentiodynamic polarization technique was employed to ascertain specific corrosion characteristics of the materials under examination. The testing parameters adhered to the guidelines established by ASTM F 2129 and ASTM F 746 [38,39], albeit with certain alterations to account for the unique specifications of the samples. Corrosion cells, featuring a reduced exposure aperture and a total volume of 20 mL, were utilized for these assays. The surface area subjected to testing remained consistent and was derived from the measured diameter of the rod specimen. Testing was conducted within an isotonic saline solution (comprising 0.9 wt.% NaCl dissolved in deionized H₂O) explicitly designed to mimic the biological environment of living tissue. No gas purging was implemented throughout the experiments, and the temperature was maintained precisely at 25 °C. A standard three-electrode configuration was implemented: the sample served as the working electrode, a saturated calomel electrode (SCE, measuring +241 mV relative to SHE) [40] functioned as the reference electrode, and a high-purity carbon rod acted as the counter (auxiliary) electrode.

A stabilization period totaling 90 min was enforced subsequent to filling the corrosion cells with the solution to allow partial corrosion reactions to settle [41]. Prior to initiating the potentiodynamic sweep, which was executed using a Voltalab PGZ 100 potentiostat (Radiometer Analytical, Copenhagen, Denmark), the starting potential was established at −100 mV relative to the established open-circuit potential (OCP) following the achievement of corrosion equilibrium, with the polarization scan rate set to 60 mV.min^{−1}. The measured current traversing the sample, in relation to the potential applied to it, was continuously recorded during the measurement phase. The potential was incrementally imposed upon the specimen, escalating steadily as a function of time according to the defined polarization rate. This entire process was managed and controlled via the Voltamaster 10 software package.

2.4. Surface Wettability Test

The wettability of the tested surfaces was evaluated using the sessile drop method, which enables direct measurement of the static contact angle and assessment of the surface's affinity toward liquids. Measurements were performed using the SEE contact angle system,

and the surface free energy was subsequently calculated with Advex Instrument software, version E (Brno, Czech Republic). Droplets of deionized water (2 μ L each) were gently deposited onto the examined surfaces, and the contact angle (θ) was obtained by fitting the tangent at the point of intersection between the liquid, solid, and vapor phases [42].

The surface free energy of the solid was derived from Young's equation (Equation (4)), in which γ_{SV} , γ_{LV} , and γ_{SL} denote the interfacial tensions at the solid–vapor, liquid–vapor, and solid–liquid boundaries, respectively [43].

$$\gamma_{sv} - \gamma_{sl} = \gamma_{lv} * \cos \theta \quad (4)$$

2.5. Drug Intercalation and Fourier-Transform Infrared Spectroscopy (FTIR) Characterization

As per test requirements, a smaller sample (i.e., 5 mm in length and diameter) had to be manufactured in order to completely suit the test chamber of the spectrometer. These samples were attached to a threaded titanium holder with an isolated surface, as illustrated in Figure 7, and one after another was anodized under the same conditions as the samples for the corrosion tests. There were two different solutions used for anodized surface intercalation. Ibuprofen 1% aqueous solution was obtained from Magistraliter Pharma Company and used as prepared. For the preparation of standard solutions for high-performance liquid chromatography (HPLC) measurements, ibuprofen (Sigma-Aldrich, Saint Louis, MO, USA, 99%) was used. There was an anhydrous dimethyl sulfoxide (Sigma-Aldrich, $\geq 99.9\%$) used as a second intercalation solution. There were samples anodized at 60 V selected for intercalation according to the results of previous corrosion and wettability tests. Anodized samples were placed in the Eppendorf flasks, and 2 mL of intercalation solutions were added to them. The holder with the Eppendorf flasks was stored in the dark for 24 h to prevent any interaction of anodized surfaces and solutions, causing decomposition of its active substances due to the possible photo-catalytic effect of nanostructured titanium surfaces [44]. After the intercalation process, the samples were removed from the Eppendorf flasks, repeatedly cleaned by high-purity distilled water, air-dried, and immediately characterized.

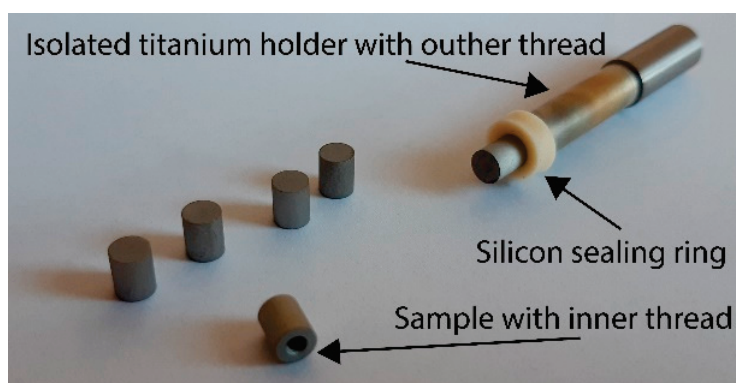


Figure 7. Schema of samples used for intercalation followed by FTIR characterization.

Fourier-transform infrared spectroscopy (FTIR) was used for the determination of surface adsorption capability. FTIR spectra were recorded on a Nicolet iS50 spectrometer (Thermo Fisher Scientific, Madison, WI, USA) using the single-reflection mode on a diamond crystal (32 scans, 4 cm^{-1} resolution). Background (ambient air) measurement and standard instrument-based polynomial baseline correction were used to record and interpret the spectra. FTIR spectra were obtained on a Nicolet iS50 spectrometer using the single-reflection mode on a diamond crystal (32 scans, 4 cm^{-1} resolution). The measure-

ments were performed in the field of mid-infrared spectroscopy in the spectral range from 2.5 μm to 20 μm (from 4000 cm^{-1} to 400 cm^{-1}).

2.6. Cell Adhesion and Proliferation

Human osteoblast-like cells (MG-63, ECACC 86051601; Sigma Aldrich, St. Louis, MO, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Biosera Europe, Nuaille, France) supplemented with 10% (*v/v*) fetal bovine serum (FBS; Biosera Europe), 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin (PAA Laboratories GmbH, Austria), and 2.5 mM L-glutamine (Diagnovum GmbH, Ebsdorfergrund, Germany). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 . Culture media were replenished regularly to maintain optimal growth conditions. The MG-63 cell line was selected because it is a widely accepted and standardized *in vitro* model for evaluating cell–material interactions on metallic implant surfaces. It provides reproducible proliferation dynamics and is highly responsive to surface chemistry and morphology, making it suitable for assessing the cytocompatibility of anodized titanium. The use of a single osteoblast-like cell line also minimized biological variability. However, to approximate *in vivo* conditions more closely, future studies will include primary osteoblasts and co-culture systems. Cell adhesion (2 h after seeding) and proliferation (48 h after seeding) were quantified using the Cell Counting Kit-8 (CCK-8; Bimake, Munich, Germany), following the manufacturer's protocol with minor volume adjustments. The experimental procedure was adapted from previous work [45]. Cells were seeded at a density of 2.5×10^5 cells/mL onto the tested surfaces.

After 2 h, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), and 550 μL of CCK-8 working solution (50 μL CCK-8 reagent + 500 μL culture medium) was added. After incubation for 16 h at 37 °C, 110 μL aliquots were transferred to a 96-well plate, and absorbance was measured at 450 nm using a Synergy H1 microplate reader (Biotek, Winooski, VT, USA). After media replacement, incubation continued. At 48 h post-seeding, 50 μL of CCK-8 reagent was added directly to each well of the 48-well plate, followed by another 16 h incubation. Absorbance was again recorded at 450 nm. Adhesion and proliferation were expressed relative to the positive control (cells grown on tissue culture polystyrene).

For fluorescence visualization, cells were stained with CellTracker™ Green (Molecular Probes, Eugene, OR, USA) and NucBlue® Live ReadyProbes® Reagent (Hoechst 33342; Life Technologies, Eugene, OR, USA), following the procedure described previously [46]. Briefly, 2 drops of NucBlue reagent were added per 1 mL of culture medium and incubated for 30 min at 37 °C. The medium was then replaced with Live Cell Imaging Solution (Life Technologies). Fluorescent imaging was performed using an Olympus CKX41 inverted microscope (Olympus, Hamburg, Germany) at 400 $\times$ magnification. In a complementary assay, cells were stained with Crystal Violet (Sigma Aldrich). After PBS washing, cells were fixed with 2.5% glutaraldehyde in PBS (pH 6.7–7.1) for 30 min at room temperature, rinsed again with PBS, and subsequently stained with 0.5% Crystal Violet for 20 min. Excess dye was removed, and samples were washed three times in deionized water. After drying, samples were observed and imaged using a Leica S9i stereomicroscope (Leica Microsystems, Wetzlar, Germany). Statistical analysis was conducted using a two-tailed Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

2.7. Practical Adhesion and Tribological Test

Unlike the previous tests, which were performed on ideal dummy samples, this test aims to investigate technology transfer from ideal samples to real implants. UFG CP Ti has been successfully used for teeth implant manufacturing [13,47,48]; therefore, a set

of Nanoimplant® [49] was machined from this material. The same holder was used for implant anodization and the protocol of anodization was applied. The samples were rinsed by deionized water and left to air dry completely in the end. Implants before and after anodization are presented in Figure 8.

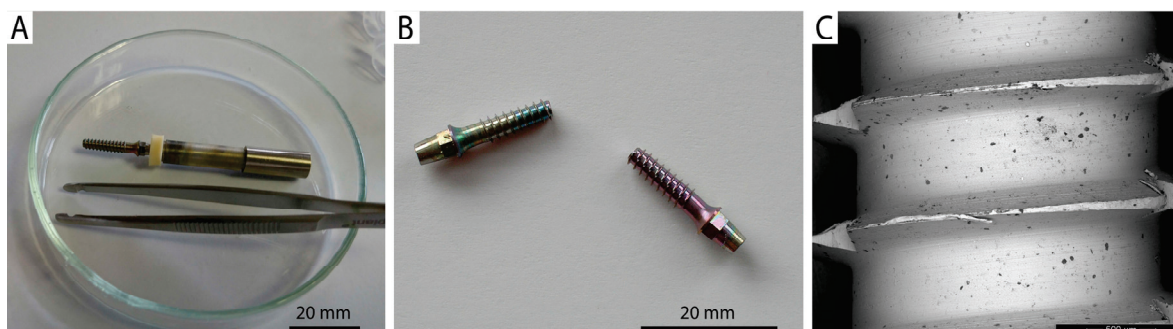


Figure 8. (A) Nanoimplant® screwed on holder before anodization; (B) Nanoimplant® after anodization at 40 V-left and 60 V-right; (C) topography of Nanoimplant® surface after anodization obtained by SEM in back-scattered electron (BSE) mode.

Conditions of the tests were selected intentionally to simulate the real process of implantation into bone. According to previous research, three different materials were used to simulate hard tissue structures of a human bone—artificial bone model [50], dried ash wood [51,52], and fresh cow rib [53,54]. The same procedure was performed as recommended for real implantation [55,56]. The only difference was that immediately after application, the implants were extracted and rinsed with deionized water to clean possible loose residues of testing material, especially tissue of cow rib. After these tests, the surfaces of extracted implants were studied by SEM to evaluate the real damage of anodized surfaces.

3. Results

3.1. Surface Morphology

After anodization, all samples were rinsed thoroughly with deionized water and dried in a desiccator at room temperature. The non-anodized CP Ti reference sample was only cleaned with acetone after final polishing and required no additional treatment. The surface morphology of anodized samples (Figure 9A–C) and the reference surface (Figure 9D) was examined using SEM in secondary electron (SE) mode.

The application of anodization completely altered the surface morphology when compared to the mechanically polished reference material (Figure 9D), where only shallow grinding and polishing scratches were visible. At 20 V (Figure 9A), the oxide layer consisted of partially separated clusters of vertically aligned TiO₂ nanotubes with an average diameter of approximately 50 nm. The enlarged inset in Figure 9A shows these nanotube bundles more clearly. Their “island-like” appearance is caused by local stress accumulation during oxide growth and slight crystallographic misorientation between underlying ultrafine grains, which leads to the partial separation of nanotube domains. This phenomenon is typical for anodized UFG titanium and has been described as stress-induced clustering of nanotubes [25].

At higher voltages (40 V and 60 V; Figure 9B,C), anodization produced a more homogeneous and compact nanoporous TiO₂ layer. In these samples, the initial surface topography is still preserved—the shallow polishing scratches remain visible—but the oxide is more uniform, as the higher electric field promotes field-assisted dissolution and suppresses the

formation of separated nanotube bundles. The pore size increases with voltage, and no pronounced island-like defects are observed.

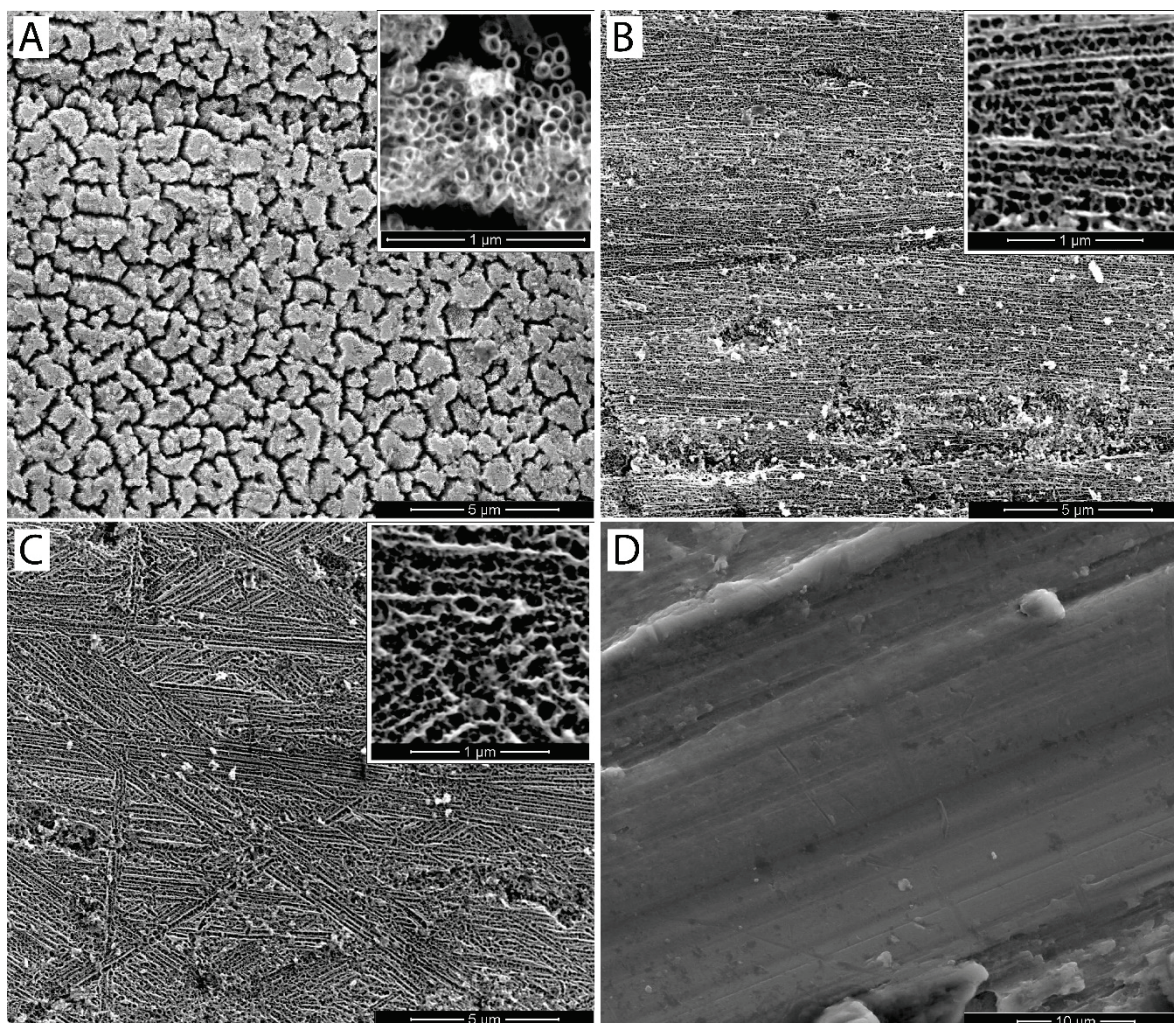


Figure 9. Surface morphology of the UFG CP Ti grade 4 anodized at (A) 20 V/300 s, (B) 40 V/300 s, and (C) 60 V/300 s, and (D) surface morphology of the reference sample CP Ti grade 3 after polishing; SEM images in SE mode.

3.2. Potentiodynamic Corrosion Test

After the time lag, when equilibrium between oxidation and reduction reactions was set [57], corrosion cells were connected to a Voltalab PGZ 100 potentiostat, and the open-circuit potential (OCP) was measured for 60 s. As the OCP remained very stable (its shift was <1 mV/min in all cases), the polarization sequence was initialized.

During the first polarization, stage transition between the cathodic and anodic states was recorded. Current density decreased to zero value and increased again. In this so-called Tafel region, a polarization curve exhibits a V-like shape, if a semilogarithmic plot is used for its representation [58]. This part of the curve was also used for the determination of corrosion potential (E_{cor}), polarization resistance (R_p), and corrosion current density (J_c) by Tafel extrapolation [59]. There was an exchange of four electrons during corrosion reactions ($\text{Ti}^0 \rightarrow \text{Ti}^{4+}$) [60]; an average material molar mass of $47.9 \text{ g}\cdot\text{mol}^{-1}$ [61] and a density of $4.5 \text{ g}\cdot\text{cm}^{-3}$ [62] were considered for calculating the corrosion rate from current density according to [63]. For the control, the Stern–Geary relation [64] was used for the determination of corrosion potential and polarization resistance as well. The results of both methods should be comparable. Potentiodynamic polarization measurements were

performed in triplicate for each surface, and all mentioned parameters were determined automatically by Voltamaster 10. Results (mean values and standard deviations) are listed in Table 3.

Table 3. Corrosion parameters obtained from the Tafel region of corrosion curves.

Sample	Taffel Method				Stearn–Geary Method	
	Corrosion potential E_{cor} (mV vs. SCE)	Polarization resistance R_p ($\text{k}\Omega\cdot\text{cm}^2$)	Cor. current density J_c ($\text{nA}\cdot\text{cm}^{-2}$)	Corrosion rate C_R ($\mu\text{m}\cdot\text{year}^{-1}$)	Corrosion potential E_{cor} (mV vs. SCE)	Polarization resistance R_p ($\text{k}\Omega\cdot\text{cm}^{-2}$)
UFG-20V	-204 ± 18	125 ± 9	143 ± 14	1.3 ± 0.15	-211 ± 21	128 ± 10
UFG-40V	-632 ± 28	76 ± 8	184 ± 19	1.7 ± 0.22	-634 ± 31	82 ± 9
UFG-60V	-652 ± 33	41 ± 6	410 ± 48	3.7 ± 0.45	-653 ± 35	48 ± 7
Ref. CP Ti gr.3	-376 ± 16	101 ± 8	137 ± 13	1.2 ± 0.14	-375 ± 17	97 ± 7

When the Tafel region was exceeded, the samples were subsequently polarized to higher potentials to evaluate the anticorrosion effect of anodically formed layers against pitting evolution on the tested surfaces. The procedure was stopped automatically when 4000 mV vs. SCE was reached. Typical corrosion curves in the semilogarithmic plot are presented in Figure 10.

According to the Tafel extrapolation results, increasing the anodization voltage led to a progressive shift in the corrosion potential towards less noble values. Higher voltages also resulted in an increased corrosion rate and a noticeable decrease in the polarization resistance, indicating a reduced protective efficiency of the anodic oxide layer.

The corrosion parameters of the reference CP Ti sample were comparable to those of the anodized UFG samples; however, the reference surface exhibited the lowest corrosion rate and therefore the most favorable corrosion performance. No sudden rises in current density were detected on the polarization curves, suggesting that pitting corrosion was not initiated and reverse scanning was not required, as all surfaces remained in the passive state throughout the experiment. Although the polarization curves did not show a clear transition into the transpassive region, the gradual increase in current density at higher potentials may indicate the onset of electrochemical degradation of the oxide layer through anodic reactions [65]. Hence, all surfaces were observed by SEM, but no signs of localized corrosion damage were found at all.

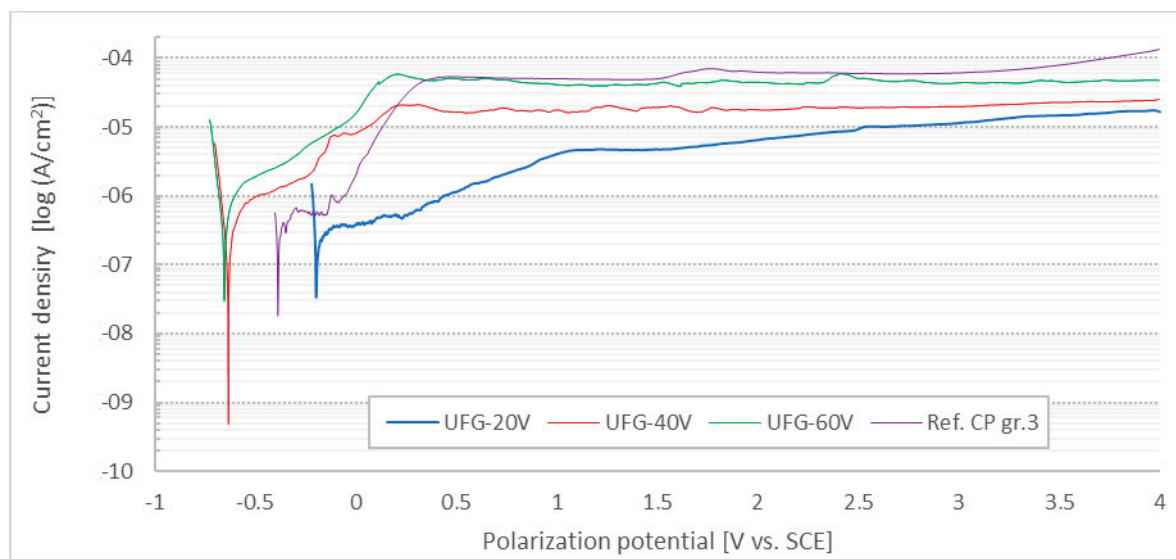


Figure 10. Polarization curves of samples (a typical curve for each sample was selected for presentation).

3.3. Surface Wettability

All surfaces were fine-polished before anodization to minimize the risk of results being affected by inconsistent surface roughness. Before the test, all samples were cleaned separately in an ultrasonic acetone bath with testing surfaces facing up to avoid being scratched. The contact angles of 10 droplets were measured for each sample. Mean values together with corresponding standard deviations are presented in Table 4. Due to the Cosine function used to calculate surface energy, its standard deviation is not symmetrical; therefore, the value was averaged too. Figure 11 illustrates images of captured droplets together with their shape extrapolation according to [66].

Table 4. Results of contact angle measurements.

Sample	Contact Angle (°)	Calculated Free Surface Energy ($\text{mJ}\cdot\text{m}^{-2}$)
UFG-20V	52.3 ± 2.1	53.6 ± 1.3
UFG-40V	67.9 ± 1.4	45.3 ± 0.8
UFG-60V	71.0 ± 1.5	39.5 ± 1.0
Ref. CP gr.3	81.4 ± 2.9	32.6 ± 1.9

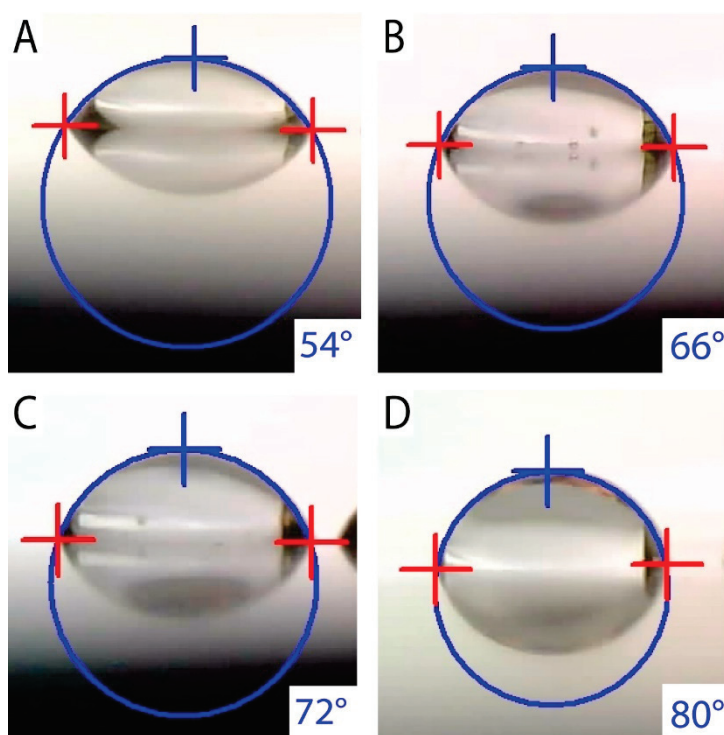


Figure 11. Typical droplets on the surface of samples (A) UFG-20V, (B) UFG-40, (C) UFG-60V, and (D) reference sample CP grade 3.

The polished reference sample exhibited the highest contact angle (81°), indicating the lowest surface wettability. In contrast, all anodized surfaces showed increased hydrophilicity, with contact angles ranging from 52° to 71° . The standard deviations of contact angle measurements were below 3° , and the calculated surface energy values showed deviations of less than $2 \text{ mJ}\cdot\text{m}^{-2}$, confirming good measurement reproducibility.

3.4. Practical Adhesion and Tribological Test

According to previous test results, only the most promising anodization protocol was used for application to Nanoimplant[®] samples, i.e., 60 V for 300 s. Considering the surface

pore size, the layer anodized at a higher voltage can be saturated by specific substances more effectively than the layer with narrow pores formed at 20 V [67].

Before the SEM observation, the samples were immersed in an ultrasonic bath with demineralized water to clean the surface of organic residues for 30 s. BSE mode was used for the surface inspection of extracted implants. Due to the material contrast, areas of the anodized layer delamination were clearly visible as they appeared brighter [68]. The surfaces of extracted implants are shown in Figure 12, where arrows point to areas of the damaged anodized layer.

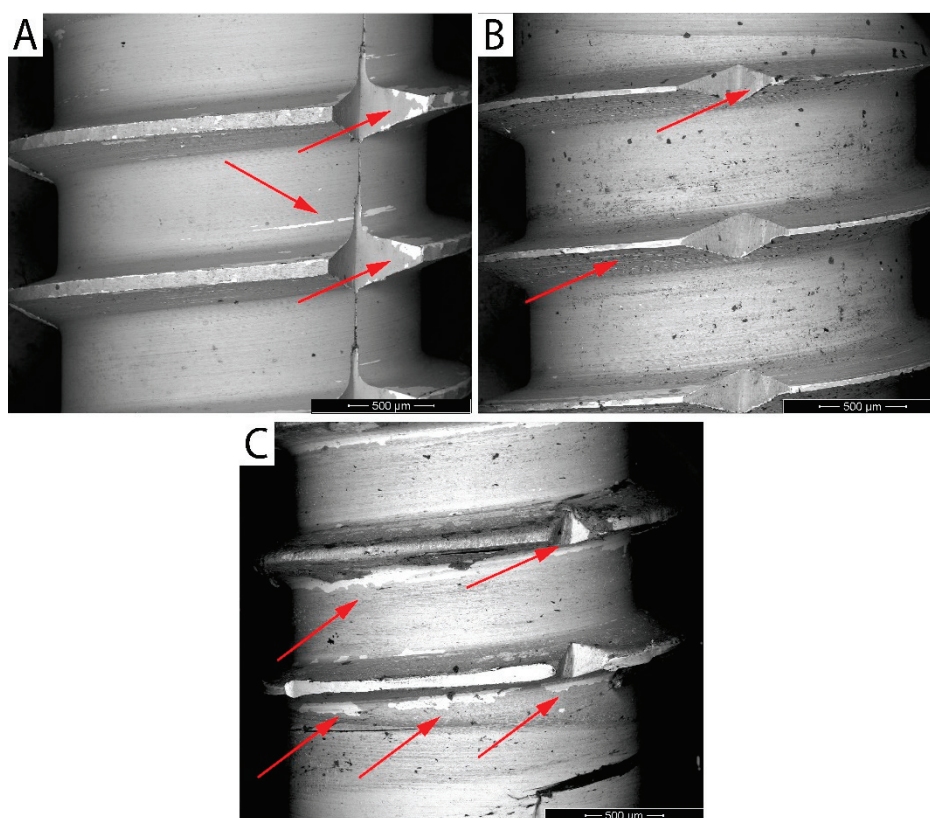


Figure 12. Implants' surfaces after their extraction from (A) artificial bone model, (B) dried ash wood, and (C) fresh cow rib; SEM images in BSE mode (material contrast).

The UFG CP Ti samples showed different levels of surface damage. The implant surface extracted from dried ash wood showed only minor surface damage. The surfaces of implants extracted from both the artificial bone model and fresh cow rib were damaged, especially along threads, while anodized surfaces among threads remained intact. The examination of implants was difficult due to organic impurities bonded to the surface, especially after extraction from fresh cow rib.

3.5. Layer Intercalation and FTIR

FTIR spectra of a pristine anodized surface (Figure 13) are dominated by a broad absorption peak around 650 cm^{-1} with a clear shoulder around 860 cm^{-1} . This corresponds well to $\nu(\text{Ti-O})$ vibrations and $\delta(\text{Ti-O-Ti})$ vibrations in titania nanotubes [69]. A less visible broad peak around 3000 cm^{-1} suggests that some O-H bonds in the form of $-\text{OH}$ termination of the TiO_2 structure or in the form of adsorbed water are also present. The area $2000\text{--}2600\text{ cm}^{-1}$ of the spectra is noised as CO_2 and other aerial impurities, together with the residual signal of the FTIR crystal, influence the spectrum [70].

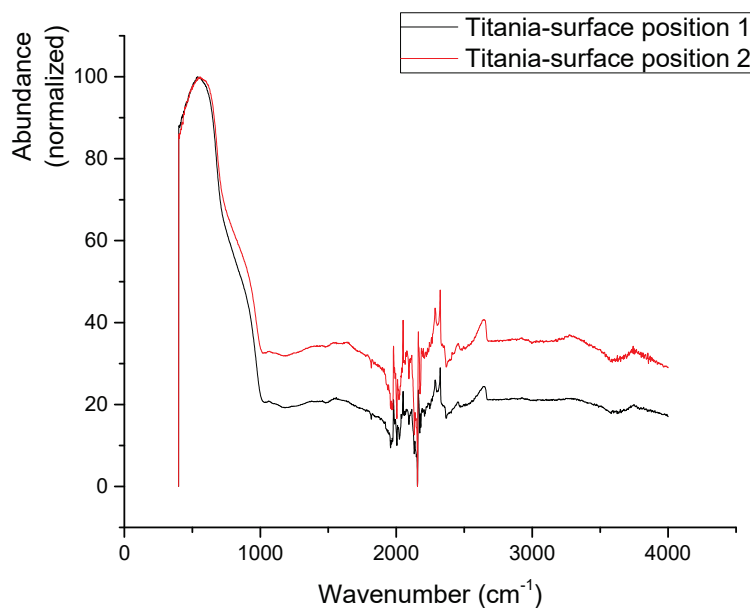


Figure 13. Infrared spectra of the anodized surface from two spots of the same sample.

DMSO-doped titania surface shows (Figure 14) most of the major peaks of DMSO: $\nu(\text{C-H})$: 2995 cm^{-1} and 2910 cm^{-1} , $\delta(\text{CH}_3)$: 1435 cm^{-1} , 1405 cm^{-1} , and 1310 cm^{-1} , and $\nu(\text{S=O})$: 1010 cm^{-1} . Further DMSO vibrations are also visible in the spectrum, such as $\nu(\text{C-S})$ 665 cm^{-1} and 695 cm^{-1} , yet they are partially hindered by strong absorptions of Ti-O bonds. Clearly visible $\delta(\text{H-O-H})$ vibration at 1650 cm^{-1} and $\nu(\text{O-H})$ $2700\text{--}3700\text{ cm}^{-1}$ vibrations suggests a significant presence of water, which is not a surprise as the DMSO is known to be hygroscopic and the surfaces were exposed to aerobic moisture. Only one DMSO vibration diminishes in DMSO-doped samples: $\nu(\text{S=O})$ 1045 cm^{-1} . No significant changes in Ti-O(-Ti) vibrations can be seen on the doped surfaces. Altogether, a comparison of DMSO and DMSO-doped surface IR spectra suggests that DMSO is mostly physisorbed on the titania surface with O-atom fixed, probably in some form of long-range $(\text{CH}_3)_2\text{S=O}\cdots\text{Ti}$ interaction.

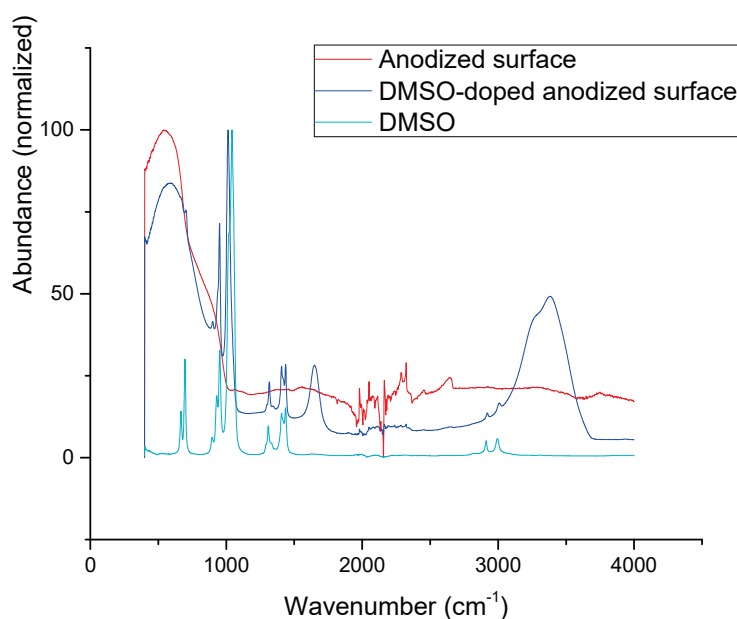


Figure 14. Infrared spectra of anodized surfaces, DMSO-doped anodized surface, and DMSO.

In ibuprofen-doped titania surfaces (Figure 15), the situation is less clear. No significant changes in Ti-O(-Ti) vibration patterns are visible. Traces of $\nu(\text{C-H})$ aromatic and $\nu(\text{C-H})$ aliphatic signals of ibuprofen can be seen in regions 2800 cm^{-1} to 3100 cm^{-1} along with the characteristic overtone and $\delta(\text{C(O)-O-H}\cdots\text{O})$ vibrations in the region of 2500 cm^{-1} to 2800 cm^{-1} and with some skeletal vibrations in the region of 1010 cm^{-1} to 1650 cm^{-1} [71]. The signals are, though, always broadened and somewhat shifted. The only signal which can be undoubtedly identified is the signal of the $\nu(\text{C=O})$ bond, always slightly shifted from the original wavenumber of ibuprofen (1700 cm^{-1} in ibuprofen vs. 1705 cm^{-1} in doped surface). It can be concluded that ibuprofen is present on the anodized surface. The nature of its adsorption is not clear though. Both physisorption and chemisorption cannot be definitely ruled out.

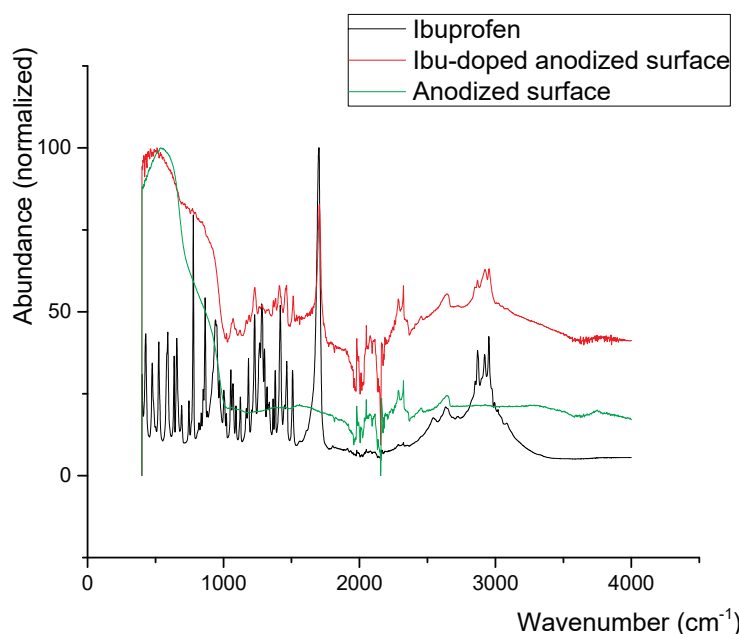


Figure 15. Infrared spectra of pristine anodized surface, Ibu-doped anodized surface, and ibuprofen.

3.6. Cell Adhesion and Proliferation

For biological evaluation, samples anodized at 40 V and 60 V were selected. All tested titanium surfaces were non-toxic to osteoblast cultures. However, significant differences were observed in cell adhesion and proliferation between etched, anodized, and control surfaces. Cell adhesion on UFG-40V was lower by 16% compared to CP Ti ($p = 0.0444$) and by 23% compared to control tissue culture plastic ($p = 0.0049$). In contrast, UFG-60 exhibited adhesion values comparable to both CP Ti and the control surface. Cell proliferation on UFG-40V remained significantly reduced compared to control cells ($p = 0.0016$). In the case of UFG-60V, proliferation was slightly higher than on CP Ti but still lower than on the control plate ($p = 0.0459$). A direct comparison of anodized surfaces showed significantly higher adhesion on UFG-60 than on UFG-40 ($p = 0.0393$). Figure 16 displays mean values and corresponding standard deviations for cell adhesion and proliferation. Corresponding p -values for all pairwise comparisons (sample vs. control, UFG vs. CP Ti, UFG vs. UFG) are summarized in Table 5. Representative microscopic images of osteoblast morphology on titanium surfaces and the control plate are shown in Figure 17.

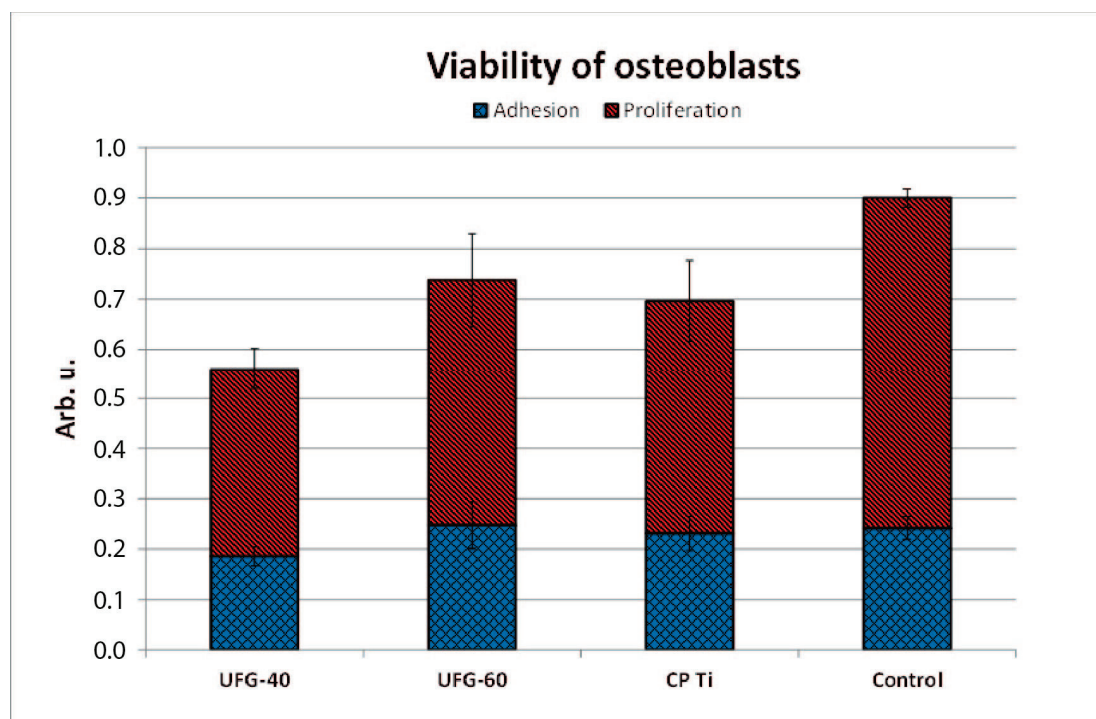


Figure 16. Comparison of cell adhesion and proliferation on the selected surfaces. Error bars indicate standard deviation.

Table 5. Summary of *p*-values (Student's *t*-test) for cell adhesion and proliferation from Figure 16.

Comparison	<i>p</i> (Adhesion)	Significance	<i>p</i> (Proliferation)	Significance
V4 vs. Control	0.0049	**	0.0016	**
V6 vs. Control	0.8411	ns	0.0459	*
CP Ti vs. Control	0.6100	ns	0.0070	**
V4 vs. CP Ti	0.0444	*	0.1585	ns
V6 vs. CP Ti	0.5899	ns	0.6392	ns
V6 vs. V4	0.0393	*	0.1017	ns

* $p < 0.05$, ** $p < 0.01$, ns—non-significant.

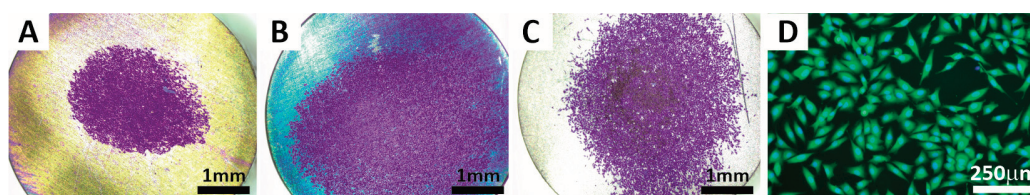


Figure 17. Osteoblasts on the surfaces of samples and control cells after 48 h of proliferation. (A) UFG-40 with cells stained by crystal violet, (B) UFG-60 with cells stained by crystal violet, (C) CP Ti with cells stained by crystal violet, (D) cells on the plastic tissue plate stained by NUC Blue and CellTracker.

4. Discussion

All anodized samples were produced from ultrafine-grained (UFG) CP Ti grade 4, which provides a combination of high mechanical strength known from Ti6Al4V and corrosion resistance typical for lower grades of commercially pure titanium [13]. The anodizing voltages and electrolyte composition were selected according to previous studies, reporting stable nanotubular oxide growth and good surface integrity [27,35,72]. Since the voltages used were above the water decomposition threshold, excessive gas evolution can reduce

oxide adhesion by forming interfacial bubbles or blisters [73]. For this reason, the water content in the electrolyte was limited to 20 wt.% to increase coating stability. Lower water content (15–25 wt.%) is also known to promote the formation of more compact and mechanically stable anodic oxide layers [74]. Surface morphology was strongly dependent on the applied voltage. At 20 V, highly ordered and vertically aligned nanotubes were formed (Figure 9A). Their organization into small clusters is associated with internal stresses during oxide growth and the limited migration of ions in UFG material [75]. At higher voltages (40–60 V), the electric field intensity caused partial dissolution and restructuring of the nanotube layer, resulting in less ordered and predominantly nanoporous surfaces (Figure 9B,C), which may enhance interfacial adhesion to the substrate [76]. Increasing voltage also led to larger pore diameters and smoother surface features, as oxide growth induces volume expansion and preferential oxidation at sharp edges [25,77]. These effects reduce surface defects from mechanical polishing and promote more uniform oxide coverage. A notable difference compared to conventional grain size titanium is the formation of nanotube clusters already at 20 V in UFG titanium, while in coarse-grained materials, similar structures only occur at higher voltages or longer anodizing times [78,79]. This can be attributed to grain refinement, higher density of grain boundaries, and local crystallographic orientation, which influence oxide growth direction and residual stress distribution. Samples anodized at 40 and 60 V predominantly showed nanoporous rather than nanotubular morphology, which correlates with reduced fluoride content and increased structural stability of the oxide layer on UFG titanium. Heat treatment of the anodized samples was not performed to avoid possible recrystallization of the UFG substrate, which can occur even below 350 °C [80] and would affect both microstructure and mechanical properties. Potentiodynamic polarization measurements confirmed that the UFG-20V surface exhibited the lowest corrosion rate ($1.3 \mu\text{m}\cdot\text{year}^{-1}$), comparable to CP Ti grade 3 ($1.2 \mu\text{m}\cdot\text{year}^{-1}$), which is commonly used in long-term hard tissue implants [35]. Samples anodized at higher voltages showed a gradual increase in the corrosion rate ($1.7 \mu\text{m}\cdot\text{year}^{-1}$ for UFG-40V and $3.7 \mu\text{m}\cdot\text{year}^{-1}$ for UFG-60V), but all values remained well below the commonly accepted limit of $20 \mu\text{m}\cdot\text{year}^{-1}$ for implantable biomaterials [81]. These results are consistent with earlier studies reporting equal or slightly lower corrosion rates for anodized UFG titanium compared to anodized conventional titanium grades [82,83]. It must be acknowledged that electrochemical parameters were calculated using a geometric surface area, which is valid for non-porous surfaces such as CP Ti. However, anodized surfaces possess a significantly higher real surface area due to porosity, especially in UFG-60V samples. As a result, corrosion current density and derived corrosion rates may be overestimated for highly porous oxides [84,85]. This effect should be considered when comparing anodized and non-anodized samples. Corrosion potential (E_{corr}) provides useful information on the electrochemical nobility of the surface. The CP Ti reference sample exhibited E_{corr} of -376 mV vs. SCE , in agreement with previously reported values [86]. Anodization at 20 V shifted the potential to more noble values (-204 mV), while anodization at 40 V and 60 V resulted in significantly less noble potentials (-632 and -652 mV , respectively). This behavior is attributed to differences in surface morphology and oxide structure, as similarly observed in other studies [26,87,88]. The UFG microstructure does not appear to have a dominant effect on the corrosion potential, since the obtained values correspond well with those reported for anodized coarse-grained titanium [35,89]. From a practical viewpoint, the lower corrosion potential of UFG-40V and UFG-60V surfaces indicates a higher risk of galvanic interactions when coupled with more noble materials in multi-material systems (e.g., NiTi, stainless steel, untreated titanium alloys) [90–92]. This may be relevant in dental or orthopedic implants where dissimilar materials are used. The improved behavior of UFG-20V samples is related to the compact and uniform nanotubular oxide, which provides

an effective barrier against ion exchange and inhibits localized corrosion [93,94]. In contrast, highly porous structures formed at higher voltages increase ionic permeability and reduce polarization resistance due to easier electrolyte penetration through open pores.

During anodic polarization up to 4 V vs. SCE, none of the samples exhibited breakdown potential or passivity loss. According to EN ISO 10993–15 [95], materials that do not reach pitting potential at 1752 mV vs. SCE are considered resistant to pitting corrosion. All anodized surfaces fulfilled this criterion, showing stable current density throughout the test, which indicates persistent passivity and only general corrosion processes [95,96]. Surface wettability is an important parameter influencing initial biological responses, including protein adsorption, cell adhesion, and tissue integration. The contact angle of CP Ti grade 3 was measured as 81.4° , indicating a moderately hydrophobic character. All anodized surfaces became more hydrophilic, with contact angles of 52.3° (UFG-20V), 67.9° (UFG-40V), and 71.0° (UFG-60V). Similar trends were reported for anodized titanium with a conventional grain size [27,35,97], confirming that anodization generally enhances hydrophilicity regardless of grain refinement. The most hydrophilic surface (UFG-20V) exhibited a contact angle significantly below 60° , which is considered favorable for osteoblast adhesion. Previous studies demonstrated that osteoblast attachment is enhanced when the contact angle is between 0° and 60° , whereas fibroblast adhesion is strongest in the 60 – 80° range [98]. This suggests that anodized UFG titanium, particularly at 20 V, may support strong bonding to bone tissue, whereas untreated titanium might exhibit weaker osseointegration and stronger fibrous encapsulation under load-bearing conditions [99]. Enhanced hydrophilicity is also associated with faster adsorption of blood proteins that initiate the early stages of healing and bone formation [100]. Although the direct influence of the UFG microstructure on wettability is still unclear, the obtained values are consistent with anodized surfaces of conventional titanium. For nanoporous surfaces, contact angles around 65 – 71° have been reported [101,102], whereas nanotubular structures typically show higher hydrophilicity (50 – 62°) [103,104], in agreement with the present results. Overall, the observed wettability behavior indicates that anodization at lower voltage (20 V) leads to surface conditions more favorable for osteoblast adhesion and osseointegration, while higher voltage anodization yields less hydrophilic surfaces that may reduce bone–implant bonding but still remain within biocompatible limits.

Adhesion of anodic oxide layers to the substrate is a key factor limiting the practical use of anodized implants [105]. For mechanical stability evaluation, only the UFG-60V surface was selected, since the oxide formed at this voltage is the thickest, most porous, and thus most prone to delamination under load [75,106]. Subsequently, the larger pore size can facilitate drug loading or biomolecule incorporation but may also reduce mechanical integrity. In contrast to standard testing procedures using simple insertion and removal in split media [107,108], a more demanding method was applied. Implants were inserted into artificial bone, dried ash wood, and fresh cow rib using a torque motion, which better simulates real implantation stresses. Only minor surface changes were observed after insertion into artificial bone (Figure 12A), and slightly more after insertion into wood (Figure 12B). The most noticeable surface damage occurred after extraction from cow rib (Figure 12C), particularly around individual threads and sharp cutting edges, where mechanical stress is highest and localized compression and shear forces act on the surface. Despite visible damage, no complete delamination, cracking, or flattening of the anodic layer was observed. This indicates better mechanical resilience compared to conventional grain-size titanium in similar studies, where surface deformation or oxide delamination was more pronounced [109]. The improved stability may be related to the refined grain size of the substrate, which enhances mechanical compatibility between the oxide layer and underlying titanium and reduces interfacial stresses. These results show that even though

UFG-60V-anodized surfaces are the most porous and electrochemically less stable among anodized samples, they maintain acceptable mechanical resistance under implantation-like conditions.

Ibuprofen and dimethyl sulfoxide (DMSO) were selected as model compounds to evaluate the ability of anodized UFG titanium to act as a local drug delivery platform. These substances differ significantly in molecular size and polarity—DMSO is a small, highly polar molecule ($M_w = 78.13 \text{ g}\cdot\text{mol}^{-1}$), while ibuprofen is larger, contains an aromatic ring and a carboxyl group, and better represents clinically relevant drugs. Their use allows an assessment of both the loading capacity of the nanoporous/nanotubular structures and the interaction mechanisms between TiO_2 and organic molecules. DMSO was used to determine whether ultrafine nanopores can accommodate very small molecules or solvents. Due to its low steric hindrance, DMSO provides a lower boundary for molecular uptake. In contrast, ibuprofen represents a bulkier molecule whose adsorption and retention can reveal which functional groups interact with the TiO_2 surface and whether steric limitations prevent infiltration into nanotubes. This information is essential for predicting which classes of drugs or antibacterial agents could be successfully immobilized on modified titanium surfaces. Previous studies have confirmed the ability of anodized titanium (with conventional microstructure) to incorporate ibuprofen within nanotubular layers [28,29,110]. Our results are in agreement—ibuprofen was successfully intercalated into UFG-anodized surfaces, suggesting that substrate grain refinement does not negatively affect the process. The mechanism of ibuprofen binding and its release kinetics are still under investigation and may depend on electrostatic interactions, hydrogen bonding, or physical entrapment within the nanotubes. Although the exact mechanism remains unclear, no degradation of ibuprofen was observed and its chemical compatibility with anodized TiO_2 was maintained. Overall, the results support the potential of anodized UFG titanium as a functional platform for local drug delivery, particularly for anti-inflammatory or antibacterial agents, while preserving biocompatibility. The biological response to titanium implants is largely determined by the physicochemical properties of the surface, including oxide composition, topography, surface energy, and chemical reactivity. Bioactive surface modification is a well-established approach to promote osseointegration and to reduce the release of potentially cytotoxic metal ions. The interaction between the material and cells is governed not only by direct cell–implant contact but also by protein adsorption, focal adhesion formation, and cell orientation mechanisms [111]. The produced anodized UFG CP Ti surfaces demonstrated good cytocompatibility, consistent with previous observations on conventional titanium grades. The nanotubular surface morphology obtained at 20 V showed enhanced cell adhesion and proliferation compared to the nanoporous morphology, which can be attributed to its combination of high surface area, hydrophilicity, and appropriate nanotube diameter. These results agree with studies reporting that nanotubular TiO_2 layers promote osteoblast functions and early bone–implant integration [29,112]. The substrate grain refinement did not negatively affect biological performance, suggesting that the primary factor influencing cell behavior is the morphology and chemistry of the anodic oxide rather than the bulk microstructure. In contrast, surfaces anodized at higher voltages (40–60 V) showed lower proliferation or weaker adhesion, which corresponds to their reduced hydrophilicity and nanoporous rather than nanotubular structure. Nevertheless, all anodized samples remained cytocompatible, confirming that none of the tested surfaces induced cytotoxic effects. Furthermore, anodized nanotube layers are capable of functioning as drug delivery reservoirs, enabling the localized administration of anti-inflammatory, antibacterial, or osteogenic agents [113]. This dual functionality—supporting osseointegration while allowing drug loading—represents a promising benefit of anodized UFG titanium surfaces. Overall, the biological results confirm that combining ultrafine-grained titanium

with optimized anodization conditions (especially at lower voltages) leads to surfaces that support osteoblast activity and provide potential for advanced implant functionality.

5. Conclusions

This research concerns a multidisciplinary study of anodized layers on CP Ti with an ultrafine-grained (UFG) microstructure. The main findings are as follows:

- Anodization at 20 V produced an oxide layer formed by clusters with typical nanotubular morphology. Anodization at 40 V and 60 V produced homogeneous TiO₂ layers with nanoporous morphology. The pore size increased with the anodization voltage.
- The morphology of oxide layers on UFG CP Ti anodized at 40 V and 60 V matched the morphology of conventional grain size CP Ti anodized in a solution with a significantly lower water content.
- The corrosion rate of the anodized surfaces was slightly higher than that of the untreated CP Ti surface, but still deep under the limit recommended for implants.
- Corrosion potential of anodized surfaces was slightly increased compared to the untreated CP Ti surface, which increases the risk of their damage in the case of coupled corrosion initiation.
- Anodized layers were not prone to pitting corrosion even if polarized to 4000 mV vs. SCE in a solution containing a medium concentration of chlorine ions.
- Anodization of UFG CP Ti decreased the surface contact angle and, at the same time, increased the surface energy, which promotes easier colonization by hard tissue-forming cells.
- The anodized layer formed at 60 V on UFG CP Ti implants exhibited sufficient resistance to mechanical stress arising during simulated implantation procedures.
- The highly porous TiO₂ surface produced at 60 V enabled the successful intercalation of DMSO and ibuprofen.
- All tested titanium surfaces were non-cytotoxic to osteoblasts. Cell adhesion and proliferation were slightly reduced on UFG-40V, but slightly enhanced on UFG-60V, when compared to the reference CP Ti. Overall, UFG-60V showed higher cell adhesion than UFG-40V and the reference titanium surface. Most parameters studied in this paper (corrosion, wettability, intercalation, biological) on UFG CP Ti and conventional grain size CP Ti anodized at similar conditions were comparable, and no significant differences were found between them.
- The results presented in this paper prove that specific electrochemical oxidation of miniaturized biomedical implants manufactured from UFG CP Ti had a positive effect on their biological performance and could also serve as a targeted drug delivery system while maintaining their excellent corrosion resistance.

6. Patents

Part of the research published in this paper is the subject of utility model 2019–36996 registered on 11th December 2019 by the Industrial Property Office of the Czech Republic.

Author Contributions: Conceptualization, J.H., D.C. and K.P.D.; Methodology, J.H., L.D. and V.B.; Validation, K.C. and J.D.; Formal Analysis, N.H.F.; Investigation, J.H., D.C. and L.D.; Resources, R.P.; Data Curation, A.V. and R.Z.V.; Writing—Original Draft, J.H. and D.C.; Writing—Review and Editing, J.H.; Visualization, J.H.; Supervision, K.P.D. and V.V.; Funding Acquisition, J.H. and K.P.D. All authors have read and agreed to the published version of the manuscript.

Funding: This paper was created with the contribution of the following projects: “No. CZ.02.01.01/00/22_008/0004631. Materials and technologies for sustainable development within the Jan Amos Komenský Operational Program financed by the European Union and from

the state budget of the Czech Republic”, SP2025/076 “Material and technological properties of construction materials in relation to their production method, processing and the effect of degradation mechanisms”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original data presented in the study are openly available in Zenodo at <https://doi.org/10.5281/zenodo.17643764>.

Conflicts of Interest: Author Ludek Dluhos was employed by the company TIMPLANT, Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Influence of Reduced Cortical Bone Compression by Implant Macrogeometry on Peri-Implant Bone Healing: An In Vitro and In Vivo Experimental Study

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Abstract

Background: Primary stability and long-term osseointegration depend on bone healing surrounding dental implants. Implant macrogeometry is crucial for controlling insertion torque and the biological reaction of peri-implant bone. This study assessed the impact of an implant design meant to lessen cortical bone compression on early bone healing. **Methods:** Forty titanium prototype implants (3 × 6 mm) were equally divided into Control (standard macrogeometry) and Test (macrogeometry with healing chambers) groups. Initial insertion torque was measured in vitro using synthetic bone blocks. Subsequently, an in vivo rabbit tibia model was used ($n = 10$ implants per group) to assess early healing. At 21 days, histological sections were analyzed for bone-to-implant contact (BIC%) at three cervical positions (C1, C2, and C3). Additionally, digital radiographs of the cervical region were evaluated using RGB color mapping, where distinct color channels quantified varying degrees of bone density. **Results:** The in vitro insertion torque for the Control group was significantly greater than the Test group (8.01 vs. 5.70 Ncm). The in vivo histomorphometric analysis indicated improved integration for the Test design, showing substantially higher BIC% at the C2 (59.30% vs. 40.30%) and C3 (42.10% vs. 17.90%) positions. Furthermore, radiographic RGB analysis revealed that the Test group possessed a higher blue channel contribution, indicating greater mineralized tissue density. **Conclusions:** These results imply that modifying implant macrogeometry to lower insertion torque and minimize cortical bone compression favorably enhances early cervical bone healing and osseointegration.

Keywords: bone healing; bone-to-implant contact; cortical bone compression; dental implants; healing chambers; insertion torque; osseointegration; rabbit model

1. Introduction

The intricate process of bone healing surrounding dental implants relies on the integration of the implant surface with the surrounding bone tissue. This mechanism dictates both primary stability and long-term success [1–5]. While the biocompatibility of the implant surface is crucial, the macrogeometry of the implant, including its design, diameter, and length, as well as the surgical technique employed, strongly influences healing efficiency [6–12]. For many years, high insertion torque values were associated with successful osseointegration [13]. However, recent evidence demonstrates that surgical methods providing adequate osteotomy dimensions lead to faster and more efficient bone regeneration [14,15]. Consequently, novel implant designs have been developed to minimize bone tissue compression during placement [13,16–22].

To understand the rationale for these novel designs, one must evaluate how cortical and medullary bone respond differently to insertion trauma [16–18,23,24]. Cortical bone is denser and less vascularized than trabecular bone, making it far more vulnerable to mechanical stress and compression. During the placement of conventional implants, mechanical friction against the cortical bone is the primary means of achieving initial stability [25–30]. However, conventional cylindrical or conical implants can cause excessive bone compression. This is especially true in regions with dense cortical bone. Such compression may result in microfractures and delay bone healing [31–33]. Ultimately, this mechanical trauma can impede appropriate regeneration, postpone the production of high-quality bone, and raise the risk of peri-implant marginal bone resorption [34].

In this case, implant macrogeometry directly affects insertion trauma and the torque needed for fixation, in addition to the surgical approach used [16,17]. Conventional cylindrical or conical implants may cause excessive bone compression after implantation, especially in regions with dense cortical bone, which could result in microfractures and postpone bone healing [31]. Because cortical bone is less vascularized and denser than trabecular bone, it is more vulnerable to mechanical stress. Cortical bone compression or microfractures may impede appropriate regeneration, postpone the production of high-quality bone tissue, and raise the possibility of peri-implant bone resorption or failure [34].

Implants featuring healing chambers offer a biomechanical alternative to mitigate this stress. These designs can limit mechanical trauma and lower insertion torque. This maintains the integrity of cortical bone, particularly in the cervical region. Preserving this bone is essential for initial stability and peri-implant bone growth [13,16,17,24,31]. According to preclinical research, maintaining cortical bone during implantation encourages the development of thick, well-organized tissue, which minimizes early resorption and improves overall bone-implant contact [16,24,31]. Previous studies in rabbits comparing implants with and without healing chambers reported higher removal torque for the chambered implants, suggesting quicker and more efficient bone mineralization. Even though increased insertion torque does not automatically trigger crestal resorption, the macrogeometry design clearly directs the pattern and quality of new bone formation.

Experimental rabbit models are frequently employed to assess the biological impact of these implant macrogeometries. Rabbits exhibit a rapid rate of bone remodeling. Furthermore, this model allows the placement of multiple implants per animal, enabling reliable intra-animal comparisons between different designs [35,36].

Higher removal torque for implants with healing chambers was found in earlier research on rabbits comparing implants with and without healing chambers, suggesting quicker and more efficient bone mineralization when compared to conventional implants. These results imply that the pattern and quality of bone healing surrounding the implant

are the primary differences, even though increased insertion torque does not always result in crestal bone resorption.

Based on this reasoning, the current study postulates that implants with healing chambers will favorably influence bone healing in the cervical region by reducing cortical compression during insertion. This should encourage increased bone density and superior bone-implant integration compared to conventional designs. Therefore, the purpose of this study was to assess differences in bone healing around titanium implants inserted with higher and lower compression (insertion torque) in rabbit tibiae, utilizing digital radiography and histological analyses with a specific focus on the cervical region.

2. Materials and Methods

2.1. Implants and Groups

Forty commercially pure titanium (grade IV) cylindrical implants, each measuring 3 mm in diameter and 6 mm in length, were created for this investigation utilizing titanium oxide microparticles and sandblasting as a surface treatment (Implacil/Osstem, São Paulo, Brazil). There were 20 implants in each group; the Test Group had healing chambers, whereas the Control Group did not. Circular holes made between the implant's threads defined the healing chambers. The two implant models and their dimensional properties are displayed in Figure 1.

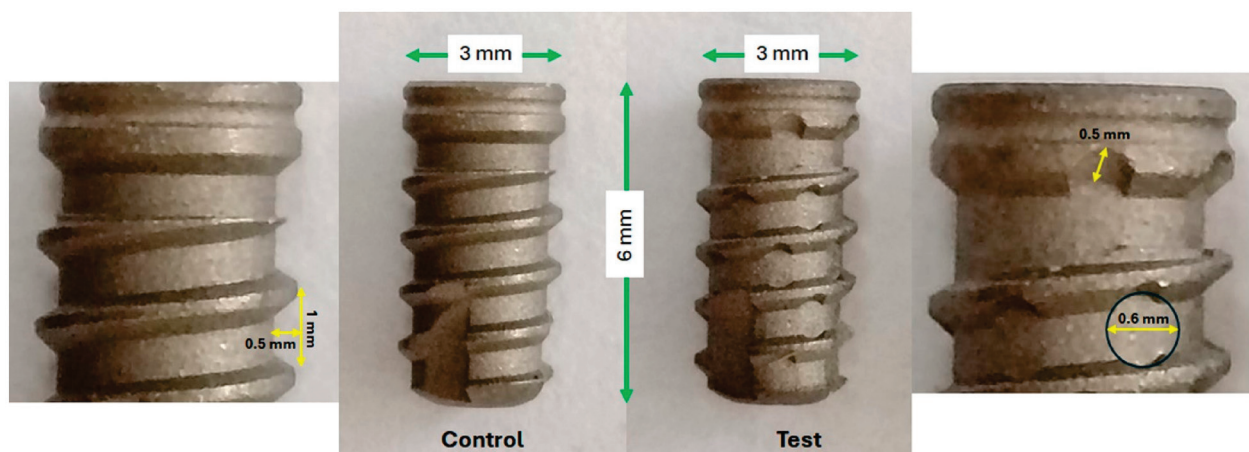


Figure 1. The two types of implants used in the study are shown schematically. The Control implant, which has a typical cylindrical shape without any alterations, is depicted in the left figure. The test implant, which has healing chambers with circular holes between the threads, is seen in the image on the right. The two implants are 6 mm long and 3 mm in diameter. Key aspects are measured in detail: both implants have a depth of 0.5 mm and a pitch of 1 mm between threads. The Test implant also has healing chambers with a diameter of 0.6 mm and a depth of 0.5 mm.

2.2. In Vitro Insertion Torque Evaluation

For the in vitro insertion torque assessment, ten implants from each group were employed. This method was chosen since most surgical torque meters are analog and frequently inaccurate, making it challenging to acquire exact and trustworthy measurements during in vivo implantation. For this test, polyurethane foam synthetic bone blocks of 95 mm × 45 mm × 35 mm (Nacional Ossos, Jaú, Brazil) were utilized to simulate human bone. The American Society for Testing and Materials (ASTM) has accepted and acknowledged these materials as standard for testing bone instruments and implants [37]. A cortical thickness of 1 mm with a high density of 40 pounds per cubic foot (PCF) and a low-density medullary part of 10 PCF were employed to simulate the properties of rabbit tibial bone. A lance-shaped drill was first used to make the perforations, and then

a cylindrical drill with a 2.8 mm diameter was used to reach a depth of 6 mm. A bench drill with a controlled speed of 800 rpm and a pressure of 1 kg was used to perform 20 similar operations (Figure 2a). This eliminated any eccentric motions that might happen while drilling manually in a clinical setting.

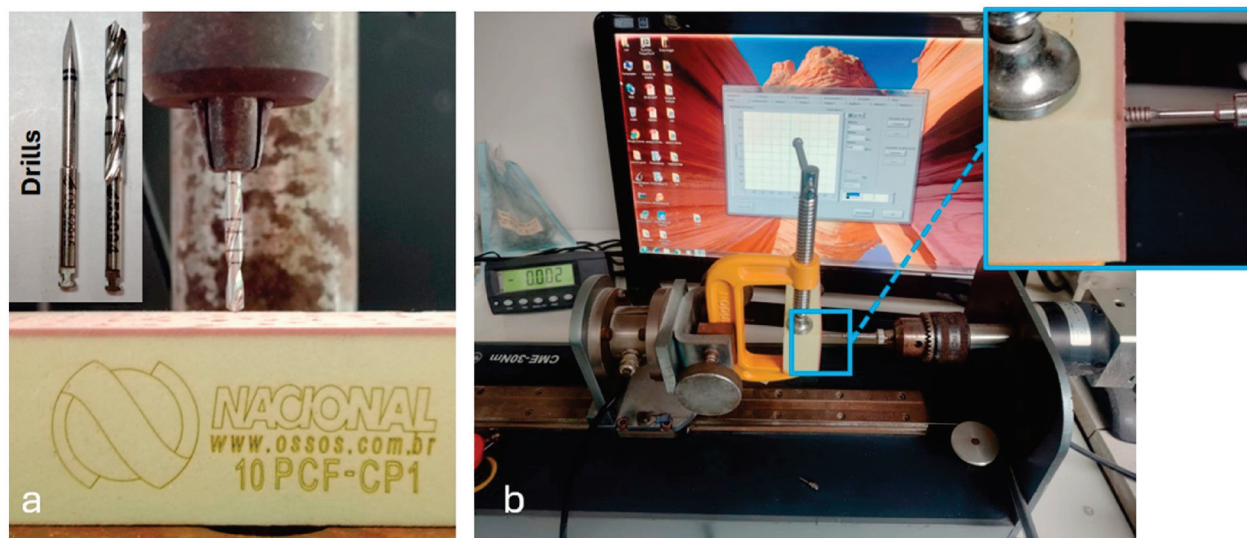


Figure 2. System for evaluating insertion torque in vitro. (a) A polyurethane foam synthetic bone block that mimics human bone is perforated using a lance-shaped drill and then a cylindrical drill (2.8 mm diameter, 6 mm depth). During the piercing process, a controlled speed of 800 rpm and 1 kg of pressure were used. (b) To ensure excellent measurement accuracy, a computerized digital torque meter was used to place the implants into the artificial bone. The measuring interface for torque monitoring during insertion is displayed on the computer's screen.

A computerized digital torque meter model CME-30 (Tecnica Industrial Oswaldo Filizola, São Paulo, Brazil) with an angular resolution of 0.002° was then used to insert the implants into the synthetic polyurethane bone block, reducing measurement errors and enhancing experimental precision (Figure 2b). Every implant was placed to the depth of the prepared perforation at the cortical bone level. For assessment and statistical analysis, the highest torque value attained during implant insertion was utilized.

2.3. Animal Model and Care

This study was initially examined and approved by the Animal Ethics Committee of the University of Rio Verde (Rio Verde, Brazil) under protocol number 04/20. All procedures strictly followed the ARRIVE guidelines and complied with Brazilian norms for animal experimentation, as recommended by the National Council for the Control of Animal Experimentation (CONCEA) resolution number 15 of 2013 [38]. Furthermore, the study design incorporated the 3Rs principles (Replacement, Reduction, Refinement) to ensure the ethical use of animals.

Following these guidelines, five adult New Zealand rabbits (*Oryctolagus cuniculus*), weighing between 3.5 and 4.0 kg, were obtained from the University of Rio Verde Animal Facility. The rabbit model is well-established and widely validated in the literature for this type of osseointegration study. Prior to the onset of the trial, the animals were dewormed and allowed to acclimate to the laboratory environment and human handling for 15 days. Throughout the acclimation period and the duration of the trial, each animal was housed individually in appropriate enclosures with ad libitum access to food and water.

To maximize statistical power while adhering to the ethical principle of Reduction (minimizing sample size), an intra-animal study design was employed. In this setup, both

the Test and Control implants were placed in the tibiae of each rabbit, allowing the animals to serve as their own controls and reducing inter-subject biological variability.

2.4. Surgical and Medicinal Procedures

2.4.1. Anesthesia and Pharmacological Protocol

The animals were initially anesthetized via an intramuscular injection of a ketamine-xylazine mixture, administered in a single syringe using 35 mg/kg of ketamine (Dopalen; Ceva Saúde Animal Ltd., Paulínia, Brazil) and 5 mg/kg of xylazine (Anasedan; Ceva Saúde Animal Ltd., Paulínia, Brazil). Local anesthesia was established by subcutaneous infiltration of 4% articaine with epinephrine 1:100,000 (DFL, Rio de Janeiro, Brazil) at the intended surgical incision site on each tibia. To provide transoperative analgesia, meloxicam at 0.2 mg/kg (Maxicam; Ouro Fino Saúde Animal Ltd., Cravinhos, Brazil) and morphine at 5 mg/kg (Dimorf; Cristália Produtos Químicos Farmacêuticos Ltd., São Paulo, Brazil) were administered subcutaneously. Additionally, prophylactic antibiotic therapy was given preoperatively via a subcutaneous dose of 84,000 IU/kg of Pentabiotic (Zoetis Indústria de Produtos Veterinários Ltd., Campinas, Brazil).

2.4.2. Surgical Procedure

Following tibial shaving, local antisepsis was performed using povidone-iodine solution and 70% alcohol. A linear incision measuring approximately 5 cm in length was created in the proximodistal direction, starting close to the joint and extending along the medial surface of each tibia. The tissues were gently elevated to expose the bone, and osteotomies were performed using the same drilling protocol described in Section 2.2. Each animal received four implants in total (two in each tibia). To ensure an equal anatomical distribution between the proximal (P) and distal (D) regions of the tibia, the implants were positioned as follows: in the right tibia, the Test Group implant was placed in the P region and the Control Group implant in the D region; in the left tibia, this arrangement was inverted.

2.5. Post-Operative Care and Euthanasia

2.5.1. Postoperative Care

Following the surgical procedures, the animals were housed in individual cages and continuously monitored until they fully recovered from anesthesia. For the first 24 h post-surgery, analgesia was maintained via subcutaneous morphine (5 mg/kg) administered every six hours. Subsequently, anti-inflammatory control was sustained using subcutaneous meloxicam (0.2 mg/kg) once daily for five consecutive days. Because the preoperatively administered Pentabiotic provides broad-spectrum antimicrobial coverage for up to seven days, no additional postoperative antibiotic doses were required.

2.5.2. Euthanasia and Sample Retrieval

At 21 days post-implantation, all animals were euthanized in strict accordance with CONCEA standards (Normative Resolution No. 37/2018). Euthanasia was performed via an intravenous overdose (50 mg/kg) of 3% pentobarbital (Euthanyle, São Paulo, Brazil). Clinical death was verified by a licensed veterinarian, who confirmed the absence of respiratory and cardiac activity, lack of a perceptible pulse, absence of the corneal reflex, and the presence of pale mucous membranes.

2.6. Sample Collection and Handling

Every sample ($n = 10$ per group) was designated for histological examination. Following collection, the samples were immediately immersed in 10% buffered formalin for

seven days. They were subsequently dehydrated in a graded series of ethanol solutions (50–100%) and embedded in historesin (Technovit 7200 VLC; Kulzer, Wehrheim, Germany).

Prior to sectioning, each intact bone block comprising a tibia was radiographed using a portable digital system (IriX-ray DX 3000; Dexcowin, Seoul, Republic of Korea) at a fixed distance of 15 cm. These initial radiographs were taken in the lateromedial (L-M) direction (i.e., with the X-ray beam passing horizontally from the lateral to the medial aspect of the tibia). Data collection and image acquisition were performed using an intraoral digital sensor (RVG First; Trophy, Toulouse, France) and Trophy Imaging software (version 7.0.19.3.d1).

Next, the tibial blocks were divided in half to isolate each implant in its own block. A second set of radiographs was then taken using the identical equipment and parameters, but in the proximodistal (P-D) direction (i.e., with the X-ray beam passing vertically along the long axis of the bone, from the proximal joint toward the distal extremity). Representative pictures of both radiographic directions are shown in Figure 3.

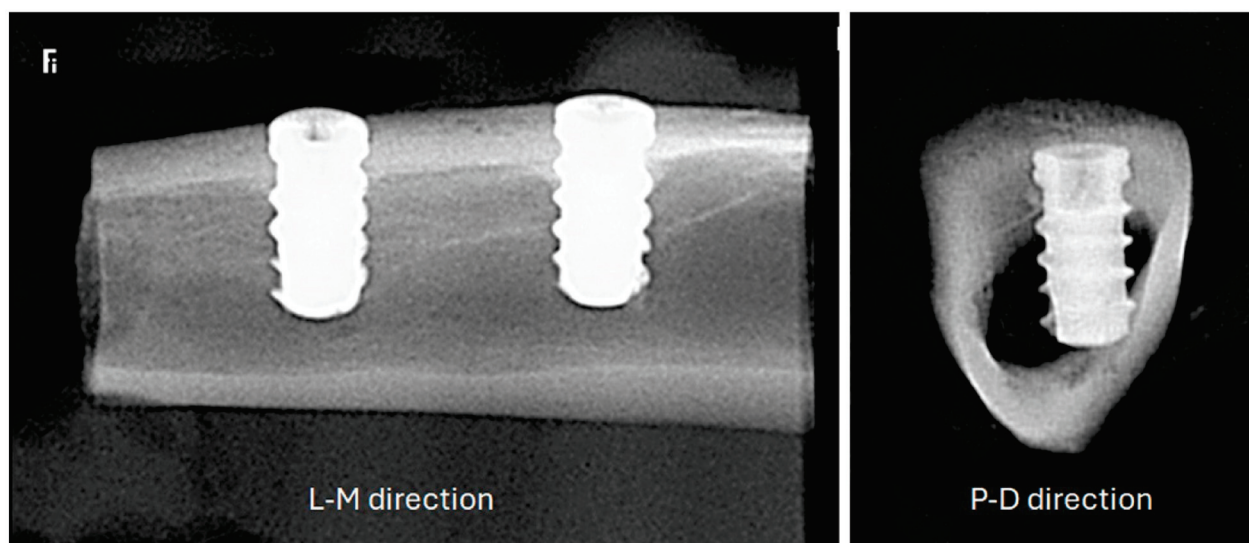


Figure 3. Digital radiographs that are representative of the bone samples in the two directions that were analyzed. The radiograph is displayed in the proximodistal (P-D) direction in the right image and in the lateromedial (L-M) direction in the left image.

Following radiographic acquisition, transverse sections were obtained using an IsoMet 1000 machine (Buehler, Lake Bluff, IL, USA). As schematically depicted in Figure 4, three slices (designated C1, C2, and C3) were performed within the first three millimeters of the cervical region of each implant.

2.7. Histomorphometric and Histological Analysis

The slides were stained using the picosirius hematoxylin method, and histological images were captured using a light microscope (E200; Nikon, Tokyo, Japan). ImageJ software (version 1.52v; National Institutes of Health, Bethesda, MD, USA) was utilized to calculate the bone-to-implant contact percentage (BIC%) for each designated transverse section level (C1, C2, and C3).

Furthermore, the area fractions of bone tissue (BT), collagen tissue (CT), and marrow spaces (MS) were quantified in each image within standardized rectangular regions of interest (4×6 mm). As depicted in Figure 5, these tissue percentages were calculated utilizing a digital color-segmentation method. Briefly, the ImageJ Color Threshold tool was applied to isolate specific hue ranges corresponding to the distinct stained tissues (red/pink = BT; yellow/orange = CT; and white/gray = MS). To ensure analytical accuracy and

validate the digital segmentation, the computer-generated threshold areas were visually cross-verified against the original histological images by a calibrated examiner prior to the final area fraction computation.

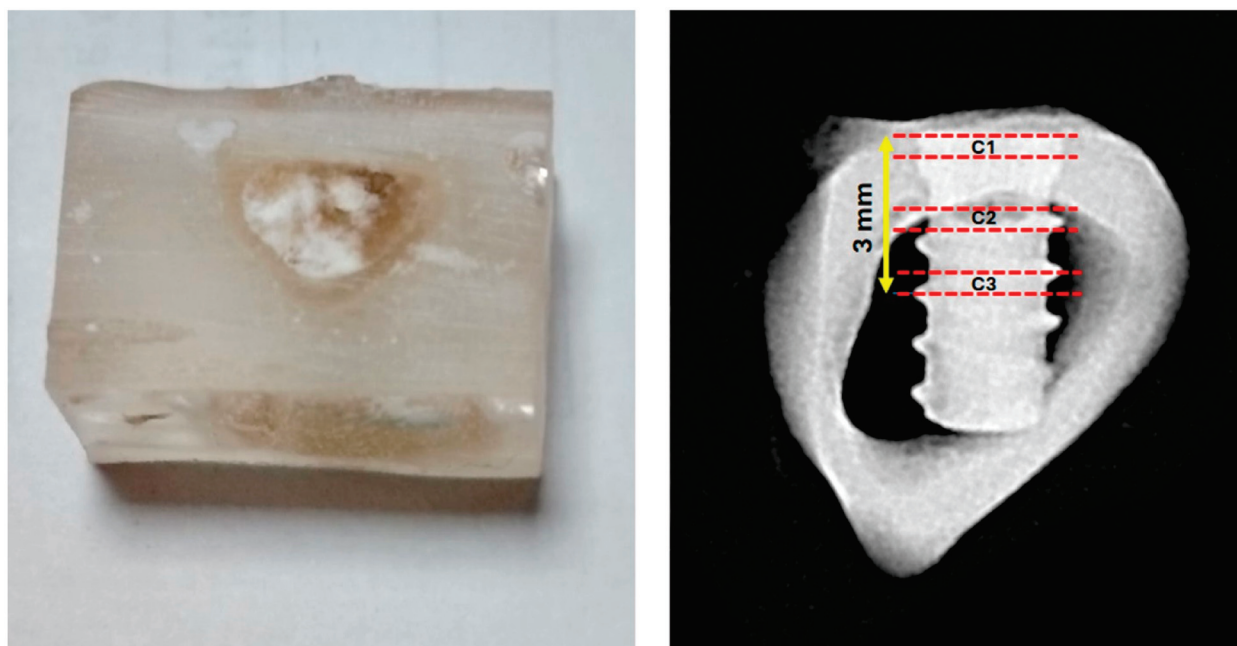


Figure 4. A unit sample is contained in a historesin block prior to sectioning. The three transverse incisions (C1, C2, and C3) within the first 3 mm of the implant were shown in the radiographic image taken in the lateromedial (L-M) direction, as shown by the red dashed lines, for further histological examination. An IsoMet 1000 machine was used to obtain the sections.

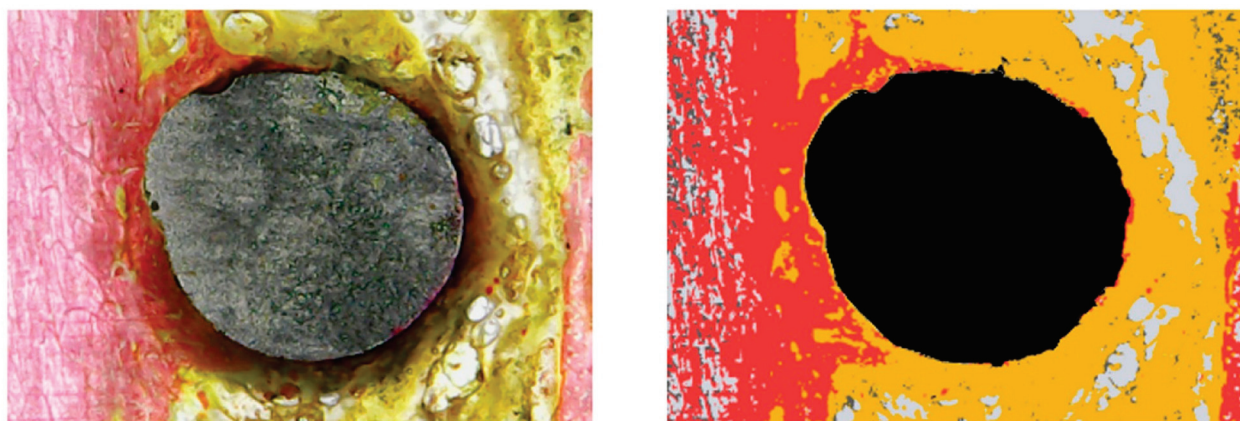


Figure 5. An illustration of the color-segmentation technique used in histomorphometric analysis. The segmented picture (**right**) and the original histology image (**left**). Bone tissue (BT), collagen tissue (CT), and marrow spaces (MS); MS: white/gray; CT: yellow/orange; BT: red/pink. The implant is represented by the black area, which was excluded from the histomorphometric analysis.

For each part, a descriptive histological study was also carried out.

2.8. Radiographic Analysis

In accordance with our research group's previously published methods [39], radiographs taken in both L-M and P-D directions were colorized using thermal pseudocoloring to depict differences in bone density in the RGB color space. Fundamentally, radiographic grayscale pixel intensity is directly proportional to the attenuation of X-ray photons by calcium and phosphate minerals; therefore, it serves as a tentative, non-invasive screening

tool for actual bone mineralization [39]. By converting these continuous grayscale values into a discrete thermal RGB color scale, subtle variations in early peri-implant mineralization become more visually and quantitatively distinguishable. The following color interpretations were assigned using the Look-Up Table (LUT):

- Red indicates a lower density (less soft tissue or mineralized bone).
- Green denotes an intermediate density.
- Blue represents a higher density (more mineralized bone).

Next, a standardized 2×3 mm region of interest (ROI) was established lateral to each implant in both imaging directions (Figure 6). This specific ROI dimension and its lateral placement were strategically selected to encompass the immediate peri-implant bone—the critical zone for evaluating early bone remodeling and the biological response adjacent to the healing chambers—while deliberately excluding the highly radiopaque titanium implant body to prevent image artifacts from skewing the density data.

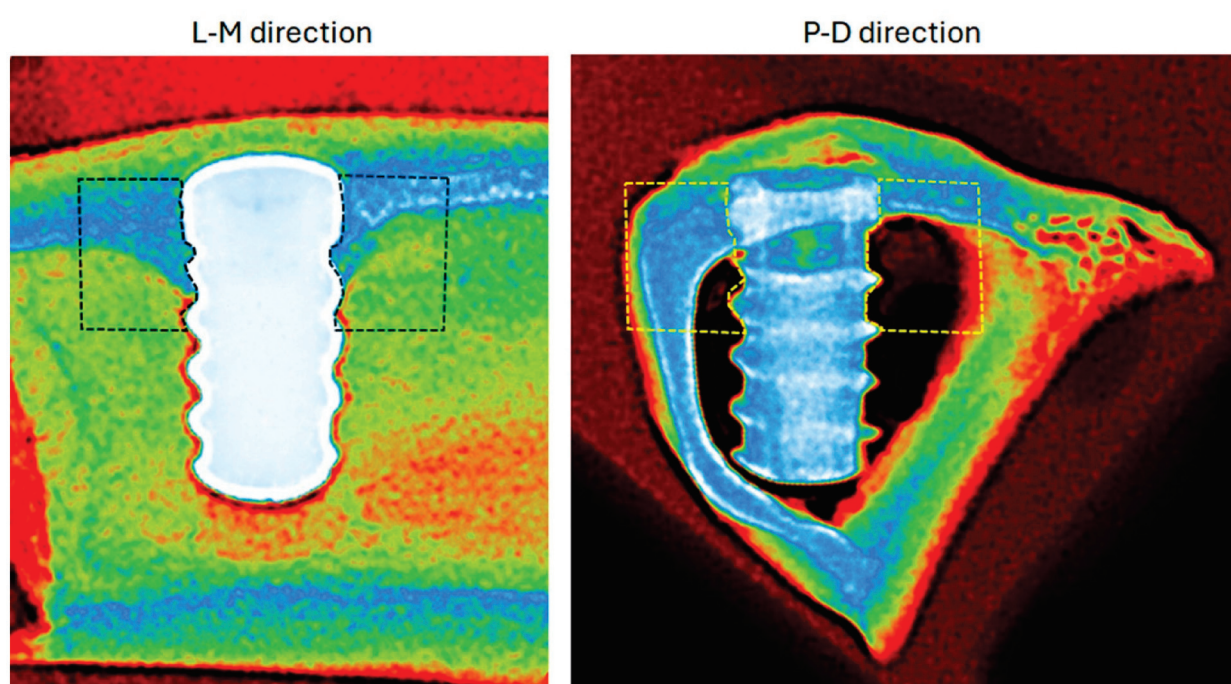


Figure 6. In order to depict changes in bone density in the RGB color space, radiographs taken in the lateromedial (L-M) and proximodistal (P-D) directions were colorized using thermal pseudocoloring. As shown by the dashed boxes, a region of interest (ROI) of 2×3 mm and situated lateral to each picture plant was defined in both image directions.

Fiji/ImageJ 2.9.0 (NIH and LOCI, University of Wisconsin, Madison, WI, USA) was used to quantitatively analyze the RGB channels. The Analyze > Color Histogram command was used to measure the presence (intensity) of each color (red, green, and blue), producing three distinct histogram graphs for each color component along with the mean value for each color. The relative color intensities inside the ROI are represented by the RGB intensity values derived from this study, which are expressed in arbitrary units. After the results were summed, the percentage contribution of each color within the ROI was computed (regarded as 100%). Evaluations and statistical analysis were conducted using these final percentage results (Figure 7).

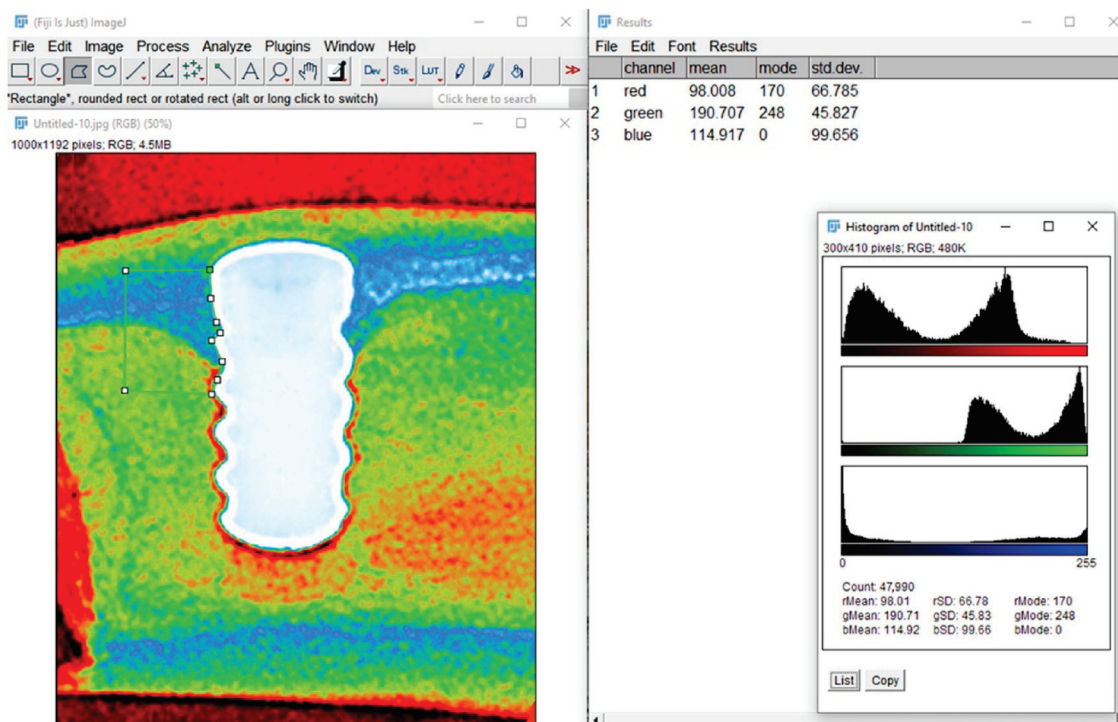


Figure 7. An illustration of RGB channel quantitative analysis using Fiji/ImageJ software (v. 2.9.0, NIH and LOCI, University of Wisconsin, Madison, WI, USA), inside a region of interest (ROI). The image with the chosen ROI is on the (left); the table with the mean intensity values for the red, green, and blue channels and the matching histograms produced by the Analyze > Color Histogram command is on the (right).

2.9. Statistical Analysis

The relevant statistical software (GraphPad Prism, v.8) was used to examine the data. The Shapiro–Wilk test was used to determine the normality of the data, and parametric tests were used because a normal distribution was found.

Because an intra-animal design was utilized, observations within the same animal are inherently correlated. To adequately account for this correlation and isolate the implant effect from inter-animal variability, paired Student’s *t*-tests were used for all comparisons between the Control and Test groups (insertion torque, BIC%, and radiographic RGB channels). This pairing ensures that the statistical model controls for subject-level clustering.

Using paired Student’s *t*-tests, the percentage values of each color channel were compared between the Control and Test groups for the radiography analysis based on RGB color decomposition, separately for the lateromedial and proximodistal directions.

When applicable, the results are displayed as mean $\pm$ standard deviation and standard error. 5% ($p < 0.05$) was chosen as the significance level.

Cohen’s *d* for paired comparisons was used to quantify the effect size, which showed significant differences between the Control and Test groups. A post hoc power analysis ($\alpha = 0.05$) showed statistical power greater than 99% for the key outcomes, and the sample size was determined based on the experimental design and viability of the animal model.

While the small sample size ($n = 5$ rabbits) limits the overall statistical power, the intra-animal paired design maximizes the detection of intra-subject differences. A post hoc power analysis ($\alpha = 0.05$) was conducted, and the sample size was determined based on the ethical principles of animal reduction (3Rs), experimental design, and viability of the animal model for exploratory studies.

3. Results

3.1. Torque Test Results

A statistically significant difference between the groups was shown by the in vitro test findings comparing insertion torque between the two implant devices. The mean insertion torque was 8.01 ± 0.82 Ncm for the Control group and 5.70 ± 0.85 Ncm for the Test group ($p < 0.0001$). Figure 8 shows the distribution of individual values for both groups.

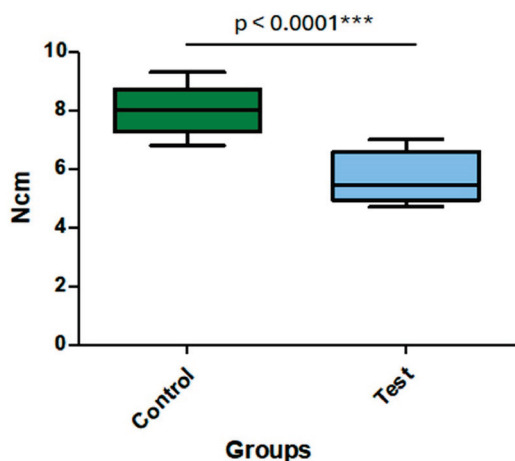


Figure 8. The distribution of insertion torque values (Ncm) obtained from the in vitro assay for implants in the Control and Test groups demonstrates the statistically significant difference between the groups.

3.2. Histological Analysis Results

3.2.1. Bone-Implant Contact

Table 1 and Figure 9 show the findings of the bone–implant contact percentage (BIC%) measurements at the three pre-selected locations (C1, C2, and C3). There were no statistically significant changes between the Control and Test groups at location C1. On the other hand, locations C2 and C3 showed statistically significant differences. At C2 (59.30% vs. 40.30%) and C3 (42.10% vs. 17.90%), the Test group’s mean BIC% values were much higher than those of the Control group; these differences were statistically significant ($p = 0.0002$).

Table 1. For the Control and Test groups, the bone-implant contact percentage (BIC%) was assessed at three predefined locations (C1, C2, and C3). Mean $\pm$ SD is used to express the values ($n = 10$ per group). Statistically significant differences between groups at the same place are indicated by different letters ($p < 0.05$).

	Control			Test		
	C1	C2	C3	C1	C2	C3
Mean	74.50	40.30 ^a	17.90 ^b	76.00	59.30 ^a	42.10 ^b
Std. Deviation	2.799	2.214	2.132	3.266	2.541	2.726
Std. Error	0.8851	0.7000	0.6741	1.033	0.8035	0.8622

Std.: Standard; a and b: different indicate statistically significant differences between the Control and Test groups at the same position ($p < 0.05$).

3.2.2. Bone Tissue, Collagen Tissue, and Medullary Spaces

Table 2 displays the results of the histomorphometric investigation. There were no discernible changes between the groups at the coronal area (C1). In comparison to the Control group, the Test group had less collagen and marrow space and more bone tissue in the more apical areas (C2 and C3). These results show that the test group’s apical regions had more bone growth.

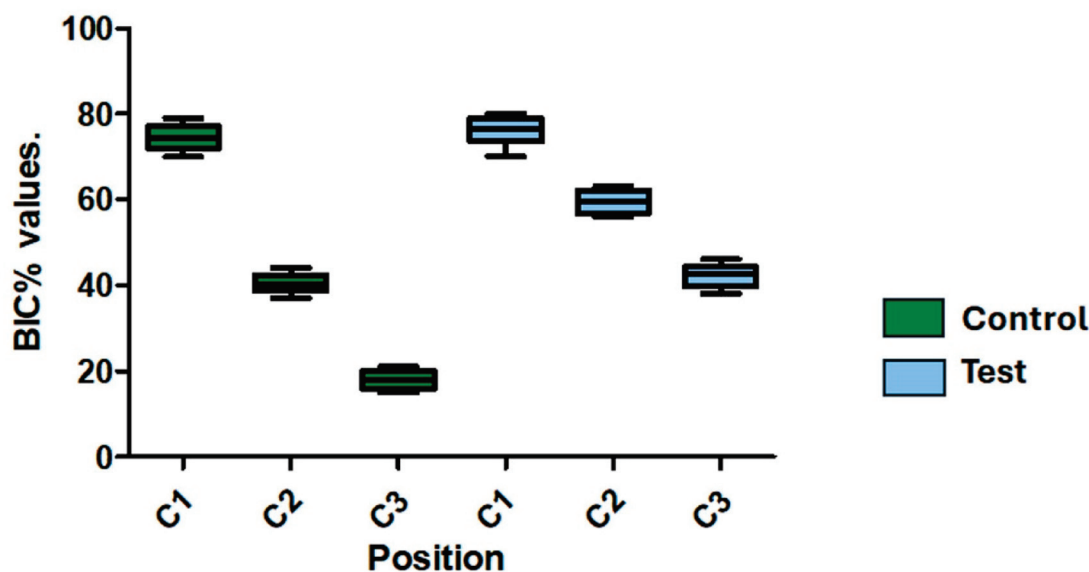


Figure 9. Bone-implant contact percentage (BIC%) values for the Control and Test groups at the three predetermined sites (C1, C2, and C3) are represented in a box plot. Whiskers show the lowest and maximum values, the middle line displays the median, and boxes show the inter-quartile range (Q1–Q3).

Table 2. Histomorphometric analysis.

Positions	C1		C2		C3	
	Control	Test	Control	Test	Control	Test
BT	94.73 ± 0.99	94.80 ± 1.09	36.48 ± 1.88 ^a	54.24 ± 1.67 ^{ba}	15.40 ± 0.99 ^a	35.48 ± 1.51 ^b
CT	2.77 ± 0.72	2.90 ± 0.77	43.97 ± 2.51 ^a	38.96 ± 2.13 ^b	45.03 ± 1.28 ^a	36.67 ± 4.46 ^b
MS	2.40 ± 0.46	2.40 ± 0.66	19.55 ± 4.15 ^a	6.80 ± 2.74 ^b	39.57 ± 3.16 ^a	27.85 ± 5.50 ^b

BT = bone tissue; CT = Collagen tissue; MS = Medullary space. Different letters (a, b) indicate statistically significant differences between the Control and Test groups at the same position ($p < 0.05$).

3.2.3. Descriptive Histological Analysis

Figure 10 displays representative histological sections from the Control and Test groups at the three predetermined locations (C1, C2, and C3). Both groups had a comparable histological appearance at position C1, which was defined by close contact between the implant surface and the surrounding tissue. There were no obvious gaps or discontinuities at the bone–implant contact in either group, and the peri-implant region was primarily populated by thick tissue that was closely fitted to the implant.

Qualitative differences between the groups became more apparent at point C2. There were interfacial voids surrounding the implant surface and areas with less tissue contact in the peri-implant region of the control group. The Test group, on the other hand, showed a greater degree of direct contact between the implant and the surrounding tissue, as well as a more continuous and uniform tissue contact along the implant perimeter with less obvious gaps.

The group differences were even more noticeable at location C3. The control specimens showed more interfacial gaps, a more uneven distribution of the surrounding material, and little contact between the implant and the surrounding tissue. On the other hand, the test group exhibited better adaptation, a more consistent distribution of tissue surrounding the implant, and a noticeably higher degree of contact along the implant surface.

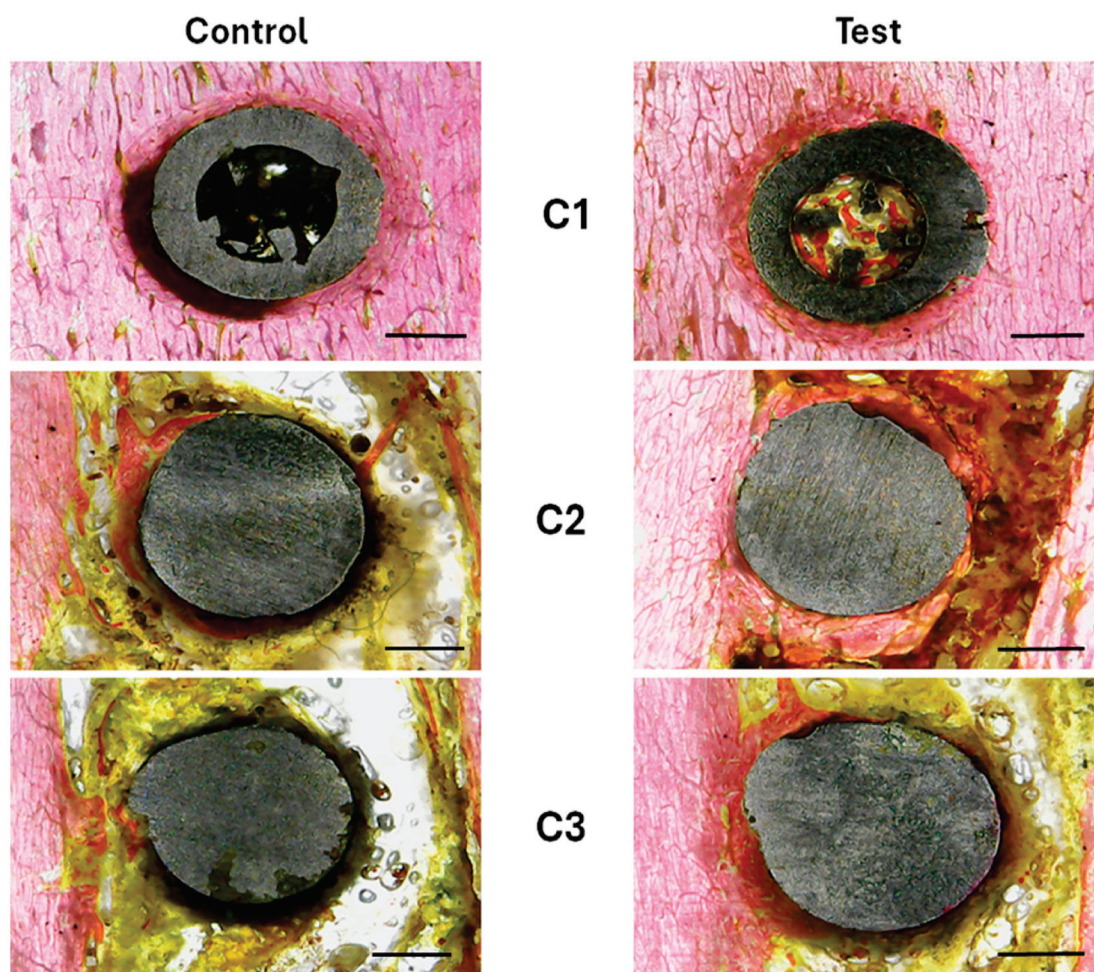


Figure 10. The Control and Test groups' representative histology pictures at the three designated locations (C1, C2, and C3). Both groups exhibit comparable tissue–implant interface characteristics at C1, with the implant surface and surrounding tissue coming into close contact. Qualitative differences between the groups can be seen at C2 and C3, where the Control group displays a less uniform interface with interfacial gaps and connective tissue interposition, while the Test group displays a more continuous and homogeneous bone contact along the implant surface. Connective tissue is yellow, whereas bone tissue is pink. 1 mm is the scale bar.

Overall, the histological observations show that the Control and Test groups exhibited similar tissue behavior at the most coronal position (C1), whereas the Test group showed a gradually improved tissue–implant interface at the middle (C2) and apical (C3) positions, which were distinguished by greater continuity and homogeneity of contact when compared to the Control group.

3.3. Radiographic Analysis Results

The RGB color decomposition-based radiography analysis results showed variations between the Control and Test groups in both assessed directions. In the lateromedial direction, the blue (B) channel had a lower mean (9.73%) while the red (R) and green (G) channels had larger means (41.96% and 48.32%, respectively) in the Control group. While the green (45.86%) and blue (27.36%) channels displayed greater values, especially the blue channel, the Test group's mean red channel value (26.78%) was lower than that of the Control group. The information is shown in Figure 11 and Table 3. With comparatively small standard deviations and standard errors, measures of dispersion showed low variability in both groups, indicating measurement consistency.

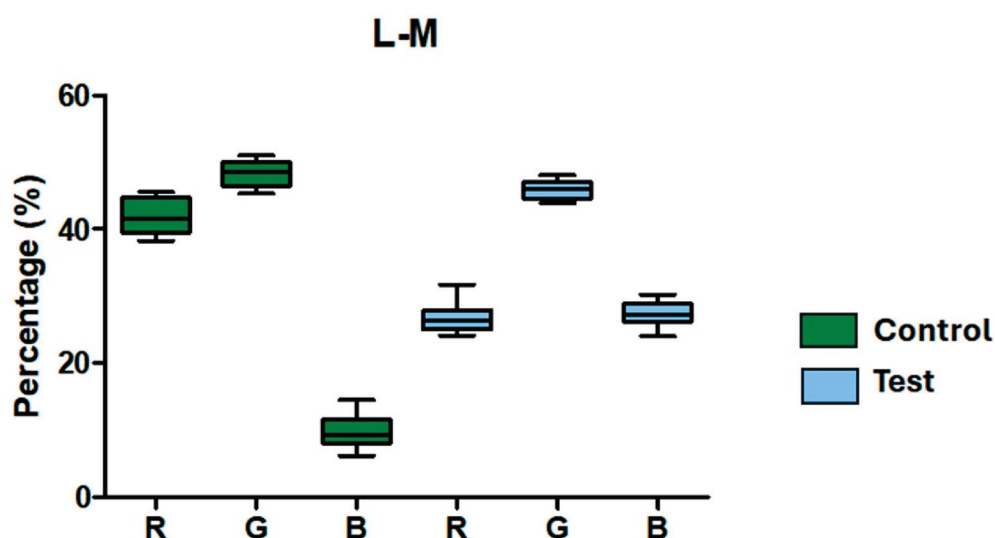


Figure 11. RGB color channel percentages for the Control (green) and Test (blue) groups obtained from radiography examinations in the lateromedial (L–M) direction. While the Test group exhibits lower R and higher G and B channel values, especially B, the Control group displays higher mean values in the R and G channels.

Table 3. Radiographic analysis based on RGB color decomposition in the lateromedial direction for Control and Test groups. Values are expressed in percentage as mean, standard deviation and standard error.

	Control			Test		
	R	G	B	R	G	B
Mean	41.96	48.32	9.730	26.78	45.86	27.36
Std. Deviation	2.811	1.836	2.363	2.226	1.327	1.901
Std. Error	0.8888	0.5806	0.7472	0.7038	0.4196	0.6011

The Control group displayed rather uniform mean values for the red (30.10) and blue (29.39) channels in the proximodistal direction, with the green channel (40.51) exhibiting greater intensity. On the other hand, the Test group showed a significant increase in the blue channel (42.59) along with declines in the mean values of the red (24.32) and green (33.08) channels. The information is shown in Figure 12 and Table 4. Standard deviations and standard errors stayed low, suggesting a consistent data distribution, just like in the lateromedial analysis.

Table 4. Radiographic analysis based on RGB color decomposition in the proximal–distal direction for Control and Test groups. Values are expressed as mean $\pm$ SD.

	Control			Test		
	R	G	B	R	G	B
Mean	30.10	40.51	29.39	24.32	33.08	42.59
Std. Deviation	2.862	2.308	2.317	3.006	2.075	1.544
Std. Error	0.9050	0.7299	0.7328	0.9506	0.6560	0.4882

Overall, the RGB-based radiography analyses showed that the Control and Test groups' chromatic intensity patterns differed, with the Test group's blue channel contribution significantly increasing in both directions assessed.

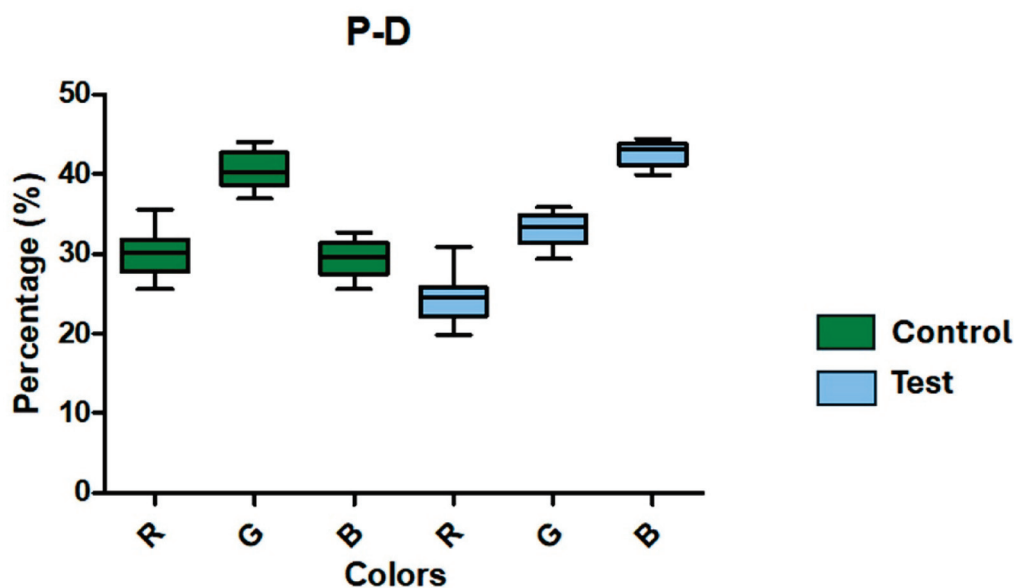


Figure 12. RGB color channel percentages for the Control (green) and Test (blue) groups derived from radiography analyses in the proximodistal (P–D) direction. While the Test group displays lower values on the red (R) and green (G) channels and a noticeable increase on the blue (B) channel, the Control group displays greater values on the green (G) channel and comparable values on the red (R) and blue (B) channels.

4. Discussion

4.1. Study Aim and Main Findings

This study compared two models—one with healing chambers (Test group) and one without (Control group)—in order to assess bone healing surrounding cylindrical titanium dental implants. In comparison to conventional implants, implants containing healing chambers improved bone–implant integration, increased bone density, and promoted superior bone healing in the cervical area [13,16,17,24,31,40–43]. RGB decomposition radiographic analyses revealed a superior bone density pattern, especially in the blue channel, which is indicative of superior bone mineralization [39,44–46], and the Test group demonstrated a significant increase in bone–implant contact percentage (BIC%) at the more apical positions (C2 and C3) [16,18,24].

4.2. Differences in Insertion Torque and Mechanical Trauma

The Control group had higher torque values than the Test group, according to the insertion torque data, which showed a significant difference between the groups. However, because insertion torque was evaluated in synthetic polyurethane blocks to ensure standardized mechanical precision, the link between these in vitro mechanical findings and the subsequent in vivo biological healing remains indirect. In line with other research demonstrating that severe cortical bone compression during conventional implant insertion can cause microfractures and hinder bone regeneration, this suggests that implants with healing chambers can lessen mechanical trauma during implantation [13,16,17,31,34]. Reduced cortical bone compression, which may have maintained bone integrity in the cervical region and encouraged improved peri-implant bone growth, is probably what caused the lower insertion torque seen in the Test group [13,16,24]. This result is consistent with cortical bone’s known function in primary implant stability and its susceptibility to mechanical damage [16–18,23–25].

4.3. Influence of Macrogeometry on Bone Healing

The regional evaluation of bone-implant contact (C1, C2, and C3) was designed to reflect the distinct clinical demands placed on different segments of the implant. The coronal region (C1), corresponding to the cervical region, is clinically paramount to the long-term success of the implant. In this experimental model, the C1 region featured the same surface treatment (titanium oxide microparticles and sandblasting) as the rest of the implant and was placed in direct contact with the crestal cortical bone. Bone stability in this crestal zone dictates the architecture of the overlying peri-implant soft tissues, provides a biological seal against the oral environment, and resists the highest marginal stress concentrations during functional loading. Consequently, the equivalent performance of both groups at C1 indicates that the Test implant safely maintains crestal bone health. Therefore, the equivalence at C1 between groups confirms that the Test group does not compromise cortical stability, while the significant gains at C2 and C3 demonstrated its superior performance in promoting osseointegration within the challenging environment of trabecular bone. The middle (C2) and apical (C3) regions are primarily responsible for deep mechanical anchorage. The statistically significant improvements in BIC% and bone tissue formation observed in the Test group at C2 and C3 suggest a strong clinical advantage for achieving primary stability. Enhanced apical osseointegration is particularly relevant for modern clinical protocols, such as immediate implant placement in extraction sockets or installations in low-density bone, where apical engagement is heavily relied upon.

Significant differences in BIC% at locations C2 and C3 were found by histological investigation, with the Test group exhibiting significantly greater values than the Control group. These variations suggest that healing chambers may encourage more bone growth surrounding the implant, particularly in the cervical region. According to the research [16–18,23,24,31], the Control group, on the other hand, may have had more severe insertion trauma, potentially impairing bone repair, as seen by lower BIC% values in the more apical direction (C2 and C3 positions). Insertion trauma, bone remodeling, and early osseointegration are directly impacted by implant macrogeometry, which includes design, diameter, and the existence of healing chambers [6,7,16,17,31]. Importantly, while reduced cortical bone compression plays a key role, the improved bone healing cannot be definitively attributed to compression alone. Outcomes are closely associated with the implant's surface topography and macrogeometry; the unique architecture of the healing chambers likely influences multiple biological factors, including clot stability, cell selection, haptotaxis, contact guidance, strain distribution, and the overall microenvironment that dictates bone ingrowth patterns.

These results were further corroborated by histomorphometric analysis. In comparison to the Control group, the Test group showed larger percentages of bone tissue (BT) and lower proportions of collagen tissue (CT) and marrow spaces (MS) in the more apical regions (C2 and C3), suggesting improved bone growth and more compact bone structure surrounding the implant. In contrast, the coronal region (C1), where bone tissue predominated in both groups, showed no discernible variations. These findings imply that the presence of healing chambers improves peri-implant tissue composition and promotes bone-implant contact, especially in areas more vulnerable to insertion-related damage.

4.4. RGB Radiographic Analysis and Implications for Healing

RGB-based radiography analysis revealed significant group differences. Used here as a tentative screening method rather than a confirmatory gold-standard analysis (such as micro-CT), the Test group displayed a significant increase in the blue channel in the lateromedial and proximodistal directions, suggesting increased bone density and miner-

alization surrounding the implant [17,24,31,39]. This probably results from better cortical bone preservation when implants with healing chambers are placed, which fosters the development of mineralized bone and effective osseointegration [13,16,17]. Particularly in cortical-rich areas, reduced cortical bone compression seems to positively regulate the bone healing pattern, supporting earlier research on the impact of implant design and insertion force on early bone response [14,15,23,24].

4.5. Concluding Remarks and Clinical Implications

Overall, the inclusion of healing chambers in the implant design promoted superior bone-implant integration and increased cervical bone density. By mitigating excessive cortical bone compression, a factor historically linked to poor regeneration and marginal bone resorption [13,16–18,31], this macrogeometric alteration yielded a more favorable biological response. Interestingly, although higher insertion torque values did not directly cause marginal bone loss in this study [13,47–49], the lower torque facilitated by the modified design still optimized the early bone-healing pattern. Despite the inherent anatomical limitations of the rabbit model, these findings suggest that minimizing cervical compression via implant macrodesign is a viable strategy to enhance early osseointegration. Further long-term clinical research is necessary to validate these benefits in human models.

5. Conclusions

Implants designed to lessen cortical bone compression showed reduced insertion torque and encouraged a more advantageous initial bone healing pattern in the cervical region. However, these findings represent strictly pre-clinical observations within the constraints of this in vitro and short-term (21 days) in vivo experimental study. In comparison to traditional implant designs, the existence of healing chambers was linked to greater bone mineralization and bone-implant contact, suggesting improved early osseointegration. While these results imply that implant macrogeometry plays a crucial role in regulating the early peri-implant bone response, immediate clinical applicability is limited. Further long-term studies are required to confirm whether these initial biological advantages translate into sustained clinical success.

Author Contributions: Conceptualization, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; methodology, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; software, S.A.G., J.A.J., T.L.E.T., A.S. and S.R.T.; validation, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; formal analysis, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; investigation, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; resources, S.A.G., J.A.J. and T.L.E.T.; data curation, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; writing—original draft preparation, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; writing—review and editing, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; visualization, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; supervision, S.A.G., J.A.J., T.L.E.T., A.S., B.F.M., M.d.C.F., S.R.T., G.C. and G.V.O.F.; project administration, S.A.G. and G.V.O.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the University of Rio Verde (Rio Verde, Brazil) (Approval Code 04/20), with the approval granted on 19 April 2021.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Biomimetic Deposition of Zn-Doped Calcium Phosphate Coatings on Surface-Activated Ti6Al4V for Multifunctional Implant Interfaces

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Abstract

The design of implant surfaces that support bone integration while limiting bacterial colonization remains a central challenge in biomaterials science and engineering. In this work, zinc-doped biomimetic calcium phosphate (CaP-Zn) coatings were fabricated on Ti6Al4V through surface activation followed by deposition in supersaturated simulated body fluid (SBF). Acid and alkali–calcium treatments produced a porous, calcium-rich interface that enabled the uniform formation of apatite-like CaP layers. Zinc incorporation was achieved without suppressing the formation of CaP phases and led to systematic changes in coating microstructure and surface chemistry. Spectroscopic and structural analyses indicated Zn incorporation within the CaP matrix, consistent with partial Ca^{2+} substitution and its association with poorly crystalline domains. These features promoted controlled ionic release and localized dissolution–reprecipitation behavior. Antibacterial testing against *Streptococcus mutans* revealed a clear Zn-dependent reduction in bacterial viability, while cytocompatibility remained within acceptable limits at moderate Zn levels. Finally, the coatings combine intrinsic bioactivity with ion-mediated antibacterial functionality, offering a multifunctional surface strategy for advanced titanium-based implants.

Keywords: biomimetic calcium phosphate coatings; Zinc-doped apatite; Ti6Al4V surface modification; antibacterial implant surfaces; controlled Zn^{2+} ion release; osteointegrative multifunctional coatings

1. Introduction

The demand for load-bearing biomedical implants has increased steadily over recent decades due to population aging and the growing prevalence of degenerative musculoskeletal diseases [1]. Metallic biomaterials, particularly titanium and its alloys, are widely

used in orthopedic and dental applications because of their favorable mechanical strength, fatigue resistance, corrosion stability in physiological environments, and excellent biocompatibility [2]. Among these, Ti6Al4V remains the most extensively employed alloy for load-bearing implants due to its balance between mechanical performance and biological acceptance [3].

Despite these advantages, titanium and its alloys are considered bioinert materials and do not form a direct chemical bond with bone tissue. As a result, their intrinsic osteoconductive capacity is limited, and bone–implant integration relies primarily on mechanical interlocking rather than true biological bonding [4]. Insufficient or delayed osseointegration may compromise primary stability and long-term implant performance, particularly in patients with low bone quality or in applications requiring rapid functional loading [5].

To overcome these limitations, extensive research efforts have focused on modifying implant surfaces to enhance their bioactivity and promote faster and stronger bone–implant bonding [6]. Surface modification strategies include physical, chemical, and biochemical approaches, among which the application of bioactive ceramic coatings has proven particularly effective [7]. Calcium phosphate (CaP) ceramics, especially hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), are widely used due to their close chemical and crystallographic similarity to the mineral phase of natural bone [8]. HA coatings have been shown to promote osteoconduction, accelerate bone apposition, and improve the interfacial bonding strength between bone and metallic substrates [9].

Several coating techniques have been developed for depositing CaP and HA layers on titanium substrates, including plasma spraying, sol–gel processing, electrophoretic deposition, electrochemical methods, and physical vapor deposition [10]. However, many of these approaches involve high processing temperatures, resulting in non-uniform microstructures, poor interfacial adhesion, or limited control over coating composition and crystallinity [11,12]. These drawbacks may negatively affect coating stability, long-term performance, and biological response.

In this context, the biomimetic coating method, originally proposed by Kokubo and co-workers, has emerged as a versatile and low-temperature alternative for producing bioactive CaP coatings under physiologically relevant conditions [13]. This method relies on the immersion of substrates in simulated body fluid (SBF), which mimics the ionic composition of human blood plasma, enabling the nucleation and growth of bone-like apatite layers at 37 °C and near-neutral pH [13]. Systematic studies have demonstrated that the formation of apatite in SBF correlates with *in vivo* bone-bonding ability, making this approach a valuable tool for evaluating and inducing bioactivity [13,14].

Subsequent refinements of the biomimetic process, including the use of concentrated SBF solutions (e.g., 5× and 10× SBF), have significantly reduced coating times and enabled better control over coating thickness, phase composition, and crystallinity [15]. It has been demonstrated in the literature that ionic strength, carbonate concentration, and solution supersaturation strongly influence CaP nucleation kinetics, coating morphology, and phase evolution [16]. These parameters are critical for tailoring coating properties relevant to biological performance. However, while bioactive CaP coatings significantly enhance osseointegration, they may also unintentionally promote bacterial adhesion and biofilm formation on implant surfaces [17]. Postoperative infections associated with implantable medical devices remain one of the most serious complications in orthopedic and dental surgery and are a leading cause of implant failure [18]. Bacterial colonization can occur within minutes after implantation, initiating a competitive process between host cell attachment and microbial adhesion, often described as the “race for the surface” [19].

Biofilms are structured microbial communities embedded in a self-produced extracellular polymeric matrix that confers strong resistance to antibiotics and host immune responses [20]. Once established, biofilms are extremely difficult to eradicate and frequently require surgical implant removal, leading to increased patient morbidity and healthcare costs [21]. Conventional systemic antibiotic therapies often fail to achieve sufficient local concentrations at the bone–implant interface and may contribute to the emergence of antibiotic-resistant strains [22]. To address this challenge, recent strategies have focused on developing multifunctional implant surfaces that simultaneously promote osseointegration while inhibiting bacterial colonization [23]. One promising approach involves the incorporation of antibacterial inorganic ions into bioactive CaP and HA coatings [24]. Among these, zinc (Zn^{2+}) has attracted considerable attention due to its dual role in bone metabolism and antimicrobial activity [25].

Zinc is an essential trace element in bone tissue and plays a crucial role in osteoblast proliferation, osteogenic differentiation, and suppression of osteoclastic bone resorption [26]. In addition, Zn^{2+} exhibits broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria and has been shown to reduce inflammatory responses [27]. Several studies have extensively reviewed the structural and biological effects of ionic substitutions in calcium phosphates, highlighting the influence of Zn^{2+} on crystallinity, solubility, and biological behavior [28]. Other studies have emphasized the potential of ion-doped calcium phosphates for imparting multifunctional properties, including antimicrobial activity and controlled ion release [29].

Despite these advantages, the biological and structural effects of zinc incorporation into CaP and HA systems remain highly dependent on concentration, substitution mechanism, and processing route [30]. Several studies have reported dose-dependent antibacterial efficacy of Zn-substituted HA powders [31]; however, reduced antibacterial performance has often been observed when Zn-HA is applied in coating form, likely due to altered ion release kinetics and coating crystallinity [32]. Some studies have further emphasized that excessive Zn incorporation may negatively affect coating crystallinity and potentially induce cytotoxic responses, highlighting the need for careful optimization [33].

Moreover, the majority of Zn-containing CaP coatings reported in the literature have been produced using high-temperature or electrochemical methods, which may suffer from adhesion issues and compositional inhomogeneity [34]. Recent advances in surface engineering have increasingly focused on the development of multifunctional coatings capable of simultaneously addressing mechanical stability, corrosion behavior, and biological performance. In this context, studies on plasma electrolytic oxidation (PEO) coatings, particularly in magnesium-based systems, have demonstrated the potential of designing gradient and composite architectures that couple controlled degradation with the release of therapeutic species. These approaches highlight the importance of tailoring coating microstructure and composition to achieve a synergistic relationship between degradation kinetics and biological functionality, including ion-mediated antibacterial effects and tissue regeneration. Although such strategies have been predominantly explored in biodegradable metals, the underlying principles (namely, the integration of controlled ion release, hierarchical coating structures, and functional responsiveness) are directly applicable to titanium-based implant systems. Therefore, these developments provide a valuable conceptual framework for the present study, where biomimetic Zn–CaP coatings are designed to achieve a similar balance between bioactivity and antibacterial performance through controlled Zn^{2+} incorporation and release under physiologically relevant conditions [35,36]. In contrast, the biomimetic method offers a unique opportunity to incorporate Zn^{2+} directly during apatite nucleation and growth, enabling homogeneous ion distribution and controlled release under mild processing conditions [37].

Despite the extensive research on Zn-doped CaP and HA coatings, several limitations remain in current approaches, particularly those based on high-temperature or electrochemical techniques. These methods often result in heterogeneous Zn distribution, limited control over the physicochemical state of the dopant, residual stresses, and restricted tunability of ion release under physiologically relevant conditions. In this context, the present work addresses a critical gap by employing a biomimetic deposition strategy that enables the *in situ* incorporation of Zn^{2+} during apatite nucleation and growth, rather than post-synthesis doping. This approach provides enhanced control over the distribution and chemical state of Zn, promoting the coexistence of structurally incorporated and surface-accessible species, which in turn leads to a dual-stage release behavior characterized by an initial antibacterial effect followed by sustained ion delivery. Furthermore, the low-temperature processing conditions ($\sim 37^\circ\text{C}$) favor the formation of carbonate-containing, poorly crystalline apatite phases that closely resemble biological mineral, thereby improving reactivity and bioactivity. Unlike previous studies that often evaluate either antibacterial or biological performance independently, this work establishes a direct correlation between coating structure, Zn incorporation, ion release kinetics, antibacterial activity, and cytocompatibility. Importantly, the results demonstrate that Zn release is governed not only by its concentration but by its physicochemical state and spatial distribution within the coating, providing new insight into the design of multifunctional implant surfaces. Thus, the biomimetic method is not only a processing alternative but a key enabler for achieving controlled ion delivery, improved interfacial integration, and biologically relevant coating functionality.

Based on these considerations, the development of biomimetic Zn-doped calcium phosphate (Zn–CaP) coatings on surface-activated Ti6Al4V substrates represents a promising strategy to simultaneously address the challenges of rapid osseointegration and infection prevention.

Accordingly, the aim of this study is to develop and systematically evaluate Zn–CaP coatings produced via biomimetic deposition, with particular emphasis on (i) the role of surface activation in promoting coating nucleation, (ii) the influence of Zn incorporation on coating morphology, phase composition, and crystallinity, and (iii) the relationship between Zn physicochemical state, ion release kinetics, antibacterial performance, and cytocompatibility. The central hypothesis is that controlled *in situ* incorporation of Zn^{2+} during biomimetic apatite formation enables the design of multifunctional coatings capable of delivering a balanced biological response, combining bioactivity, antibacterial efficacy, and cytocompatibility at the bone–implant interface.

2. Materials and Methods

2.1. Substrate Material and Sample Preparation

Commercial Ti6Al4V ELI alloy bars were used as the substrate material in this study. The material was supplied by United Performance Metals Inc., Hamilton, OH, USA. Cylindrical specimens were machined using a CNC micromachining lathe (Nanoform X, Moore Nanotechnology Systems, Keene, NH, USA) to obtain discs with a diameter of 6.35 mm and a thickness of 3.50 mm. To remove residual cutting fluids, oils, and surface contaminants introduced during machining, all specimens were subjected to a standardized cleaning protocol. Samples were first washed in a 2 wt.% Alconox detergent solution (Alconox Inc., White Plains, NY, USA) for 15 min using a rotary washing system. Subsequently, specimens were thoroughly rinsed with running distilled water to eliminate detergent residues. Final cleaning was performed by ultrasonic treatment in reagent-grade distilled water for 5 min. All experiments were conducted using reagent-grade distilled water (CTR Scientific, Monterrey, NL, Mexico). After cleaning, samples were dried using a stream of warm air and stored in a clean environment prior to surface treatments.

2.2. Acid Etching Treatment

Surface modification by acid etching was performed following a procedure adapted from Wen et al. [38]. The Ti6Al4V specimens were immersed in a mixed acid solution composed of hydrochloric acid (HCl) and sulfuric acid (H₂SO₄) at a volumetric ratio of 1:1, corresponding to an overall acid concentration of 67%. The etching treatment was carried out at 80 °C for 20 min under static conditions. The acids were supplied by CTR Scientific, Monterrey, NL, Mexico. This process was designed to remove the native oxide layer, generate microscale surface roughness, and introduce surface porosity. Upon completion of the etching period, specimens were removed from the acid solution using plastic tweezers and immediately rinsed with copious amounts of distilled water to stop the reaction and remove residual acids. Samples were subsequently dried at room temperature under ambient conditions.

Alternative surface modification methods were carefully considered, including single-acid treatments (e.g., HCl or H₂SO₄ alone), alkaline treatments (e.g., NaOH), and physical methods such as sandblasting or grit blasting. However, these approaches present certain limitations depending on the intended application. For instance, single-acid treatments often result in less homogeneous and less reactive surfaces, while alkaline treatments tend to form sodium titanate layers that require additional heat treatment to become bioactive and are typically used in bioactive glass-like surface formation, not immediate CaP nucleation. Mechanical methods (e.g., grit blasting), although effective in increasing roughness, may introduce surface contamination (e.g., alumina residues) or residual stresses and do not provide the same level of chemical activation, which can negatively affect coating uniformity and reproducibility.

In contrast, the combined HCl/H₂SO₄ etching at elevated temperature promotes the formation of micro- and submicron-scale roughness, while simultaneously increasing surface energy and hydroxyl group density. Crucially, in the present work, the acid etching step is not an isolated treatment, but part of a multi-step surface activation strategy (acid → alkali → Ca²⁺ exchange → thermal → water treatment). The initial HCl/H₂SO₄ etching plays a determinant role in enabling the subsequent formation of a reactive titanate layer, which ultimately governs the nucleation kinetics, adhesion, and uniformity of the biomimetically deposited CaP coatings. This sequential synergy is widely recognized as essential for achieving highly bioactive titanium surfaces [39–43].

The selection of the HCl/H₂SO₄ etching treatment at 80 °C for 20 min is firmly grounded in established surface engineering protocols for Ti6Al4V alloys, particularly in the context of biomimetic calcium phosphate (CaP) deposition and bioactive implant interfaces.

The use of mixed strong acids (HCl/H₂SO₄) has been consistently demonstrated to be significantly more effective than single-acid systems in generating a hierarchically roughened and chemically active titanium surface. This is attributed to the synergistic effect between chloride ions (promoting localized dissolution) and sulfate ions (favoring controlled oxidation–reprecipitation phenomena), which together produce a uniform micro/submicron topography and high density of Ti–OH functional groups (both critical for nucleation of CaP phases). It has been demonstrated that dual-acid treatments produce more homogeneous and bioactive surfaces compared to single-acid etching [44,45]. Similarly, in the literature, it was reported that mixed-acid treatments significantly enhance apatite-forming ability due to increased surface hydroxylation and surface energy [46–48].

The temperature of 80 °C is a crucial parameter, not merely a processing convenience. Elevated temperature accelerates the dissolution–reprecipitation kinetics, enabling the formation of well-defined microcavities and increased surface area within controlled timeframes, while avoiding excessive or uncontrolled corrosion. This has been validated in multiple studies where temperatures in the range of 60–90 °C were identified as optimal

for achieving reproducible surface activation without compromising the mechanical integrity of Ti6Al4V [49,50]. Lower temperatures typically result in insufficient activation, whereas excessively high temperatures or prolonged exposure can induce detrimental surface weakening.

Regarding etching duration (20 min), this parameter was carefully selected and experimentally validated to strike a balance between: (i) effective removal of the native TiO₂ passive layer, (ii) generation of controlled micro-roughness, and (iii) preservation of substrate integrity. Over-etching is well known to produce irregular morphologies, excessive material loss, and even microcrack initiation, which can adversely affect coating adhesion and long-term implant performance [51,52]. Therefore, the selected duration aligns with optimized conditions reported in the literature and confirmed through our preliminary trials.

2.3. Surface Activation by Alkali and Calcium Treatments

Following acid etching, the specimens were subjected to a multi-step surface activation procedure involving alkaline treatment, calcium ion exchange, thermal treatment, and water immersion. All reagents in this stage were supplied by Sigma-Aldrich (now Merck), St. Louis, MO, USA.

2.3.1. Alkali Treatment

A 10 M NaOH solution was prepared by dissolving 40 g of NaOH pellets in 100 mL of distilled water. The solution was heated to 60 °C using a hot plate equipped with temperature control. Each specimen was immersed individually in glass tubes containing 5 mL of the NaOH solution and maintained under these conditions for 24 h. After completion of the alkali treatment, the samples were removed and thoroughly rinsed with distilled water to eliminate residual NaOH and reaction by-products.

2.3.2. Calcium Treatment

A 1 M CaCl₂ solution was prepared by dissolving 11.1 g of CaCl₂ in 100 mL of distilled water. The alkali-treated specimens were immersed individually in the CaCl₂ solution at 40 °C for 24 h to promote Na⁺/Ca²⁺ ion exchange and formation of calcium-containing titanate species. After treatment, specimens were rinsed with distilled water and dried at room temperature.

2.3.3. Thermal Treatment and Water Immersion

The samples were subsequently subjected to thermal treatment in a muffle furnace (Thermo Fisher Scientific, Waltham, MA, USA) at 600 °C for 1 h to stabilize the surface layer and promote partial crystallization of titanate phases. After thermal treatment, specimens were allowed to cool slowly inside the furnace to room temperature. Finally, the samples were immersed in deionized water at 80 °C for 24 h (denoted as H₂O treatment). This step was applied to further hydroxylate the surface and enhance the formation of Ti–OH functional groups. After water treatment, the samples were rinsed with distilled water and dried at room temperature.

2.4. Biomimetic Deposition of Calcium Phosphate (CaP) Coatings

2.4.1. Preparation of Supersaturated CaP Solutions

Biomimetic CaP coatings were deposited using modified supersaturated solutions [53]. Two solution variants were prepared by increasing the calcium ion concentration to 7 and 10 times that of conventional simulated body fluid (SBF), referred to as SBFX7 and SBFX10, respectively. Calcium chloride (CaCl₂), monosodium phosphate (NaH₂PO₄), and sodium bicarbonate (NaHCO₃) were used as sources of calcium, phosphate, and carbonate ions,

respectively [54]. All reagents used in this stage were supplied by Sigma-Aldrich (Merck, St. Louis, MO, USA). The mass and molar concentrations of the salts used to prepare 500 mL of each solution are summarized in Table 1.

Table 1. Mass of salts used to prepare 500 mL of CaP solutions and corresponding molar concentrations.

Solution	Weight of salts (g)		
	CaCl ₂	NaH ₂ PO ₄	NaHCO ₃
SBFX7	0.9711	0.2625	0.1278
SBFX10	1.3873	0.3749	0.1890
	Molar concentration of salts (mmol/L)		
	CaCl ₂	NaH ₂ PO ₄	NaHCO ₃
SBFX7	17.50	4.4	3.04
SBFX10	25	6.25	4.50

2.4.2. Coating Deposition Procedure

For coating deposition, 500 mL of distilled water (CTR Scientific, Monterrey, NL, Mexico) was placed in a glass beaker and heated to 36.5 °C on a hot plate with continuous magnetic stirring at 100 rpm. CaCl₂ and NaH₂PO₄ were added sequentially, with a 5 min interval between additions to allow partial equilibration. Five minutes after the addition of the last salt, the activated Ti6Al4V specimens were immersed in the solution. After 30 min from the final salt addition, NaHCO₃ was added to introduce carbonate species and adjust the solution chemistry. Temperature and stirring conditions were maintained constant throughout the deposition period. At the end of the deposition time, specimens were removed from the solution, rinsed with deionized water (CTR Scientific, Monterrey, NL, Mexico), and dried at room temperature.

2.4.3. Deposition Variants

To evaluate the influence of solution concentration and immersion time, four deposition conditions were investigated, as summarized in Table 2.

Table 2. Deposition variants evaluated in this study.

Deposition Variants	Solution	Deposition Time (h)
VD1	SBFX7	4
VD2	SBFX7	6
VD3	SBFX10	4
VD4	SBFX10	6

Based on coating morphology, coverage, and integrity, the optimal deposition variant was selected for subsequent Zn incorporation.

2.5. Preparation of Zn-Containing CaP Coatings

Zn-containing CaP coatings were prepared following the same deposition procedure described above. Zinc chloride (ZnCl₂, supplied by Sigma-Aldrich, St. Louis, MO, USA) was used as the Zn²⁺ source and was added 3 min after the addition of the last salt. Three Zn²⁺ concentrations were investigated to evaluate the effect of Zn content on coating chemistry, cell viability, antibacterial activity, and Zn²⁺ release behavior. The ZnCl₂ masses and corresponding Zn²⁺ concentrations are summarized in Table 3.

Table 3. Deposition variants with Zn²⁺ incorporation.

Deposition Variants	ZnCl ₂ (mg)	Zn ²⁺ Concentration (mmol/L)
VDZn1	22	0.3
VDZn2	82	1.2
VDZn3	136	2.0

The Zn²⁺ concentrations employed in this study were selected based on a combination of literature evidence and preliminary screening experiments. Specifically, the chosen range was designed to: (i) remain within biologically relevant limits, where Zn²⁺ is known to promote osteogenic activity without inducing significant cytotoxic effects. Previous studies indicate that Zn concentrations in the low ppm range can enhance osteoblast function while maintaining acceptable cell viability, (ii) enable differentiation between incorporation regimes, from low Zn content (favoring surface-adsorbed species) to higher concentrations (increasing the likelihood of structural incorporation into the CaP lattice). This was essential to evaluate how the physicochemical state of Zn influences release kinetics and biological response, (iii) avoid excessive supersaturation or uncontrolled precipitation in solution, which could lead to uncontrolled phase formation or compositional inhomogeneity during biomimetic deposition.

Additionally, preliminary trials were conducted to identify concentration thresholds that produce measurable antibacterial effects without compromising coating integrity or cytocompatibility. These preliminary tests conducted in this study indicated that Zn concentrations above 2.0 mmol/L led to unstable coatings with reduced structural integrity and non-uniform morphology. Therefore, 2.0 mmol/L was selected as a practical upper limit that ensures a balance between effective Zn incorporation, coating stability, and biological compatibility.

The selected conditions (VDZn1, VDZn2, VDZn3) therefore represent a controlled compositional gradient that directly supports the central hypothesis regarding the relationship between Zn incorporation, release behavior, and multifunctional performance.

2.6. Post-Deposition Thermal Treatment

After coating deposition, all samples were subjected to a post-deposition thermal treatment in a muffle furnace. The heating protocol consisted of the following steps: (i) heating to 500 °C and holding for 30 min, (ii) increasing temperature to 800 °C and holding for 1 h, and (iii) cooling inside the furnace to room temperature. This thermal treatment was applied to improve coating cohesion, interparticle bonding, and coating–substrate adhesion.

2.7. Surface and Chemical Characterization

2.7.1. Field Emission Scanning Electron Microscopy (FESEM)

Surface morphology and topography were examined using a field emission scanning electron microscope (FESEM, SU8000, Hitachi High-Technologies Corporation, Tokyo, Japan). Images were acquired using an accelerating voltage of 2 kV and an in-lens secondary electron detector.

The thickness of the coatings was determined through cross-sectional analysis using field emission scanning electron microscopy (FESEM). Representative cross-sectional micrographs were obtained by mechanically grinding the edge of the coated Ti6Al4V specimens to expose the coating–substrate interface, allowing its direct observation. Multiple measurements were taken at different locations along the cross-section to account for local variations and ensure statistical reliability.

2.7.2. Energy Dispersive X-Ray Spectroscopy (EDS)

Elemental surface composition was analyzed by energy dispersive X-ray spectroscopy (EDS) scanning electron microscope (JSM-6510LV, JEOL Ltd., Tokyo, Japan) equipped with an EDS detector. Analyses were performed at an accelerating voltage of 20 kV to obtain semi-quantitative elemental information.

2.7.3. X-Ray Photoelectron Spectroscopy (XPS)

Surface elemental composition and chemical states were analyzed by X-ray photoelectron spectroscopy (XPS, K-Alpha™, Thermo Fisher Scientific, East Grinstead, UK) using Al K α radiation ($h\nu = 1486.68$ eV). Binding energies were calibrated using the C1s peak at 284.8 eV to correct for possible charging effects, ensuring accurate binding energy assignment. Survey spectra and high-resolution spectra for P, Zn, Na, Ca, O, and C were collected for all samples. The XPS data were processed using appropriate background subtraction (Shirley-type) and peak deconvolution procedures with well-established fitting parameters, including consistent full width at half maximum (FWHM) and Gaussian–Lorentzian peak shapes. Depth profiling was performed by Ar⁺ ion sputtering at a rate of 1.19 nm/s for 30 s, followed by repeated spectral acquisition. Data processing was performed using Thermo Scientific™ Avantage™ software (version 5.9925, Thermo Fisher Scientific, East Grinstead, UK).

2.7.4. X-Ray Diffraction (XRD)

Crystalline phases were identified by X-ray diffraction (XRD, Empyrean, Malvern Panalytical Ltd., Malvern, UK). Patterns were recorded over a 2θ range of 10–90°, operating at 45 kV and 40 mA, with a scanning speed of 0.013° per 10 s. Cu K α radiation was used as the X-ray source.

2.7.5. Surface Roughness

Surface roughness was measured using a contact profilometer (SJ-210, model 178-563-01A, Mitutoyo Corporation, Kawasaki, Japan). Measurements were performed on both treated and coated surfaces to assess the effect of surface modification and coating deposition.

The surface roughness parameters Ra, Rz, and Rq were evaluated. Ra represents the average absolute deviation of the surface profile from the mean line and provides a general indication of overall surface texture. Rq is the square root of the mean of the squared deviations, giving greater weight to larger surface irregularities and thus being more sensitive to outliers than Ra. Rz (maximum height of the profile) describes the vertical distance between the highest peak and the deepest valley within the evaluation length, providing information about extreme surface features and peak-to-valley variations. Together, these parameters allow a more comprehensive characterization of surface topography changes induced by the treatments.

2.8. In Vitro Biological Characterization

2.8.1. Cytotoxicity Assay (MTT)

Cytotoxicity was evaluated using the MTT reduction assay according to ISO 10993-5:2009, Annex C (*Biological Evaluation of Medical Devices—Part 5: Tests for In Vitro Cytotoxicity*; International Organization for Standardization (ISO): Geneva, Switzerland, 2009). Primary human gingival fibroblasts (HGF, ATCC® PCS-201-108™) and human dermal fibroblasts (HDF, ATCC® PCS-2018-012) were used. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell suspensions were prepared at concentrations of approximately 4.0×10^4 cells/mL (HGF) and 5.0×10^4 cells/mL (HDF). One milliliter

of cell suspension was seeded per well and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO₂ to allow cell attachment. After incubation, the test materials were placed in the wells along with negative controls (culture medium) and positive controls (5% Triton X-100). After 24 h of exposure, samples and culture medium were removed. Fresh medium (200 µL) and MTT solution (60 µL) were added to each well, and plates were incubated for 4 h in the dark. The resulting formazan crystals were dissolved using 200 µL of dimethyl sulfoxide (DMSO) per well. Absorbance was measured at 570 nm using a microplate reader (Thermo Scientific MultiSkan GO, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell viability was calculated as:

$$\text{Cell viability (\%)} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100 \quad (1)$$

where OD = optical density.

2.8.2. Antibacterial Activity Assay

Antibacterial activity was evaluated against the Gram-positive bacterium *Streptococcus mutans*. Bacterial cultures were grown in Brain Heart Infusion (BHI) medium at 37 °C for 24 h. The bacterial concentration was determined using a Neubauer counting chamber and adjusted to 1×10^6 CFU/mL. Each coated specimen was incubated in tubes containing 200 µL of bacterial suspension for 24 h. After incubation, samples were removed and rinsed with phosphate-buffered saline (PBS) to remove loosely attached bacteria. Serial dilutions (1:10) were prepared, and 100 µL from the final dilution was plated onto nutrient agar plates. Plates were incubated for 24 h at 37 °C prior to colony counting.

Colonies were counted, and CFU/mL was calculated as:

$$\text{CFU/mL} = \frac{(\text{number of colonies} \times \text{dilution factor})}{\text{sample volume (mL)}} \quad (2)$$

The percentage reduction in bacterial colonies relative to the undoped CaP control was calculated.

$$\text{Percentage reduction (\%)} = 100 - \left[\frac{(\text{CFU of the control surface})}{(\text{CFU of the coated surface})} \times 100 \right] \quad (3)$$

2.9. Zn²⁺ Ion Release Test

Zn²⁺ release from Zn-containing coatings was evaluated in phosphate-buffered saline (PBS, 1×, pH 7.4). Samples were immersed in 50 mL of PBS at room temperature. The solution was replaced with fresh PBS, and Zn²⁺ concentrations were measured after 1, 3, and 7 days. Zn²⁺ concentrations were determined by atomic absorption spectroscopy (AAS, iCE™ 3000, Thermo Fisher Scientific, Cambridge, UK). Measurements were performed at an emission wavelength of 213.9 nm, with a detection limit of 0.002 µg/mL. Results were reported as µg/mL of Zn²⁺ released into the solution.

2.10. Numbers of Repetition, Statistical Analysis, and Experimental Reproducibility

All experiments were conducted in triplicate ($n = 3$) unless otherwise specified. This includes: surface characterization (SEM/EDS, XRD, XPS, roughness), ion release measurements (AAS), antibacterial assays (CFU counting), and cytotoxicity tests.

For each condition, independent samples were prepared and analyzed to account for sample-to-sample variability. The reproducibility of the biomimetic coatings was evaluated through consistent morphological features, phase composition, and Zn incorporation trends across replicates.

We observed low variability between samples, with standard deviations generally below 10% for quantitative measurements such as Zn release and biological assays, indicating good reproducibility of the deposition process.

The experimental data were analyzed using standard statistical methods to ensure reliability and significance: results are reported as means $\pm$ standard deviation (SD). Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA). When significant differences were detected, post hoc Tukey tests were applied. A significance level of $p < 0.05$ was considered statistically significant.

The reproducibility of the coatings was confirmed through: (i) consistent surface morphology observed by SEM across independent samples, (ii) stable phase composition verified by XRD patterns showing reproducible apatite-related peaks, (iii) comparable Zn incorporation trends confirmed by EDS and XPS analyses, and (iv) reproducible ion release profiles with similar kinetic behavior across replicates.

The biomimetic deposition method, combined with controlled surface activation and solution chemistry, contributed to a high level of process stability. Minor variations observed are attributed to the intrinsic heterogeneity of nucleation processes in biomimetic systems, which is a known characteristic of this technique.

3. Results

3.1. Baseline Characterization of the Ti6Al4V Substrate

Prior to surface modification, the Ti6Al4V ELI alloy was characterized to establish a reference microstructural, compositional, and crystallographic state for assessing the effects of subsequent chemical and thermal treatments (Figure 1A–C). FESEM images in Figure 1A revealed a typical machined surface morphology dominated by parallel grooves and tool marks, resulting in an anisotropic topography. Such as-machined surfaces are well known to exhibit low surface energy, a limited density of chemically active sites, and reduced wettability, which collectively limit heterogeneous nucleation of calcium phosphate phases [55]. From a biological and bioactivity perspective, this surface condition is considered essentially bioinert and exhibits a limited intrinsic ability to induce apatite formation in simulated body fluid (SBF). This behavior is consistent with extensive reports showing that untreated Ti6Al4V surfaces display poor apatite-forming ability compared to chemically activated, hydroxylated, or microtextured surfaces [56].

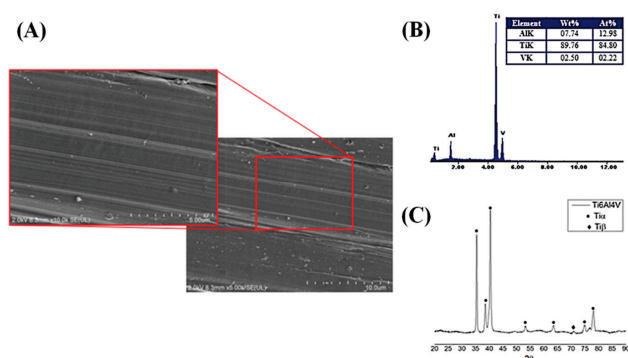


Figure 1. Ti6Al4V ELI alloy machined surface prior to surface modification. (A) FESEM images, 5000 $\times$ and 10,000 $\times$. (B) EDS spectrum. (C) XRD pattern.

The EDS analysis in Figure 1B confirmed the expected elemental composition of the alloy, with dominant Ti, Al, and V signals and no detectable contamination. Meanwhile, the XRD patterns in Figure 1C revealed the coexistence of α -Ti (hcp) and β -Ti (bcc) phases, confirming the typical biphasic microstructure of Ti6Al4V used in biomedical applications. The baseline characterization confirms that the as-machined substrate provides a chemically

passive and weakly bioactive surface, which requires surface activation to enable effective biomimetic coating nucleation and growth [57].

3.2. Effect of Surface Activation Treatments

3.2.1. Acid Etching

Figure 2A–C shows the microstructural, compositional, and crystallographic state of Ti6Al4V ELI alloy after acid etching. According to the FESEM images in Figure 2A, acid etching using an $\text{H}_2\text{SO}_4/\text{HCl}$ mixture produced a marked transformation of the surface topography. The machining marks were effectively removed and replaced by a homogeneous network of micropores. This microtextured morphology originates from preferential chemical dissolution at high-energy regions such as grain boundaries, phase interfaces, and dislocation-rich areas, leading to a substantial increase in surface roughness, specific surface area, and the density of chemically active sites [58].

Such microporosity is known to enhance both mechanical interlocking and heterogeneous nucleation of calcium phosphate phases by increasing the number of energetically favorable nucleation sites. Importantly, no significant microcracks were observed, indicating that the selected etching conditions achieved effective surface activation without inducing detrimental structural damage, in agreement with previous reports on acid-treated titanium alloys [59]. The EDS analysis in Figure 2B revealed an increase in oxygen content associated with the spontaneous formation of a thin titanium oxide layer upon exposure to air. Finally, the XRD analysis in Figure 2C detected the presence of titanium hydride (TiH_2), which can form during strong acid treatments due to hydrogen uptake. The formation of TiH_2 has been reported to modify surface chemistry and surface energy and can influence subsequent alkaline treatments by altering dissolution and reprecipitation kinetics [60].

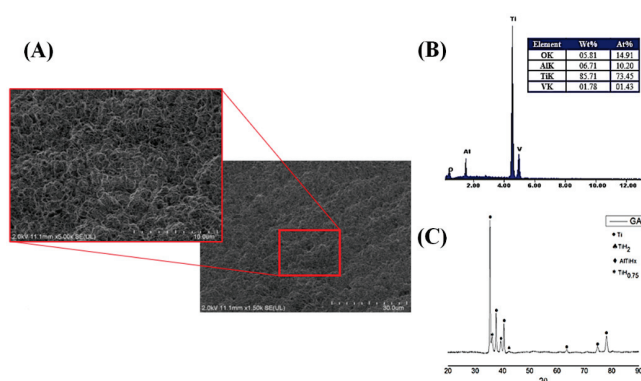


Figure 2. Ti6Al4V ELI alloy after acid etching. (A) FESEM images, 1500 $\times$ and 5000 $\times$. (B) EDS spectrum. (C) XRD pattern.

3.2.2. Alkaline and Calcium Activation (NaOH – CaCl_2 – TT – H_2O)

The morphological evolution of the Ti6Al4V ELI surface after the sequential application of surface activation treatments is shown in Figures 3–5. Following alkaline treatment in NaOH , FESEM images in Figure 3A–B revealed the formation of a highly porous, fibrillar, or feather-like network structure, characteristic of hydrated sodium titanate layers. This morphology results from partial dissolution of the native TiO_2 layer followed by reprecipitation of sodium titanate species, leading to the formation of a gel-like network enriched in Ti-OH groups. Such titanate gel layers are widely recognized as key intermediates for bioactivation, as the high density of surface hydroxyl groups provides negatively charged sites that strongly promote Ca^{2+} adsorption and subsequent apatite nucleation [61]. EDS analysis performed after NaOH treatment (Figure 3C) confirmed the substantial incorporation of sodium and oxygen, in addition to the substrate elements Ti, Al, and V. The

simultaneous detection of Na and O supports the formation of a sodium titanate layer (commonly described as Na_2TiO_3 or structurally related sodium titanate species), which has been consistently identified as an essential phase responsible for inducing bioactivity on titanium surfaces. Approximately 6 at.% Na was detected at the outer surface, indicating the effective development of a sodium-rich hydrated titanate layer. This compositional modification is consistent with the fibrillar gel-like morphology observed by FESEM and reflects the partial dissolution–reprecipitation mechanism induced under alkaline conditions. The resulting titanate network behaves as an ion-exchangeable reservoir, where loosely bound Na^+ ions can be replaced by H_3O^+ upon subsequent immersion in aqueous environments. This exchange process increases the density of surface Ti–OH groups and enhances the negative surface charge, thereby strengthening electrostatic interactions with Ca^{2+} ions from the surrounding medium. These physicochemical features are critical for initiating calcium accumulation and, ultimately, for driving heterogeneous apatite nucleation during subsequent biomimetic mineralization.

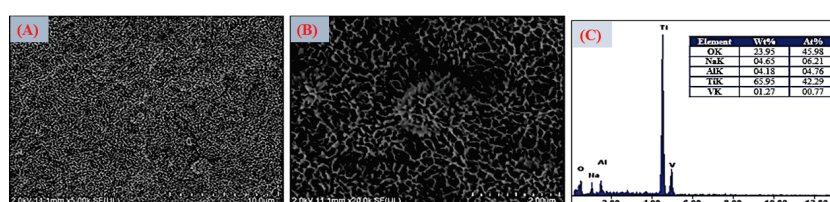


Figure 3. Ti6Al4V ELI alloy after alkaline treatment in NaOH. (A) FESEM image, 5000 $\times$. (B) FESEM image, 20,000 $\times$. (C) EDS spectrum.

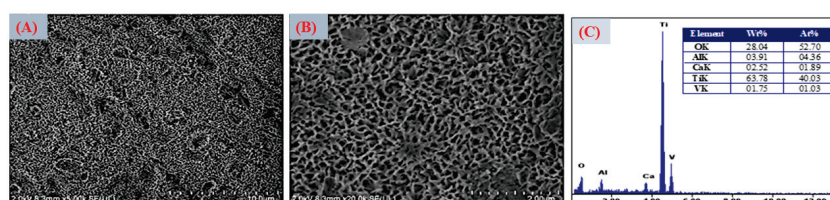


Figure 4. Ti6Al4V ELI alloy after CaCl_2 treatment. (A) FESEM image, 5000 $\times$. (B) FESEM image, 20,000 $\times$. (C) EDS spectrum.

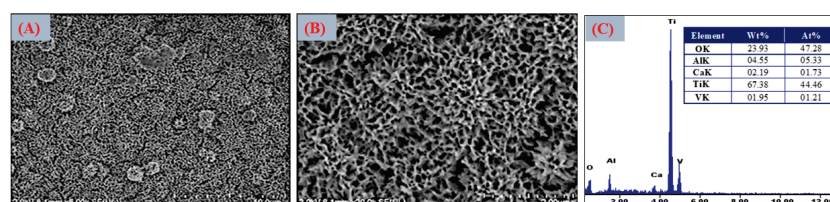


Figure 5. Ti6Al4V ELI alloy after thermal treatment, 600 $^{\circ}\text{C}$ for 1h. (A) FESEM image, 5000 $\times$. (B) FESEM image, 20,000 $\times$. (C) EDS spectrum.

After CaCl_2 treatment, the fibrillar morphology was preserved, as shown in the FESEM images in Figure 4A,B, while globular precipitates appeared, indicating the formation of calcium-rich titanate species. The EDS analysis in Figure 4C confirmed the disappearance of Na and the appearance of Ca, demonstrating an effective $\text{Na}^+/\text{Ca}^{2+}$ ion exchange. This ion-exchange process leads to the formation of calcium titanate or calcium hydrogen titanate layers, which are recognized as highly effective precursors for rapid apatite formation in SBF [62].

FESEM images in Figure 5A,B revealed that thermal treatment induced partial densification and the formation of microcracks, attributed to dehydration and shrinkage of the gel-derived layer. Although microcracking may appear undesirable, such features can enhance ionic permeability and facilitate ion exchange with the surrounding medium, thereby accelerating interfacial reactions and apatite nucleation kinetics [63].

Subsequent thermal treatment did not produce significant changes in the elemental composition detected by EDS, indicating that calcium remained stably incorporated within the surface layer. The persistence of Ca after heat treatment suggests that calcium species were not merely adsorbed but were structurally integrated into the titanate matrix, likely through the formation of calcium titanate-type phases or Ca-associated titanate domains [64]. This compositional stability is consistent with the partial densification observed in FESEM images and implies that thermal consolidation promoted structural rearrangement rather than elemental loss. During heating, dehydration of the gel-derived sodium/calcium titanate layer facilitates condensation reactions between Ti–OH groups, leading to a more interconnected oxide network while preserving incorporated Ca species. The retention of calcium is particularly relevant, as Ca-enriched titanate surfaces exhibit enhanced chemical affinity for phosphate ions in physiological environments [65]. In combination with the microcrack network generated during shrinkage, this stable Ca-containing layer can further promote ionic transport and accelerate the initial stages of calcium phosphate nucleation during subsequent biomimetic immersion.

Treatment in deionized water (TH₂O) did not lead to noticeable morphological changes, which is consistent with its primary role in hydrating and chemically stabilizing the surface layer rather than altering its topography. This step mainly promotes further ion exchange and the formation of additional surface hydroxyl groups, contributing to the chemical conditioning of the titanate layer without significantly affecting its microstructural features.

A slight reduction in Ca content after the deionized water treatment (Figure 6) was detected by EDS. This decrease can be attributed to the partial dissolution of weakly bound calcium species, likely those superficially adsorbed or associated with more labile domains of the hydrated titanate layer [66]. Importantly, this reduction did not compromise the overall presence of calcium at the surface, indicating that a significant fraction remained structurally integrated within the titanate network.

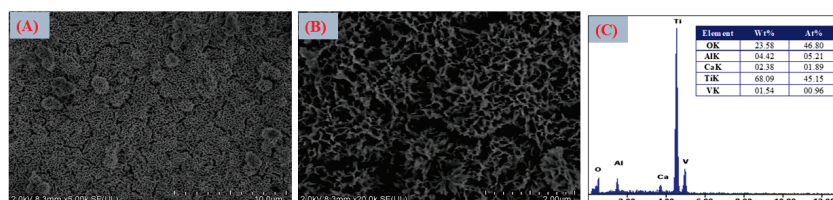


Figure 6. Ti6Al4V ELI alloy after deionized water treatment. (A) FESEM image, 5000 $\times$. (B) FESEM image, 20,000 $\times$. (C) EDS spectrum.

Such behavior is consistent with the role of the TH₂O step as a chemical conditioning stage rather than a structural modification process. Immersion in deionized water promotes further Na⁺/H₃O⁺ exchange and stabilization of Ti–OH groups, while simultaneously removing loosely attached ionic species. The retention of a Ca-enriched surface after this mild leaching step suggests that calcium is at least partially incorporated through stronger chemical interactions, which may enhance the surface's readiness for subsequent phosphate adsorption and calcium phosphate nucleation under biomimetic conditions [67].

Figure 7A,B presents the XPS characterization of the Ti6Al4V ELI surface after the full sequence of activation treatments (NaOH–CaCl₂–TT–TH₂O). The survey spectrum (Figure 7A) displays well-defined signals corresponding to Ti, O, and Ca. The absence of intense metallic Ti signals and the predominance of oxidized species indicate that the substrate is fully covered by a reaction layer formed during the alkaline and calcium treatments. High-resolution analysis of the Ca 2p region (Figure 7B) reveals the characteristic Ca 2p_{3/2} and Ca 2p_{1/2} doublet, consistent with Ca²⁺ chemical states.

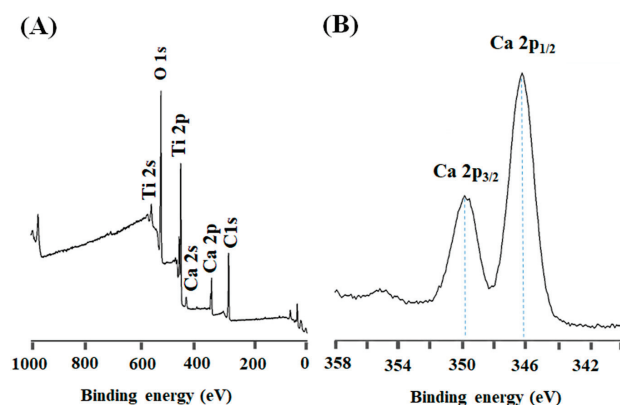


Figure 7. (A) Full XPS spectrum and (B) High-resolution Ca 2p spectrum of the Ti6Al4V surface subjected to NaOH-CaCl₂-TT-TH₂O activation treatments.

The two peaks in the Ca 2p region with binding energies of 346.8 eV and 350.3 eV are in excellent agreement with the characteristic positions of Ca 2p_{1/2} and Ca 2p_{3/2}, respectively. The binding energy positions and peak symmetry suggest that calcium is not present as a simple surface precipitate but is chemically associated with oxygen within a titanate-based matrix, likely through Ca–O–Ti linkages or calcium titanate-like environments. This interpretation supports the EDS findings and indicates that Ca is stably incorporated within the outer nanometric layer rather than weakly adsorbed. Importantly, XPS (being inherently surface-sensitive) confirms that calcium enrichment occurs precisely at the interface where biomimetic reactions are initiated [68].

XRD analysis (Figure 8) provides detailed insight into the phase evolution occurring at each stage of the activation sequence applied to Ti6Al4V. In the acid-etched condition, the diffraction pattern was dominated by α -Ti reflections from the substrate, together with peaks indexed to TiH_{0.75}, AlTiH_x, and calcium hydrogen titanate. The formation of titanium hydrides is commonly associated with hydrogen uptake during acid exposure, where cathodic reactions at the metal surface promote hydrogen absorption into the near-surface region. Although typically confined to shallow depths, these hydride phases can introduce lattice distortion and defect sites, potentially influencing subsequent ion exchange and chemical reactivity.

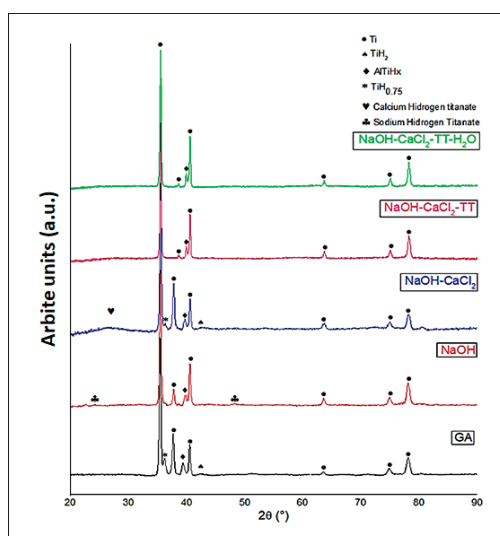


Figure 8. XRD patterns of the surfaces after the application of the activation treatments.

After NaOH treatment, reflections corresponding to Ti and AlTiH_x remained, while sodium hydrogen titanate phases became detectable. The emergence of sodium titanate

confirms the dissolution–reprecipitation mechanism previously inferred from FESEM, EDS, and XPS analyses. Under strongly alkaline conditions, partial dissolution of the native TiO_2 layer is followed by reprecipitation of a poorly crystalline sodium titanate hydrogel. The weak and broadened nature of these reflections indicates small crystallite size and significant structural disorder, consistent with a gel-derived surface layer. For the NaOH – CaCl_2 sequence, calcium hydrogen titanate reflections were identified along with $\text{TiH}_{0.75}$, Ti , AlTiH_x , and TiH_2 . This phase assemblage evidences partial $\text{Na}^+/\text{Ca}^{2+}$ exchange within the titanate network, resulting in the formation of Ca-containing titanate species. The persistence of hydrides suggests that calcium treatment primarily modifies the outer titanate structure without fully eliminating hydrogen-enriched domains. Following thermal treatment (NaOH – CaCl_2 –TT), only Ti and AlTiH_x peaks were clearly observed. According to the literature, heat treatment can transform hydrogen and calcium titanates into low-crystallinity calcium titanate phases and rutile. However, such phases were not distinctly identified in the present study. This apparent discrepancy can be rationalized by considering that (i) the activated layer is extremely thin, (ii) it exhibits low crystallinity or partial amorphous character, and (iii) its diffraction signal is masked by the intense contribution of the metallic substrate. Similar limitations of XRD for detecting nanometric calcium titanate layers have been widely reported, with surface-sensitive techniques such as XPS and EDS demonstrating greater sensitivity for these modified interfaces. The final NaOH – CaCl_2 –TT– TH_2O condition exhibited a comparable diffraction pattern, further supporting the conclusion that the outer layer is nanometric and structurally disordered. Collectively, the progressive attenuation of titanate-related peaks confirms the formation of a thin, calcium-enriched, highly hydroxylated, and chemically reactive surface. This activated interface provides an optimal physicochemical platform for subsequent biomimetic calcium phosphate deposition and controlled Zn^{2+} incorporation, enhancing nucleation kinetics, coating adhesion, and interfacial stability. These characteristics are central to achieving both bioactivity and antibacterial functionality, in line with the core hypothesis of the study [69].

3.3. Biomimetic Deposition of CaP Coatings

The influence of ionic concentration (SBFX7 vs. SBFX10) and immersion time (4 h vs. 6 h) on CaP deposition was systematically evaluated. The results clearly demonstrate that both parameters strongly control coating morphology, surface coverage, thickness, and mechanical integrity, in agreement with classical biomimetic deposition models [70].

FESEM micrographs in Figure 9A,B corresponding to the VD1 condition (SBFX7) reveal the formation of spherical particles arranged in bouquet-like clusters, heterogeneously distributed across the substrate surface.

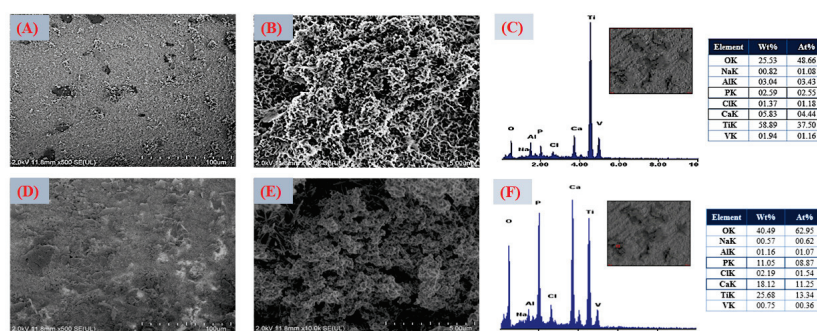


Figure 9. (A,B) FESEM images of the coated surface with VD1 (SBFX7-4 h), 500 $\times$ and 10,000 $\times$. (C) EDS spectra of the surfaces coated with VD1. (D,E) FESEM images of the thermally treated coated surface with VD1, 500 $\times$ and 10,000 $\times$. (F) EDS spectra of the surfaces coated with thermally treated VD1.

This morphology is typical of the early stages of biomimetic apatite nucleation, where nanoscale crystallites initially aggregate into globular assemblies that progressively coalesce into more continuous layers. Such features indicate that nucleation is occurring at discrete active sites rather than uniformly over the entire surface, reflecting local variations in interfacial chemistry [71].

The presence of larger lamellar structures underlying or supporting the bouquet-like aggregates suggests the coexistence of distinct growth mechanisms. These lamellae may originate from preferential nucleation in regions with higher surface hydroxyl density or locally increased calcium concentration within the activated titanate layer. Surfaces enriched in calcium titanate are known to promote heterogeneous nucleation by facilitating electrostatic interactions with phosphate species, which can lead to localized crystal growth and morphological differentiation. Elemental composition was evaluated by EDS. The average atomic percentages (Figure 9C) confirmed the presence of Ca (4.44 at.%), P (2.55 at.%), and O (48.66 at.%), consistent with the formation of a thin calcium phosphate layer.

After thermal treatment (Figure 9D,E), the coating appears more compact, with no evident cracking. This behavior indicates that the relatively low thickness of the deposited layer allows accommodation of thermal stresses generated during heating [72].

Nevertheless, the coating remains partially discontinuous, suggesting that the immersion time (4 h) and the ionic concentration of the supersaturated solution (SBFX7) were insufficient to achieve full surface coverage. From a functional perspective, incomplete homogeneity may affect coating adhesion, bioactivity reproducibility, and the subsequent uniform incorporation of Zn^{2+} . Localized analyses performed on the lamellar regions (Figure 9F) showed significantly higher Ca and P contents (11.25 and 8.87 at.%, respectively; O: 62.95 at.%), confirming that these structures correspond to CaP-rich domains. The increase in Ca and P after heat treatment suggests slight densification and partial structural rearrangement, without fundamentally altering the coating chemistry. The compositional and morphological evidence supports the formation of a thin, apatite-like calcium phosphate layer on the Ti6Al4V substrate.

For the VD2 condition (Figure 10A,B), increasing the immersion time to 6 h while maintaining the same ionic concentration (SBFX7) resulted in a higher density of bouquet-like aggregates and the formation of more compact clusters distributed over a larger fraction of the surface. This morphology reflects further progression of secondary nucleation and crystal growth, leading to increased surface coverage compared with VD1. As immersion proceeds, previously formed nuclei act as templates for additional ion accumulation, promoting radial growth and coalescence of adjacent globular structures. Despite this improvement, lamellar features and partially uncovered regions are still evident, indicating that although extended time enhances growth kinetics, the ionic supersaturation remains a limiting factor for achieving a fully continuous layer. In other words, time alone promotes thickening and local densification but does not completely overcome constraints imposed by ion availability and diffusion [73]. EDS analysis (Figure 10C) showed increased atomic concentrations of Ca (6.26 at.%) and P (3.30 at.%) relative to VD1, confirming that longer immersion favors greater CaP accumulation. After thermal treatment (Figure 10D,E), the coating exhibits further densification without visible cracking, suggesting that the layer thickness remains within a range capable of accommodating thermally induced stresses. Heat treatment likely promotes partial dehydration and structural rearrangement of the initially precipitated calcium phosphate, improving interparticle cohesion without causing delamination.

EDS analysis (Figure 10F) showed increased atomic concentrations of Ca and P relative to VD1, confirming that longer immersion favors greater CaP accumulation. Additionally,

EDS analysis (Figure 10F) indicates that the increase in Ca and P content after heat treatment is consistent with slight densification and partial structural rearrangement of the coating. Significantly higher Ca and P atomic concentrations (12.58 and 8.18 at.%, respectively; O: 50.4 at.%) were measured in these regions, confirming that they correspond to CaP-rich domains. These results further support that longer immersion times promote greater CaP accumulation and local thickening of the deposited layer.

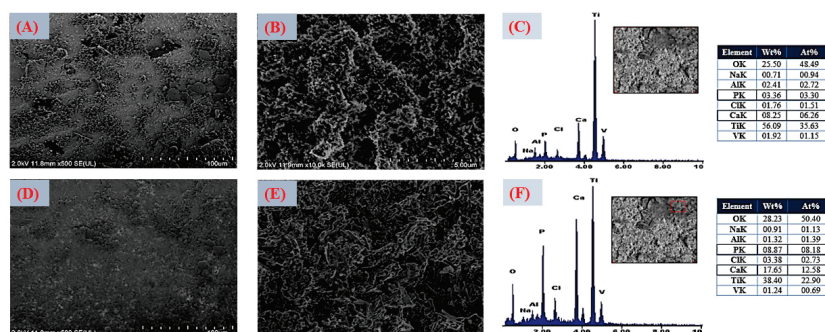


Figure 10. (A,B) FESEM images of the coated surface with VD2 (SBFX7- 6 h), 500 $\times$ and 10,000 $\times$. (C) EDS spectra of the surfaces coated with VD2. (D,E) FESEM images of the thermally treated coated surface with VD2, 500 $\times$ and 10,000 $\times$. (F) EDS spectra of the surfaces coated with thermally treated VD2.

From a process optimization standpoint, these findings indicate that extending deposition time without increasing ionic supersaturation only partially improves surface coverage. Achieving a continuous and homogeneous layer (critical for reliable bioactivity and subsequent Zn functionalization) likely requires adjustments to both immersion time and solution chemistry.

FESEM micrographs corresponding to the VD3 condition (Figure 11A,B) show a clear improvement in coating uniformity and continuity compared with VD1 and VD2. Increasing the Ca^{2+} and PO_4^{3-} concentrations in the solution (SBFX10) enhances the degree of supersaturation, which in turn raises the heterogeneous nucleation rate and promotes the formation of a thicker and more homogeneous layer, even at a relatively short immersion time (4 h). Under these conditions, the higher ionic activity reduces the energy barrier for nucleation and increases the density of stable nuclei formed on the activated titanate surface. This behavior is consistent with classical biomimetic deposition models, where supersaturation governs both nucleus density and crystal growth kinetics. A higher supersaturation level accelerates ion clustering at reactive surface sites, leading to more uniform spatial coverage and faster lateral growth of calcium phosphate domains [74]. EDS analysis (Figure 11C) revealed increased Ca (7.11 at.%) and P (3.71 at.%) contents compared with the previous variants, confirming greater CaP deposition. After thermal treatment (Figure 11D,E), the layer exhibits more pronounced densification, with visible “necks” forming between adjacent particles. This feature is indicative of solid-state sintering or partial crystal coalescence, suggesting improved interparticle bonding. Such microstructural evolution can enhance the internal cohesion of the coating and strengthen its adhesion to the substrate, which are crucial factors for mechanical stability under physiological loading.

EDS analysis (Figure 11F) revealed increased Ca (12.39 at.%) and P (6.94 at.%) contents, confirming greater CaP deposition, which can promote possible continuous CaP layer formation. From a coating design perspective, VD3 represents a favorable balance between thickness, uniformity, and crack-free morphology. These characteristics are particularly advantageous for subsequent Zn^{2+} incorporation and for achieving controlled, reproducible ionic release in multifunctional implant applications.

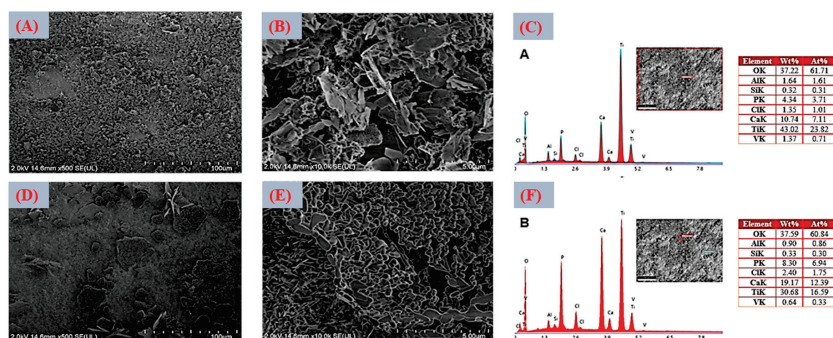


Figure 11. (A,B) FESEM images of the coated surface with VD3 (SBFX10- 4 h), 500× and 10,000×. (C) EDS spectra of the surfaces coated with VD3. (D,E) FESEM images of the thermally treated coated surface with VD3, 500× and 10,000×. (F) EDS spectra of the surfaces coated with thermally treated VD3.

The VD4 condition (Figure 12A,B), which combines higher ionic concentration (SBFX10) with extended immersion time (6 h), produced the thickest and most compact layer among all the variants evaluated. The surface exhibits nearly complete coverage, with densely packed particle agglomerates indicative of sustained secondary nucleation and continuous crystal growth. Under these highly supersaturated conditions, both the nucleation density and the growth rate are maximized, resulting in substantial accumulation of calcium phosphate across the activated substrate [75]. However, localized cracking is evident in certain regions. This behavior suggests that excessive layer thickness generates residual stresses, likely arising from mismatches in thermal expansion coefficients between the CaP coating and the Ti6Al4V substrate, as well as shrinkage during drying and subsequent heat treatment. EDS analysis (Figure 12C) revealed the highest Ca (12.17 at.%) and P (5.98 at.%) concentrations among all variants, confirming that VD4 yields the greatest CaP deposition. After thermal treatment (Figure 12D,E), cracking becomes more pronounced, although the formation of interparticle “necks”, similar to those observed in VD3, indicates partial sintering and improved local cohesion. While densification enhances particle bonding, excessive thickness compromises the overall mechanical integrity of the coating. The post-heat-treatment EDS (Figure 12F) confirmed increased Ca (15.19 at.%) and P (7.47 at.%) contents, confirming greater CaP deposition that might promote a CaP continuous layer homogeneously distributed with full surface coverage.

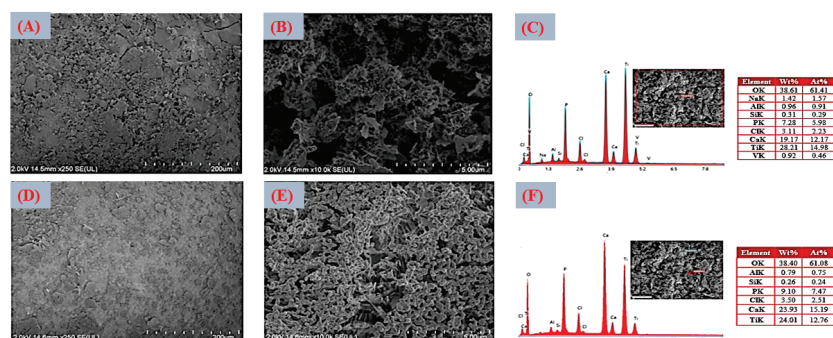


Figure 12. (A,B) FESEM images of the coated surface with VD4 (SBFX10- 6 h), 500× and 10,000×. (C) EDS spectra of the surfaces coated with VD4. (D,E) FESEM images of the thermally treated coated surface with VD4, 500× and 10,000×. (F) EDS spectra of the surfaces coated with thermally treated VD4.

Despite maximizing CaP loading, the presence of cracks represents a critical limitation of the VD4 variant. Such defects may promote coating delamination under mechanical stress and lead to uncontrolled ionic release [76]. Therefore, although VD4 achieves the

highest deposition efficiency, it does not necessarily represent the optimal balance between thickness, structural integrity, and functional reliability required for biomedical applications.

A comparative assessment of the four deposition variants clearly demonstrates that both ionic concentration and immersion time play a decisive role in controlling coating morphology, uniformity, thickness, and structural stability. VD1 and VD2 resulted in relatively thin and heterogeneous layers with incomplete surface coverage, indicating that limited supersaturation and/or insufficient growth time restrict the formation of a fully continuous calcium phosphate network. Although increasing immersion time (VD2) enhanced CaP accumulation compared with VD1, the coatings remained partially discontinuous. In contrast, VD3 (SBFX10, 4 h) produced a continuous and homogeneous coating with extensive surface coverage and no significant cracking. The higher ionic activity increased supersaturation with respect to apatite, promoting rapid heterogeneous nucleation and sustained lateral crystal growth. As a result, a coherent CaP layer developed within a relatively short deposition time. Post-deposition thermal treatment induced partial densification and the formation of interparticle necks, suggesting improved cohesion and potentially stronger adhesion to the substrate [77]. These features are consistent with optimized biomimetic deposition windows reported in the literature [78]. VD4 (SBFX10, 6 h) generated the thickest coatings and the highest Ca and P contents. However, pronounced cracking was observed, attributed to excessive thickness and residual stresses associated with drying shrinkage and thermal expansion mismatch. This behavior reflects the well-known trade-off between maximizing CaP deposition and maintaining mechanical reliability [79].

VD3 emerges as the most suitable condition for subsequent Zn^{2+} incorporation. It offers an optimal balance of high surface coverage, good homogeneity, absence of significant cracking, and sufficient thickness to accommodate Zn without compromising structural integrity. These characteristics are essential to ensure uniform Zn distribution, controlled ion-release kinetics, and reproducible antibacterial performance, in agreement with the central hypothesis of this study.

3.4. Zn-Containing CaP Coatings (Zn-CaP)

Three deposition variants were prepared using different zinc concentrations in the solution (low—VDZn1, intermediate—VDZn2, and high—VDZn3) in order to systematically assess the influence of Zn on coating morphology, composition, and homogeneity, as well as its potential impact on subsequent bioactivity and antibacterial performance. By gradually increasing the Zn^{2+} content in the biomimetic medium, it was possible to examine how this trace element modulates nucleation and crystal growth mechanisms within calcium phosphate (CaP) systems.

Figure 13A–F shows FESEM micrographs at different magnifications of the surface coated with the lowest zinc concentration (VDZn1) after post-deposition heat treatment. The resulting coating is characterized by clusters of particles forming lamellar assemblies arranged in flower-like configurations. On top of these primary structures, secondary globular precipitates can be observed. This morphology is typical of calcium phosphates produced via biomimetic routes, where heterogeneous nucleation at the substrate–solution interface initially leads to amorphous or poorly crystalline calcium phosphate phases [80]. With time and thermal treatment, these phases tend to reorganize into more ordered apatite-like structures. The radial growth pattern observed in the flower-like structures suggests a diffusion-controlled mechanism combined with anisotropic crystal growth. At low zinc concentrations, the presence of Zn^{2+} does not significantly suppress the formation of these apatite-like assemblies. However, zinc is known to interact with calcium phosphate at the

atomic scale, potentially altering surface energy and slightly refining the microstructure. Because Zn^{2+} has a smaller ionic radius than Ca^{2+} and a different electronic configuration, partial substitution within the apatite lattice or adsorption at active growth sites may subtly influence crystal habit and grain size [81].

For the intermediate concentration variant (VDZn2, Figure 14A–F), the morphology remains comparable to that of VDZn1.

Nevertheless, a slight reduction in average particle size and a greater degree of agglomeration fragmentation can be observed. This effect is consistent with the role of Zn^{2+} as a crystallization modifier. Several studies have reported that zinc can partially inhibit apatite crystal growth by competing with Ca^{2+} for lattice positions and inducing local structural distortions. Such distortions may slow down crystal propagation along specific crystallographic directions, resulting in finer and more dispersed particles [82].

In the case of the highest zinc concentration (VDZn3, Figure 15A–F), no drastic qualitative morphological changes are detected when compared with the previous variants. However, a lower surface coverage density and a less homogeneous particle distribution are evident. This behavior suggests that elevated Zn^{2+} concentrations in the solution may limit both nucleation density and subsequent apatite layer growth. Excess zinc can reduce the effective supersaturation with respect to calcium phosphate phases and may stabilize intermediate amorphous species, delaying their transformation into crystalline apatite. As a consequence, the coating appears less continuous and more heterogeneous [83].

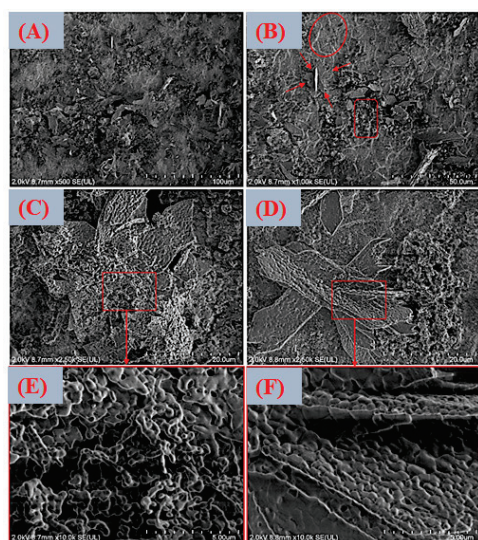


Figure 13. FESEM images of the surface coated with zinc inclusion at the lowest concentration (VDZn1). (A) 500 $\times$, (B) 1000 $\times$, (C,D) 2500 $\times$, and (E,F) 10,000 $\times$.

The three Zn-containing variants share several microstructural features: (i) non-uniform particle arrangements, (ii) a tendency toward agglomerate formation, (iii) flower-like structures associated with biomimetic apatite, and (iv) increasing surface heterogeneity with higher Zn concentration.

These microstructural characteristics have direct implications for biological performance. Surface roughness, particle size distribution, and heterogeneity strongly influence protein adsorption, early cell adhesion, and subsequent *in vivo* apatite formation. Moderate roughness and nanoscale features are often beneficial for osteoblastic activity, whereas excessive heterogeneity or poor coverage may compromise mechanical stability and long-term integration [84]. Therefore, controlling zinc concentration is not only a compositional adjustment but also a strategy to tailor surface architecture for optimized biological response.

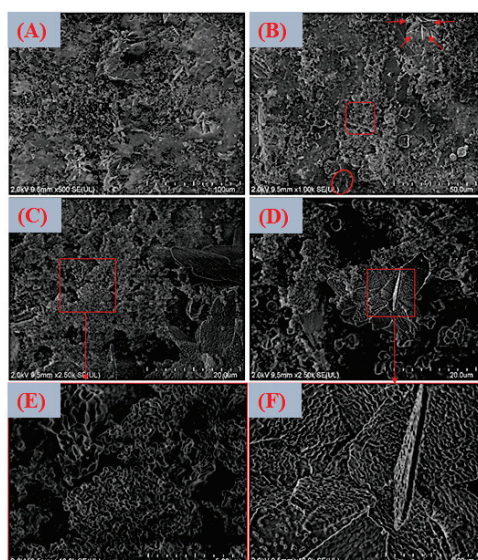


Figure 14. FESEM images of the surface coated with zinc inclusion at the intermediate concentration (VDZn2). (A) 500 $\times$, (B) 1000 $\times$, (C,D) 2500 $\times$, and (E,F) 10,000 $\times$.

Figure 16A–C presents the EDS spectra obtained from the surfaces coated with VDZn1, VDZn2, and VDZn3, respectively. In all cases, peaks corresponding to Ca, P, and O are clearly identified, confirming the formation of a calcium phosphate layer. Signals from Ti and Al originate from the Ti6Al4V substrate and indicate that the electron beam partially interacts with the underlying alloy, particularly in regions where the coating is thinner. Importantly, a Zn signal is detected in all variants, confirming successful incorporation of zinc into the coating through the biomimetic process.

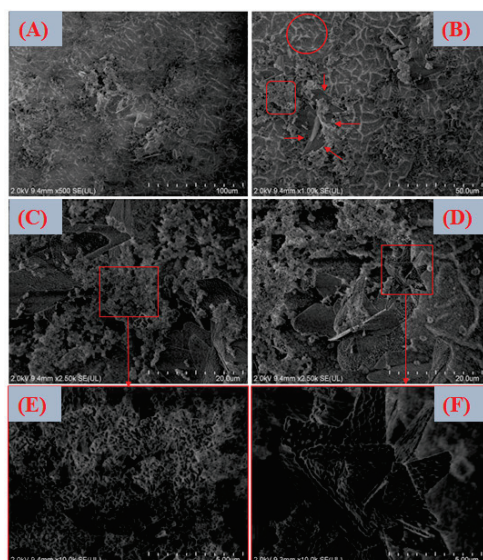


Figure 15. FESEM images of the surface coated with zinc inclusion at the highest concentration (VDZn3). (A) 500 $\times$, (B) 1000 $\times$, (C,D) 2500 $\times$, and (E,F) 10,000 $\times$.

The semi-quantitative EDS results (Table 4) reveal a progressive increase in atomic Zn content with increasing zinc concentration in the deposition solution: 0.4 at% for VDZn1, 0.7 at% for VDZn2, and 0.9 at% for VDZn3. This trend demonstrates that the method enables controlled zinc incorporation. Although the absolute Zn content remains relatively low, even trace levels of zinc are known to significantly influence osteogenic differentiation and antibacterial activity due to their role as an essential cofactor in numerous enzymatic processes [85].

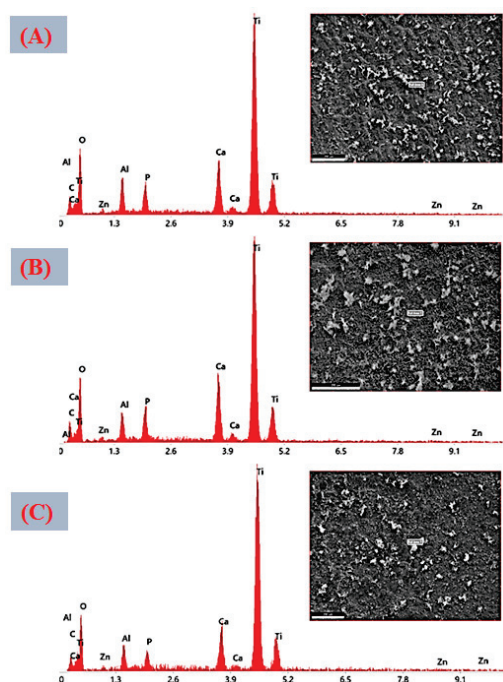


Figure 16. EDS spectra of the surfaces of Ti6Al4V coated with (A) VDZn1, (B) VDZn2, and (C) VDZn3.

Table 4. Semi-quantitative area EDS analysis of Zn-containing coating variants (atomic %).

Deposition Variant	Elements (at%)						
	C	O	Al	P	Ca	Ti	Zn
VDZn1	24.2	51.5	2.4	1.6	3.3	16.6	0.4
VDZn2	22.4	51.6	2.0	1.9	4.2	17.2	0.7
VDZn3	21.5	51.6	2.1	1.2	3.1	19.6	0.9

A slight increase in Ca and P content, particularly for VDZn2, suggests the existence of an optimal zinc concentration window in which apatite nucleation and Zn^{2+} incorporation occur simultaneously without severely inhibiting CaP growth. Moderate Zn levels may enhance surface reactivity by modifying crystal surface energy, potentially facilitating interaction with physiological fluids [86]. In contrast, excessive Zn appears to reduce overall CaP deposition efficiency, as reflected by the relative compositional changes and reduced surface coverage observed in VDZn3.

Point EDS analysis (Figure 17A–C, VDZn1, VDZn2, and VDZn3, respectively) confirms that the flower-like structures are rich in Ca, P, and O, consistent with apatite precipitates. The simultaneous detection of Zn within these regions indicates that zinc is directly associated with the CaP phase. This association may occur through partial substitution of Ca^{2+} within the apatite lattice, interstitial incorporation, or surface adsorption onto crystal facets. Each mechanism can influence dissolution behavior and ion-release kinetics, which are critical parameters for achieving a balanced osteogenic and antibacterial response [87].

The Ca/P atomic ratio provides valuable insight into the chemical nature and structural maturity of the calcium phosphate phase formed in each Zn-containing coating. For reference, stoichiometric hydroxyapatite (HA) exhibits a Ca/P ratio of approximately 1.67. Deviations from this value often indicate the presence of non-stoichiometric phases, such as calcium-deficient apatite (CDA), amorphous calcium phosphate (ACP), or mixtures of different CaP phases [88]. These variations are particularly relevant in biomimetic systems, where precipitation typically occurs under near-physiological conditions and rarely yields perfectly stoichiometric HA. In the present study (Table 5), the Ca/P ratios are 1.44 for

VDZn1, 1.51 for VDZn2, and 1.80 for VDZn3, revealing distinct compositional trends as a function of zinc concentration.

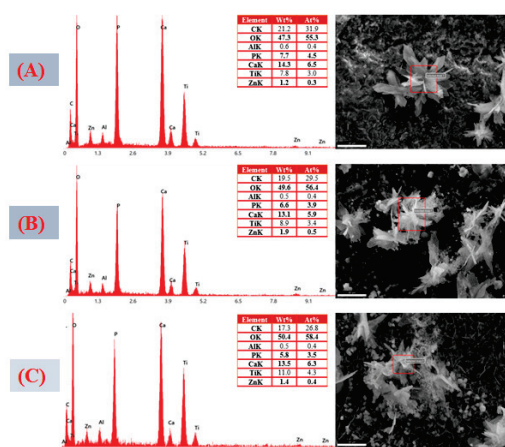


Figure 17. Point EDS analysis of the surfaces of Ti6Al4V coated with (A) VDZn1, (B) VDZn2, and (C) VDZn3.

Table 5. Semi-quantitative point EDS analysis of Zn-containing coating variants (atomic %).

Deposition Variant	Elements (at%)							Ca/P Ratio
	C	O	Al	P	Ca	Ti	Zn	
VDZn1	21.9	55.3	0.4	4.5	6.5	3.0	0.3	1.44
VDZn2	29.5	56.4	0.4	3.9	5.9	3.4	0.5	1.51
VDZn3	26.8	58.4	0.4	3.5	6.3	4.3	0.4	1.8

The Ca/P ratio of 1.44 (VDZn1) is clearly below the stoichiometric HA value. Such a reduced ratio suggests the formation of a calcium-deficient apatite or the presence of amorphous calcium phosphate as a precursor phase. In biomimetic deposition processes, ACP frequently forms during the early stages of nucleation and subsequently transforms into poorly crystalline apatite. A Ca/P ratio in the range of 1.3–1.5 is commonly associated with these metastable or calcium-deficient structures [89]. From a biological standpoint, calcium-deficient apatite is generally more soluble than stoichiometric HA. This higher solubility can enhance ion exchange with the surrounding environment, potentially promoting faster apatite reprecipitation in simulated body fluid and improved *in vivo* bioactivity. The relatively low zinc content (0.3 at%) in VDZn1 likely allows CaP nucleation to proceed with minimal structural disruption, resulting in a coating that retains the typical characteristics of biomimetic apatite, albeit in a slightly Ca-deficient form [90].

The Ca/P ratio increases to 1.51 in VDZn2, approaching more closely the stoichiometric HA value but still remaining within the calcium-deficient apatite range. This intermediate ratio is particularly interesting because it suggests a more balanced incorporation of calcium and phosphate during crystal growth. The moderate Zn content (0.5 at%) may contribute to this effect. Zinc ions are known to influence nucleation kinetics and crystal growth by interacting with active sites on growing CaP nuclei. At moderate concentrations, Zn^{2+} may slightly retard crystal growth without completely suppressing Ca incorporation, allowing a more controlled precipitation process [91]. The result is a composition closer to that of crystalline apatite while still retaining some degree of calcium deficiency. From a functional perspective, a Ca/P ratio around 1.5 is often considered advantageous in biomedical coatings. Calcium-deficient apatite with this composition tends to combine adequate structural stability with enhanced biological reactivity. It can dissolve gradually under

physiological conditions, releasing Ca^{2+} and Zn^{2+} ions that may stimulate osteoblastic activity while maintaining sufficient coating integrity.

In contrast, VDZn3 exhibits a Ca/P ratio of 1.80, exceeding the stoichiometric HA value of 1.67. Such an elevated ratio suggests either a relative deficiency in phosphorus, an excess of calcium-rich phases, or the coexistence of secondary calcium-containing compounds (e.g., CaO or other Ca-rich precipitates) [92]. It may also reflect partial inhibition of phosphate incorporation during crystal growth due to higher Zn^{2+} concentrations. At elevated zinc levels, Zn^{2+} can compete more strongly with Ca^{2+} and may interfere with the normal arrangement of phosphate tetrahedra in the apatite lattice. This disruption can alter precipitation kinetics, potentially leading to non-stoichiometric compositions or heterogeneous phase formation. The morphological observations for VDZn3 (reduced surface coverage and greater heterogeneity) are consistent with such compositional imbalance. From a biological standpoint, a Ca/P ratio significantly higher than 1.67 may influence dissolution behavior and mechanical stability [93]. Calcium-rich phases may exhibit different solubility profiles and could lead to less predictable ion-release kinetics. Moreover, compositional heterogeneity may generate localized differences in dissolution rate, which could affect long-term coating performance.

To verify zinc incorporation and examine its chemical state at the outermost surface, X-ray photoelectron spectroscopy (XPS) was carried out (Figure 18).

The analysis confirmed the presence of Zn in all three variants, with surface atomic concentrations of 0.24%, 0.59%, and 0.93% for VDZn1, VDZn2, and VDZn3, respectively. These values are in very good agreement with the semi-quantitative EDS results, indicating consistent Zn incorporation both at the micrometric scale (EDS) and within the first few nanometers of the surface (XPS).

The high-resolution spectra (Ti 2p, O 1s, Ca 2p, P 2p, and Zn 2p) have been analyzed with clearer labeling of the corresponding chemical states. In particular, the Ti 2p region confirms the presence of Ti^{4+} , consistent with a TiO_2 surface layer. For the oxidation state Ti^{4+} , the main peaks are found at Ti 2p_{3/2} (458.3 and 458.8 eV) and the Ti 2p_{1/2} peak (~463.9 eV–464.5 eV). Meanwhile, the Ca 2p and P 2p signals are characteristic of calcium phosphate phases. The two peaks in the Ca 2p region with binding energies of ~346.8 eV and ~350.3 eV are in perfect agreement with the characteristic positions of Ca 2p_{1/2} and Ca 2p_{3/2}. The Zn 2p_{3/2} peak, centered at approximately ~1021–1022 eV, supports the presence of Zn^{2+} species, with no evidence of metallic zinc or other oxidation states. Additionally, the O 1s spectrum has been deconvoluted into contributions associated with lattice oxygen, hydroxyl groups, and adsorbed species, providing further insight into surface chemistry and potential nucleation sites.

The binding energies summarized in Table 6 are characteristic of calcium phosphate systems containing divalent zinc: (i) O1s (~529–531 eV), associated with O–P–O bonds and hydroxyl groups typical of hydroxyapatite; (ii) Ca2p (~346–347 eV), corresponding to Ca^{2+} in an apatite environment; (iii) P2p (~133.1 eV), characteristic of phosphate (PO_4^{3-}) groups; (iv) Zn2p (~1021–1022 eV), attributable to Zn^{2+} coordinated with oxygen in oxide or phosphate environments.

These values are consistent with previously reported data for zinc-doped hydroxyapatite and modified calcium phosphate coatings. The absence of significant shifts toward metallic Zn binding energies indicates that zinc is present in its oxidized state (Zn^{2+}), rather than as metallic clusters [94]. Moreover, the relatively narrow variation in Ca2p and P2p positions among the three variants suggests that the overall apatite framework remains chemically stable despite the introduction of Zn. A closer look at the O1s signal reveals a slight shift toward lower binding energies as Zn content increases (from 531.1 eV in VDZn1 to 529.2 eV in VDZn3). This subtle change may reflect differences in the local chemical en-

vironment of oxygen, possibly due to Zn–O coordination or variations in hydroxyl content within the apatite lattice. Similarly, small variations in the Ca2p peak positions could be related to minor lattice distortions induced by Zn incorporation, given the smaller ionic radius of Zn^{2+} compared with Ca^{2+} . Even limited substitution can generate measurable electronic perturbations detectable by surface-sensitive techniques such as XPS [95].

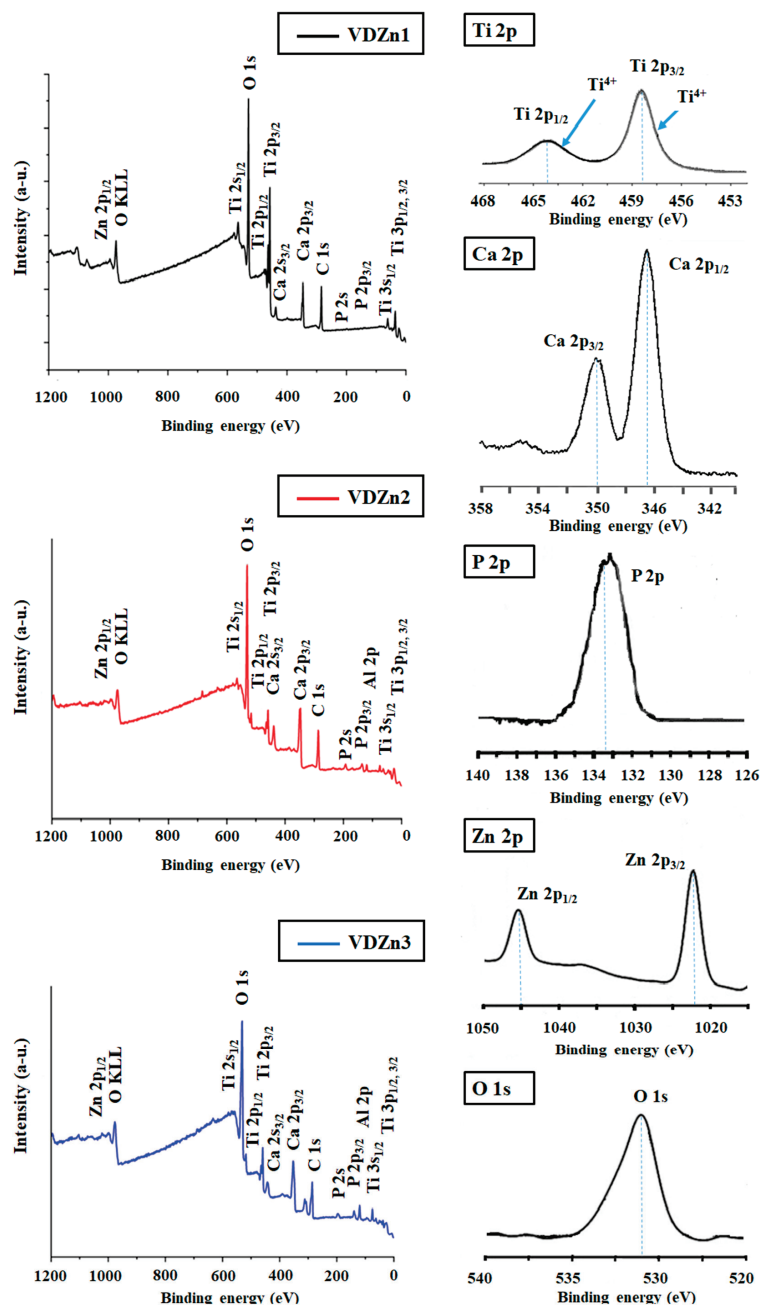


Figure 18. XPS energy analysis of Ti6Al4V surfaces coated with Zn-CaP.

The incorporation of zinc into the CaP coatings through the biomimetic route represents one of the central contributions of this study. Both EDS and XPS analyses confirm the successful introduction of small but measurable amounts of Zn in all variants. Importantly, XPS demonstrates that zinc is present as Zn^{2+} in an oxygen-coordinated environment, consistent with its chemical association with the calcium phosphate phase rather than simple surface contamination. This finding confirms that the biomimetic process enables effective chemical integration of zinc within the coating [96].

Table 6. XPS peak positions (binding energy, eV) for Zn–CaP coatings deposited on Ti6Al4V.

Deposition Variant	C1s (eV)	O1s (eV)	Ca2p (eV)	P2p (eV)	Zn2p (eV)	Zn (at%, Surface)
VDZn1	284.6	531.1	347.1	133.1	1021.9	0.24
VDZn2	284.6	530.0	346.5	133.1	1020.7	0.59
VDZn3	284.6	529.2	346.1	133.1	1021.3	0.93

Beyond confirming its presence, the results show that zinc actively influences the precipitation chemistry of calcium phosphates. The progressive variation in the Ca/P atomic ratio from VDZn1 to VDZn3 demonstrates that Zn concentration modifies phase development and crystal growth dynamics. At low and intermediate Zn levels (VDZn1 and VDZn2), the Ca/P ratios correspond to calcium-deficient apatite, a phase known for its higher solubility and enhanced biological reactivity compared with stoichiometric hydroxyapatite. Under these conditions, the typical apatite morphology and satisfactory surface coverage are largely preserved, indicating that moderate Zn incorporation does not disrupt the nucleation–growth balance of the biomimetic system.

Among the studied conditions, VDZn2 emerges as the most balanced formulation. It combines moderate zinc incorporation with a Ca/P ratio closer to apatite stoichiometry, suggesting controlled crystal growth without compromising phase stability. This compositional and microstructural balance is particularly relevant for biomedical applications. Calcium-deficient apatite can promote ion exchange and surface reactivity, while maintaining sufficient structural integrity to ensure coating stability. In contrast, the highest Zn concentration (VDZn3) shifts the composition toward a Ca-rich regime (Ca/P = 1.80), indicating altered precipitation dynamics and possible phase heterogeneity. This deviation correlates with reduced coating homogeneity and surface coverage, suggesting that excessive Zn begins to destabilize the optimal biomimetic deposition window [97].

From a biological perspective, the controlled presence of Zn in the CaP layer is highly significant. Zinc is an essential trace element involved in key enzymatic pathways related to bone metabolism. It stimulates osteoblastic differentiation, enhances alkaline phosphatase activity, and supports extracellular matrix mineralization. At the same time, Zn²⁺ exhibits antibacterial activity against common implant-associated pathogens such as *Staphylococcus aureus* and *Escherichia coli*, partly through membrane destabilization and interference with microbial metabolism. Zinc has also been reported to modulate the local inflammatory response, which may contribute to improved early-stage healing. By integrating zinc into an osteoconductive CaP matrix, these coatings are designed as multifunctional systems capable of simultaneously promoting osseointegration and reducing infection risk [98].

Although XPS confirms the chemical state of zinc at the surface, it does not fully resolve whether Zn²⁺ is incorporated substitutionally within the apatite lattice or predominantly adsorbed onto crystal surfaces. Because XPS is limited to the near-surface region and primarily provides information on oxidation state and local chemical environment, complementary structural studies are necessary to clarify the incorporation mechanism. X-ray diffraction with Rietveld refinement could reveal subtle changes in lattice parameters; FTIR or Raman spectroscopy could detect vibrational shifts associated with phosphate or hydroxyl groups; and ion release studies in physiological media would help determine dissolution behavior and Zn²⁺ release kinetics [99].

In summary, this study demonstrates that zinc incorporation through a biomimetic approach is not merely a compositional modification but a strategy to tune microstructure, phase composition, and biological functionality. The results highlight the existence of an optimal Zn concentration range (represented here by VDZn2) where structural stability, controlled ion release, enhanced bioactivity, and antibacterial potential can be achieved

simultaneously. This balance underscores the scientific and practical relevance of the present research for the development of next-generation multifunctional implant coatings.

3.4.1. The Titanium Oxide Layer After Surface Treatment

As is well known, the titanium oxide layer formed after surface treatment plays a critical role as an interfacial layer, directly influencing the nucleation, growth, adhesion, and overall performance of the biomimetic CaP coatings. In this study, the surface activation of Ti6Al4V substrates via acid etching (HCl/H₂SO₄ at 80 °C for 20 min) was specifically designed to modify both the surface topography and the chemistry of the native oxide layer. Although the oxide layer thickness was not directly measured as an individual parameter, its presence and functional characteristics were indirectly assessed through a combination of surface-sensitive techniques and coating behavior.

From a physicochemical viewpoint, acid etching partially removes the native passive film and promotes the rapid reformation of a thin, hydrated titanium oxide layer (primarily TiO₂) upon exposure to air and aqueous media. This regenerated oxide layer is typically nanometric in thickness and is known to be amorphous or poorly crystalline, enriched with hydroxyl (–OH) groups. These surface hydroxyl groups are essential, as they act as active sites for ionic interaction and might serve as nucleation centers for calcium phosphate during immersion in SBF.

The chemical nature of this interfacial layer was evaluated by X-ray photoelectron spectroscopy (XPS), which confirmed the presence of Ti⁴⁺ species consistent with TiO₂, along with surface hydroxylation. Previous studies have demonstrated that such hydroxylated TiO₂ surfaces significantly enhance apatite nucleation by increasing surface energy and facilitating electrostatic interactions with Ca²⁺ and PO₄^{3–} ions.

Regarding morphology, the acid treatment generated a micro-roughened surface, as confirmed by SEM analysis. This topographical modification is intrinsically linked to oxide layer formation, as it increases the effective surface area and promotes mechanical interlocking with the subsequently deposited CaP coating.

While we acknowledge that direct measurement of oxide layer thickness (e.g., via TEM or ellipsometry) and crystallographic characterization (e.g., grazing-incidence XRD) would provide additional insight, the combined evidence from surface chemistry (XPS), morphology (SEM), and coating growth behavior collectively supports the formation of a thin, hydroxylated TiO₂ interlayer with high bioactivity. Additionally, the uniform and dense nucleation of CaP observed in SEM images provides strong indirect evidence but strong evidence of a chemically active and homogeneous oxide layer.

3.4.2. The Thickness of Biomimetic Zn-Containing CaP Coatings (Zn-CaP)

Figure 19 shows the thickness of Zn-CaP variants (VDZn1, VDZn2, and VDZn3) determined through cross-sectional analysis using field emission scanning electron microscopy (FESEM). Representative cross-sectional micrographs were obtained, allowing direct visualization of the coating–substrate interface. The results indicate that the coatings exhibit a relatively uniform thickness, with average values in the range of approximately 9–11 µm. This thickness range is consistent with biomimetically deposited CaP layers obtained under similar conditions, where growth is governed by nucleation and ion diffusion processes in simulated body fluid (SBF). The observed uniformity also reflects the effectiveness of the prior surface activation treatment in promoting homogeneous nucleation across the substrate.

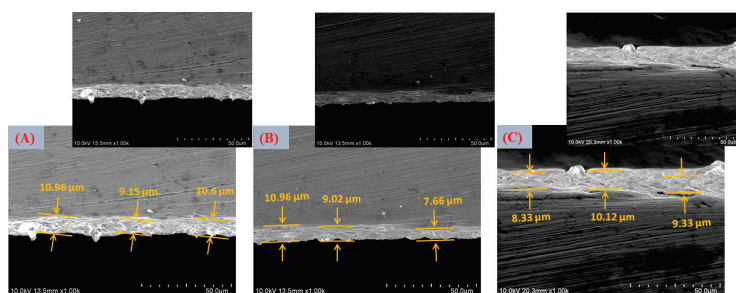


Figure 19. FESEM images of the thickness of Zn-CaP variants (and). (A) VDZn1, 1000 $\times$, (B) VDZn2, 1000 $\times$, and (C) VDZn3, 1000 $\times$.

From a functional viewpoint, the measured thickness is sufficient to provide full surface coverage while maintaining good interfacial adhesion and avoiding excessive internal stresses. Moreover, coatings within this thickness range are known to support controlled ion release and bioactive behavior without compromising mechanical stability.

To further validate the compositional homogeneity and Zn incorporation, the study was complemented by elemental mapping using FESEM–EDS for each Zn–CaP variant (see Figure 20). The EDS results (see Table 7) confirmed that all coatings are primarily composed of O, Ca, and P, consistent with calcium phosphate phases, with the additional presence of Zn in doped samples.

Table 7. Semi-quantitative elemental mapping (EDS) analysis of Zn-containing coating variants (atomic %).

Deposition Variant	Elements (at%)			
	O	P	Ca	Zn
VDZn1	85.4	2.4	11.6	0.6
VDZn2	82.7	4.2	12.4	0.7
VDZn3	86.4	4.6	8.0	1.0

For VDZn1, the composition showed 70.3 wt% O, 23.9 wt% Ca, 3.8 wt% P, and 1.9 wt% Zn (0.6 at%), indicating successful incorporation of Zn at low levels. A similar compositional trend was observed for VDZn2, confirming the reproducibility of the biomimetic process and suggesting that Zn incorporation at intermediate conditions remains relatively stable without significantly altering the CaP matrix composition.

In contrast, VDZn3 exhibited a higher Zn content (3.6 wt%, 1.0 at%), accompanied by a decrease in Ca content (16.7 wt%) and an increase in oxygen concentration (72.2 wt%). This compositional shift suggests that higher Zn incorporation modifies the precipitation chemistry, potentially promoting the formation of more hydrated or defect-rich calcium phosphate phases. The reduction in Ca content, together with relatively stable P levels, indicates a deviation from stoichiometric apatite toward a more Ca-deficient or structurally perturbed phase, which is consistent with the Ca/P trends observed in previous sections. Elemental mapping further revealed a relatively homogeneous spatial distribution of Ca, P, and Zn across the coating thickness for all variants, with no evidence of Zn-rich agglomerates or phase segregation. This uniform distribution supports the hypothesis that Zn is incorporated both within the CaP matrix and at surface-accessible sites, rather than forming separate Zn-based phases.

From a microstructural perspective, the combination of uniform thickness and homogeneous elemental distribution indicates that the biomimetic deposition process enables controlled and reproducible incorporation of Zn without compromising coating continuity or integrity. However, the compositional variations observed at higher Zn levels (VDZn3)

suggest that excessive Zn incorporation may alter crystal growth kinetics and local stoichiometry, which could influence both dissolution behavior and biological response. The integration of cross-sectional FESEM analysis with EDS compositional and mapping data provides a more comprehensive understanding of the structure–composition relationship in Zn–CaP coatings. These results confirm that coating thickness, elemental distribution, and Zn incorporation are strongly interrelated and must be carefully balanced to achieve optimal functional performance.

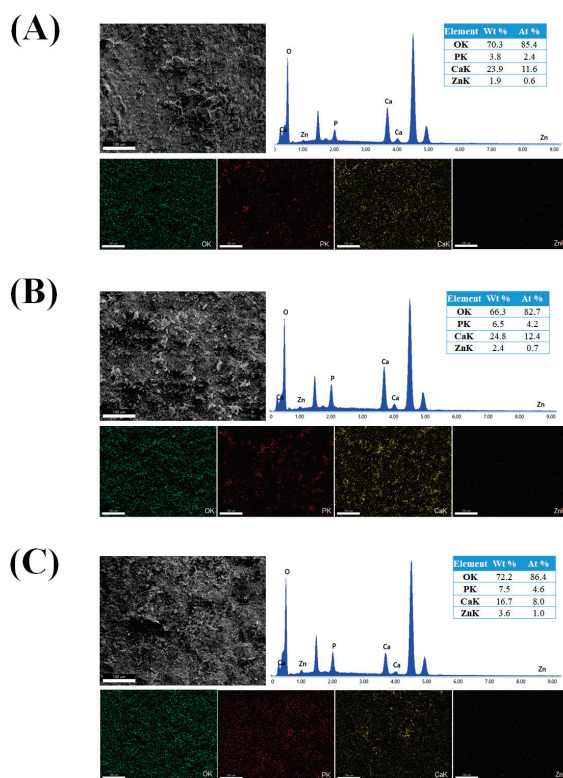


Figure 20. Elemental mapping study of Zn–CaP variants using FESEM–EDS. (A) VDZn1, (B) VDZn2, and (C) VDZn3.

3.4.3. The Adhesion of the Biomimetic Zn–CaP Coatings

The adhesion of the biomimetic Zn–CaP coatings was qualitatively assessed through complementary observations of coating integrity after mechanical handling and ultrasonic cleaning. The coatings exhibited no signs of delamination, cracking, or spallation under these conditions, suggesting strong interfacial bonding with the Ti6Al4V substrate. Additionally, SEM cross-sectional analysis revealed a continuous and well-integrated interface without observable gaps or defects, which is typically associated with good adhesion in biomimetic systems (see Figure 21).

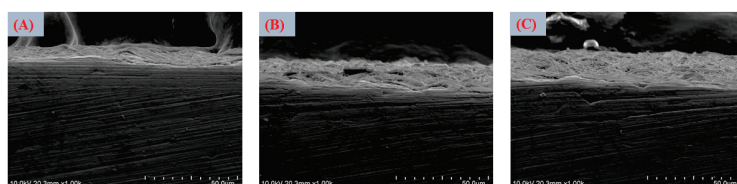


Figure 21. FESEM images of the adhesion stability of Zn–CaP variants after mechanical handling and ultrasonic cleaning. (A) VDZn1, 1000×, (B) VDZn2, 1000×, and (C) VDZn3, 1000×.

From a mechanistic perspective, the good adhesion can be attributed to two main factors: (i) the surface activation treatment, which generates a micro-roughened and hydroxylated TiO₂ layer that enhances both mechanical interlocking and chemical bonding,

and (ii) the low-temperature biomimetic deposition process, which promotes gradual nucleation and growth of CaP directly on the substrate, minimizing residual stresses commonly observed in other coating processes like plasma spraying. Further quantitative work needs to be addressed in order to study the mechanical properties of this coating.

Although a standardized quantitative adhesion test (e.g., pull-off test according to ASTM C633 (*Standard Test Method for Adhesion or Cohesion Strength of Thermal Spray Coatings*; ASTM International: West Conshohocken, PA, USA, 2013) or scratch test according to ASTM C1624-05 (*Standard Test Method for Adhesion Strength and Mechanical Failure Modes of Ceramic Coatings by Quantitative Single Point Scratch Testing*; ASTM International: West Conshohocken, PA, USA, 2005) was not initially included, the interface and coating morphologies obtained in this study are similar to those of other authors, who reported values of adhesion resistance in a range between ~10 and 30 MPa [100–103]. These values, while lower than those of some high-temperature coatings, are generally considered sufficient for dental implant applications, where load transfer is primarily supported by the metallic substrate and long-term stability is achieved through biological fixation (osseointegration) rather than solely by coating adhesion.

It is also important to note that excessively high adhesion strength is not always required or even desirable for bioactive coatings, as partial dissolution and remodeling at the interface are integral to their biological function. In this context, the combination of adequate initial adhesion and progressive bone bonding is more relevant for clinical performance.

3.5. Cytocompatibility

The cytotoxic behavior of the Zn–CaP coatings was evaluated using the MTT colorimetric assay, a widely accepted method that measures the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble formazan crystals through NAD(P)H-dependent mitochondrial oxidoreductase activity. Since the formation of formazan is directly linked to mitochondrial integrity and cellular metabolic function, this assay provides sensitive information about early cytotoxic effects and overall cell viability [104]. After 24 h of direct contact between the samples and the cells, a clear purple coloration of the culture medium was observed for all variants, including VDZn1, VDZn2, and VDZn3 (Figure 22).

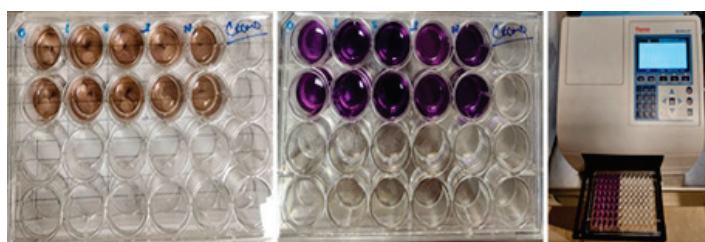


Figure 22. Change in the coloration of the cell culture that was in direct contact with the samples of the treatment variants VDZn0, VDZn1, VDZn2, and VDZn3. Microplate reader.

This qualitative change confirms efficient MTT reduction and indicates that mitochondrial activity was preserved in both primary human gingival fibroblasts (HGF) and human dermal fibroblasts (HDF). Even in the presence of zinc, none of the coatings caused complete inhibition of cellular metabolism. Quantitative evaluation was subsequently performed by spectrophotometric measurement of formazan absorbance using a microplate reader. Cell viability percentages were calculated relative to negative (NC) and positive (PC) controls following standardized protocols. The results for primary human gingival fibroblasts (HGF) and human dermal fibroblasts (HDF) are summarized in Figure 23 and Table 8.

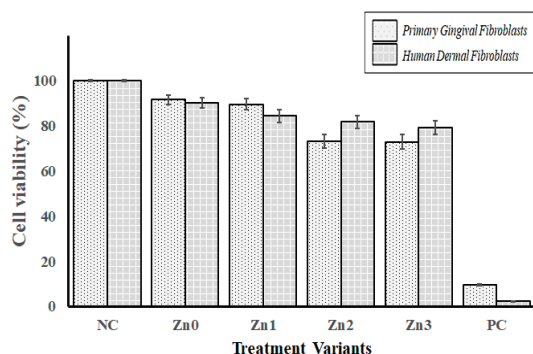


Figure 23. Cell viability percentage of Primary Gingival Fibroblasts (HGF-ATCC® PCS-201-018TM) and Human Dermal Fibroblasts (ATCC® PCS-2018-012) cultured in contact with the studied surfaces (NC-Negative Control, PC-Positive Control).

The undoped CaP coating (VDZn0) exhibited high viability values (approximately 90–92%), confirming the excellent biocompatibility of calcium phosphate materials and aligning with the well-documented cytocompatibility of biomimetic apatite coatings [105]. When zinc was incorporated at low levels (VDZn1), cell viability remained high (>85%), indicating that small amounts of Zn^{2+} do not induce significant cytotoxic effects. This behavior is consistent with previous studies reporting that zinc, at controlled concentrations, can even exert beneficial biological effects.

Table 8. Cell viability (%) determined by MTT assay after 24 h of direct contact with Zn–CaP coatings.

Deposition Variant	Gingival Fibroblasts (HGF) (%) ± SD	Dermal Fibroblasts (HDF) (%) ± SD
NC	100.00 ± 0.50	100.00 ± 0.50
VDZn0 (CaP)	91.68 ± 2.10	90.26 ± 2.35
VDZn1	89.55 ± 2.45	84.36 ± 2.80
VDZn2	73.19 ± 3.10	81.74 ± 2.95
VDZn3	72.86 ± 3.25	79.07 ± 3.05
PC	9.690 ± 0.40	2.260 ± 0.10

As an essential trace element, Zn^{2+} functions as an enzymatic cofactor and plays a role in regulating gene expression associated with mineralization and tissue regeneration. At low doses, it may stimulate alkaline phosphatase activity and support extracellular matrix formation. In contrast, the intermediate and high zinc variants (VDZn2 and VDZn3) showed moderate reductions in viability. Compared with the positive control, gingival fibroblasts exhibited relative decreases of approximately 26–27%, while dermal fibroblasts showed reductions of about 18–21%. This pattern reflects a clear dose-dependent cellular response to Zn^{2+} , as widely described in the literature [106]. While zinc is essential at physiological levels, elevated local concentrations may increase Zn^{2+} release into the culture medium, potentially disturbing intracellular ionic balance. Excess Zn^{2+} has been associated with oxidative stress, mitochondrial dysfunction, and interference with metabolic pathways when present above optimal thresholds [43].

All experiments were performed in triplicate ($n = 3$), and the results are presented as means ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) to evaluate the effect of Zn incorporation on cell viability. Prior to ANOVA, data normality and homogeneity of variances were verified using the Shapiro–Wilk and Levene tests, respectively. Differences were considered statistically significant at $p < 0.05$. The ANOVA results revealed statistically significant differences among the experimental groups ($p < 0.001$). Post hoc comparisons were performed using Tukey’s test. As expected, the positive control (PC) showed significantly lower cell viability compared

to all other groups ($p < 0.001$), confirming the sensitivity of the assay, while the negative control (NC) exhibited the highest viability values. No statistically significant differences were observed between NC and the Zn-free CaP coating (VDZn0) ($p > 0.05$), indicating excellent cytocompatibility of the base coating. Similarly, the low-Zn variant (VDZn1) did not show significant differences compared to VDZn0 ($p > 0.05$), suggesting that low Zn incorporation does not adversely affect cell viability. In contrast, the intermediate and high Zn-containing coatings (VDZn2 and VDZn3) exhibited a statistically significant reduction in cell viability compared to NC and VDZn0 ($p < 0.05$), confirming a dose-dependent effect of Zn^{2+} on cellular response. However, no statistically significant difference was found between VDZn2 and VDZn3 ($p > 0.05$), indicating a plateau in the cytotoxic effect at higher Zn concentrations. Despite this reduction, all Zn-containing samples maintained viability values above the 70% threshold defined by ISO 10993-5 (*Biological Evaluation of Medical Devices—Part 5: Tests for In Vitro Cytotoxicity*; International Organization for Standardization (ISO): Geneva, Switzerland, 2009), confirming that the coatings remain non-cytotoxic within the evaluated range. The relatively low standard deviation values (<5%) further support the reproducibility and reliability of the MTT assay results.

Importantly, all Zn-containing coatings maintained viability values above 70% for both cell lines. According to ISO 10993-5 (*Biological Evaluation of Medical Devices—Part 5: Tests for In Vitro Cytotoxicity*; International Organization for Standardization (ISO): Geneva, Switzerland, 2009) criteria, materials with cell viability above this threshold are considered non-cytotoxic or slightly cytotoxic. Therefore, despite the observed dose-dependent reduction, the Zn–CaP coatings developed in this study can still be regarded as cytocompatible within the evaluated range.

Taken together, these findings highlight a critical balance in the design of multi-functional implant coatings. On one hand, zinc incorporation is intended to provide antibacterial properties and potentially enhance osteogenic responses; on the other hand, excessive Zn^{2+} release may compromise cellular viability. The results indicate that moderate zinc incorporation (particularly in VDZn1 and potentially VDZn2) offers a favorable compromise. These variants combine high or acceptable cell viability with effective Zn incorporation, preserving the biological compatibility of the CaP matrix while introducing the possibility of antibacterial and bioactive functionality.

This balance between biological safety and functional enhancement is central to the contribution of the present work. By demonstrating controlled Zn incorporation, dose-dependent cellular responses, and compliance with international cytotoxicity standards, the study establishes a solid foundation for subsequent in vitro bioactivity and antibacterial evaluations, where controlled Zn^{2+} release is expected to promote apatite formation while limiting bacterial colonization at the implant–tissue interface [107].

As is well known, cellular response in direct-contact cytotoxicity tests is governed by a combination of factors, including not only Zn^{2+} release but also surface-related properties such as roughness, surface chemistry/charge, wettability, and stiffness.

The acid-etched Ti6Al4V substrates exhibit increased micro-scale roughness and a hydroxylated TiO_2 surface, which are known to enhance protein adsorption and initial cell attachment. After biomimetic deposition, the CaP coatings present a moderately rough, porous morphology and a hydrophilic character, both of which favor cell adhesion and spreading. At the same time, the incorporation of Zn^{2+} modifies surface chemistry and can influence local surface charge and ion exchange at the interface. Therefore, the observed cellular behavior is interpreted as the result of a coupled effect: favorable topographical and physicochemical features that promote adhesion, together with Zn^{2+} release that may modulate cell metabolism in a dose-dependent manner.

3.6. Antibacterial Activity

The antibacterial behavior of Ti6Al4V substrates coated with calcium phosphate (CaP) layers, with and without zinc incorporation, was evaluated using a standard plate counting method. The Gram-positive bacterium *Streptococcus mutans* was selected as the model microorganism due to its well-established role in oral biofilm formation and its clinical relevance in dental and implant-associated infections. This strain is characterized by strong surface adhesion, acidogenic metabolism, and a high capacity for early-stage colonization on biomaterials, making it particularly suitable for assessing the antibacterial performance of functionalized coatings [108].

After 24 h of incubation on nutrient agar, the number of bacterial colonies formed on each Petri dish was carefully quantified. Both original plates and their replicas were analyzed to ensure reproducibility and statistical reliability. Representative images of bacterial growth at the highest dilution are shown in Figure 24.

In addition, images corresponding to prolonged incubation (7 days) for VDZn2* and VDZn3* are included to qualitatively evaluate the persistence of the antibacterial effect over time.

The average colony number for each treatment (VDZn0, VDZn1, VDZn2, and VDZn3) was used to calculate colony-forming units (CFU) according to Equation (2), and the results are summarized in Table 9. A clear and progressive decrease in CFU values was observed as the zinc content in the coating increased, from 76×10^7 CFU for the Zn-free CaP control (VDZn0) to 40×10^7 CFU for the highest Zn-containing variant (VDZn3). When expressed as a percentage reduction relative to the control surface (Figure 25), the data reveal a well-defined concentration-dependent antibacterial response.

Table 9. Colony-forming units by treatment variant.

Deposition Variants	CFU $\pm$ SD
VDZn0	$(76 \pm 2.5) \times 10^7$
VDZn1	$(68 \pm 2.4) \times 10^7$
VDZn2	$(52 \pm 2.1) \times 10^7$
VDZn3	$(40 \pm 1.8) \times 10^7$

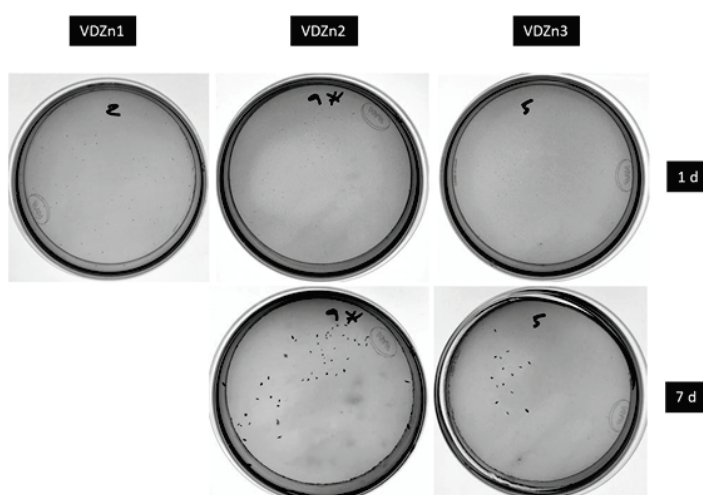


Figure 24. Bacterial growth of *S. mutans* colonies in contact with treatment variants VDZn1, VDZn2, and VDZn3 after one day of inoculation on agar. VDZn2* and VDZn3* observed after 7 days of incubation.

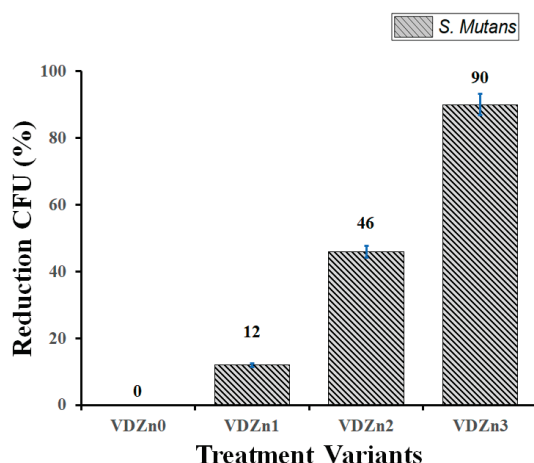


Figure 25. Percentage reduction of *S. mutans* colonies 24 h after being plated on nutrient agar. VDZn0 was used as the control surface.

All antibacterial experiments were performed in triplicate ($n = 3$) using independent samples, and the results are expressed as means $\pm$ standard deviation (SD). Colony-forming units (CFU) were quantified after incubation, and statistical analysis was carried out using one-way analysis of variance (ANOVA) to evaluate the effect of Zn incorporation on bacterial viability. Prior to ANOVA, the assumptions of normality and homogeneity of variances were verified using the Shapiro–Wilk and Levene tests, respectively. Differences were considered statistically significant at $p < 0.05$. The ANOVA results indicated statistically significant differences in CFU values among the different coating variants ($p < 0.05$). Post hoc multiple comparisons were performed using Tukey’s test to identify specific differences between groups. The Zn-free CaP coating (VDZn0) exhibited significantly higher CFU values compared to all Zn-containing variants ($p < 0.01$), confirming the antibacterial effect of Zn incorporation. Furthermore, a progressive and statistically significant reduction in CFU was observed with increasing Zn content from VDZn1 to VDZn3 ($p < 0.05$), demonstrating a clear dose-dependent antibacterial response. Significant differences were also identified between intermediate and high Zn concentrations (VDZn2 vs. VDZn3, $p < 0.05$), indicating that higher Zn levels further enhance bacterial inhibition. The relatively low standard deviation values ($<5\%$) confirm good experimental reproducibility and reliability of the microbiological assay, supporting the robustness of the observed antibacterial trends.

Zn-containing coatings therefore exhibited a clear, dose-dependent antibacterial effect against *S. mutans*. The systematic reduction in viable colonies confirms that zinc incorporation effectively imparts antibacterial functionality to the CaP matrix. This behavior is widely attributed to the controlled release of Zn^{2+} ions, which can disrupt bacterial membrane integrity, interact with thiol groups in essential metabolic enzymes, induce intracellular oxidative stress, and inhibit biofilm formation and maturation [109].

In the specific case of *S. mutans*, Zn^{2+} has been reported to interfere with glycolytic pathways and acid production, both of which are critical for its cariogenic activity and adhesion capacity. Zinc can also impair extracellular polysaccharide synthesis, reducing biofilm stability and limiting bacterial colonization on implant surfaces. These mechanisms are particularly relevant in dental and orthopedic applications, where early bacterial adhesion is a decisive factor in peri-implant infection.

At the same time, an inverse relationship between antibacterial activity and cell viability was observed as the zinc content increased. While higher Zn incorporation enhanced bacterial inhibition, it also led to a moderate decrease in fibroblast viability, although all values remained above the 70% threshold commonly accepted for non-cytotoxic materials. This trade-off between antimicrobial efficacy and cytocompatibility is consistent with previ-

ous studies on metal-ion-functionalized CaP systems, where an optimal zinc concentration range provides antibacterial benefits while preserving acceptable host cell responses [110].

From a mechanistic perspective, Zn^{2+} release kinetics play a central role in balancing these biological effects. Not only the total zinc content, but also its distribution within the apatite lattice, chemical state, and degree of substitution for Ca^{2+} influence the effective concentration of Zn^{2+} at the interface. Structural incorporation within the apatite network tends to favor a more sustained and controlled release profile, whereas loosely bound zinc may lead to a faster initial release, enhancing antibacterial performance but increasing the risk of cytotoxic effects. Therefore, precise control over zinc incorporation is essential for optimizing multifunctional coatings [111].

Among the evaluated variants, VDZn3 exhibited the strongest antibacterial effect, whereas VDZn1 provided the highest level of cytocompatibility with moderate antibacterial activity. In this context, VDZn2 appears to represent the most balanced configuration, combining a substantial reduction in CFU with acceptable cell viability.

These findings support the design strategy of multifunctional CaP-based implant coatings in which controlled zinc incorporation enables simultaneous osteoconductive and anti-infective properties. The results also provide a solid framework for correlating ion release behavior, biomimetic apatite formation, and antibacterial performance, contributing to the rational development of implant surfaces that promote tissue integration while minimizing infection risk.

3.7. Zn Ion Release into the Surrounding Medium

3.7.1. Physicochemical Basis of Ion Release in CaP Coatings

Calcium phosphate (CaP) coatings obtained by biomimetic routes are typically characterized by a partially crystalline and often carbonated apatite structure, closely resembling the mineral phase of bone. This structural similarity is not only compositional but also functional, as it confers high surface energy and a strong tendency to interact with ionic species present in physiological environments [112]. In aqueous media, these coatings do not behave as inert layers; instead, they participate in a dynamic dissolution–reprecipitation process. The initial release of calcium and phosphate ions increases local supersaturation, which in turn promotes the nucleation of a secondary apatite layer with improved structural organization. This sequence is widely regarded as a crucial indicator of *in vitro* bioactivity and is often correlated with the ability of a material to bond to living bone.

When Zn^{2+} is introduced into the CaP system, its incorporation is not restricted to a single structural position. Depending on synthesis conditions and local chemistry, zinc may substitute for Ca^{2+} within the apatite lattice, be trapped within amorphous calcium phosphate domains, or remain weakly bound at the surface through electrostatic interactions with phosphate and hydroxyl groups. The smaller ionic radius of Zn^{2+} compared with Ca^{2+} generates local lattice distortions, which tend to reduce crystallinity and increase defect density. From a thermodynamic standpoint, this destabilization raises the free energy of the solid phase, making it more prone to dissolution under physiological conditions [113].

This effect is particularly relevant in biomimetic coatings, which already exhibit a relatively low degree of crystallinity. In such systems, even small compositional changes can significantly alter dissolution kinetics. As a result, Zn-containing CaP coatings often display enhanced ion release compared with their undoped counterparts. Importantly, this increased solubility is not necessarily detrimental; rather, it can be advantageous when it enables a controlled and sustained release of biologically active ions [114]. In the present case, Zn^{2+} release becomes a decisive parameter linking the structural features of the coating with its biological performance.

3.7.2. Zinc Release Kinetics and Coupling with Biomimetic Bioactivity

The release of Zn^{2+} into a physiological medium (PBS) revealed a consistent trend across all compositions, characterized by an early-stage release followed by a progressive reduction in ion flux. This type of profile is commonly reported for ion-doped calcium phosphates and reflects the heterogeneous distribution of dopant species within the coating [115].

The Zn^{2+} release profiles for the VDZn1, VDZn2, and VDZn3 are shown in Figure 26. A notable observation is that the sample with the lowest nominal zinc content (VDZn1) exhibited the highest Zn^{2+} concentration at 24 h.

This apparent inconsistency suggests that zinc is not uniformly incorporated into the apatite structure. Instead, a significant fraction of Zn in VDZn1 is likely present in surface-accessible or weakly bound states, which are rapidly released upon immersion [116]. In contrast, samples with higher zinc content (VDZn2 and VDZn3) may contain a larger proportion of structurally incorporated Zn, resulting in a slower and more diffusion-controlled release.

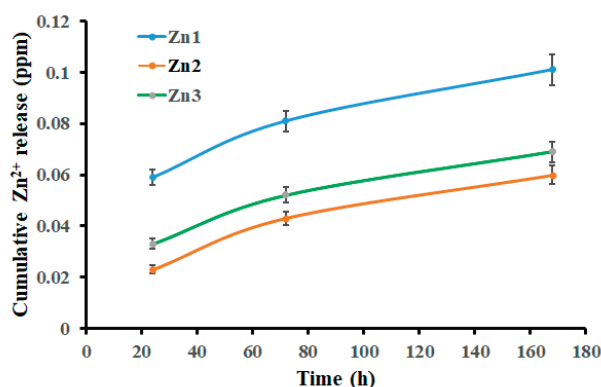


Figure 26. Zn^{2+} release profile in 50 mL phosphate-buffered saline (PBS).

After 72 h, the convergence of Zn^{2+} concentrations across all samples indicates that the contribution of easily exchangeable species becomes negligible, and the release mechanism transitions toward one governed by matrix dissolution. The stabilization observed after 7 days further supports this interpretation, suggesting the establishment of a quasi-equilibrium between dissolution and reprecipitation processes at the coating–solution interface. Table 10 shows the cumulative Zn^{2+} release ($\text{mg}\cdot\text{L}^{-1}$) in PBS at different incubation times.

Table 10. Cumulative Zn^{2+} release ($\text{mg}\cdot\text{L}^{-1}$) in PBS at different incubation times.

Time (h)	VDZn1 ($\text{mg}\cdot\text{L}^{-1}$) $\pm$ SD	VDZn2 ($\text{mg}\cdot\text{L}^{-1}$) $\pm$ SD	VDZn3 ($\text{mg}\cdot\text{L}^{-1}$) $\pm$ SD
24	0.059 ± 0.003	0.023 ± 0.0015	0.033 ± 0.0020
72	0.081 ± 0.004	0.043 ± 0.0025	0.052 ± 0.0030
168	0.101 ± 0.006	0.060 ± 0.0035	0.069 ± 0.0040

Statistical analysis was performed using one-way analysis of variance (ANOVA) to evaluate the effect of Zn concentration and incubation time on Zn^{2+} release. Prior to ANOVA, data normality and homogeneity of variances were verified using the Shapiro–Wilk and Levene tests, respectively. All experiments were conducted in triplicate ($n = 3$), and results are presented as means $\pm$ standard deviations. The ANOVA results revealed statistically significant differences ($p < 0.05$) in Zn^{2+} release among the different coating variants (VDZn1, VDZn2, and VDZn3) at each incubation time. Post hoc multiple comparisons were performed using Tukey’s test to identify specific differences between groups.

At 24 h, VDZn1 exhibited significantly higher Zn^{2+} release compared to VDZn2 ($p < 0.01$) and VDZn3 ($p < 0.05$), confirming the presence of a higher fraction of surface-accessible Zn species. At 72 h and 168 h, the differences between VDZn2 and VDZn3 were reduced and, in some cases, not statistically significant ($p > 0.05$), indicating convergence toward a diffusion-controlled release regime. Additionally, a two-way ANOVA considering both Zn concentration and incubation time as independent factors confirmed that both variables, as well as their interaction, had a statistically significant effect on Zn^{2+} release ($p < 0.05$). This result supports the interpretation that ion release is governed by a coupled mechanism involving both compositional and kinetic factors. These statistical findings reinforce the reliability of the observed trends and confirm that the differences in Zn^{2+} release are not due to random variability but are intrinsically linked to the physicochemical characteristics of the coatings.

The cumulative release values reinforce this interpretation, showing that total Zn^{2+} release is not directly proportional to the initial zinc content. This behavior highlights the importance of considering not only composition but also the physicochemical state of the dopant. From a design perspective, it suggests that optimizing Zn functionality requires precise control over its spatial distribution within the coating rather than simply increasing its concentration [117].

The release behavior can be described by a two-stage mechanism: (i) a rapid initial release dominated by desorption and dissolution of loosely bound Zn species, and (ii) a slower stage controlled by diffusion through the CaP matrix and its gradual degradation. This dual mechanism is particularly advantageous for biomedical applications, as it provides an early antibacterial effect followed by sustained ion delivery [118].

In this context, recent advances in surface engineering further support the relevance of coupling controlled degradation with functional ion release as a crucial design principle for next-generation implant coatings. Studies on plasma electrolytic oxidation (PEO)-based and composite coatings, particularly in magnesium systems, have demonstrated that the integration of gradient architectures and tailored microstructures enables a fine regulation of degradation kinetics and therapeutic ion delivery. These systems highlight that the biological performance of a coating is not solely dictated by its composition but also by the dynamic interplay between dissolution behavior, ion release rates, and interfacial reactions with the surrounding environment. Although such approaches have been primarily explored in biodegradable substrates, the same principles are directly applicable to biomimetic CaP coatings. In the present study, the observed two-stage Zn^{2+} release mechanism and the establishment of a quasi-equilibrium state are consistent with these concepts, where controlled degradation governs both antibacterial activity and bioactive response. Therefore, the results reinforce the idea that optimizing coating performance requires precise control over both structural features and ion release kinetics, rather than simply increasing dopant concentration [35,36].

3.7.3. Biological Implications of Zn^{2+} Release

The biological response associated with Zn^{2+} release reflects a balance between its beneficial and potentially adverse effects. On one hand, zinc is well known for its antimicrobial activity. Its action is not limited to a single pathway; rather, it involves membrane destabilization, interference with enzyme activity through binding to thiol groups, and the generation of oxidative stress within bacterial cells. These combined effects impair bacterial viability and reduce their ability to form stable biofilms.

In the case of *Streptococcus mutans*, a crucial microorganism in oral biofilm formation, Zn^{2+} can disrupt carbohydrate metabolism and reduce acid production, which in turn affects adhesion and colonization. This mechanism is particularly relevant for implant-

related infections, where early bacterial attachment plays a critical role in the establishment of biofilms [119].

The experimental results obtained here are consistent with these mechanisms. The reduction in colony-forming units, especially for the VDZn3 condition, indicates that the local concentration of Zn^{2+} reached levels sufficient to inhibit bacterial growth. At the same time, the absence of excessively high release suggests that cytotoxic thresholds were not exceeded.

Nevertheless, the observed decrease in cell viability with increasing zinc content points to a narrow therapeutic window [120]. While moderate Zn^{2+} levels can stimulate osteogenic activity and improve antibacterial performance, excessive concentrations may impair cell proliferation. This highlights the need for careful optimization of Zn incorporation, ensuring that release rates remain within a range that supports tissue integration while preventing bacterial colonization.

These observations are consistent with recent advances in multifunctional coating design, where the synergy between material degradation and biological response has been identified as a critical factor governing implant performance. In particular, studies on plasma electrolytic oxidation (PEO)-based coatings in magnesium systems have demonstrated that corrosion-driven ion release can be strategically tuned to simultaneously promote antibacterial activity and tissue regeneration. Although the present system is not based on biodegradable metals, a clear parallel can be established: in Zn–CaP coatings, dissolution-mediated Zn^{2+} release plays an analogous role to corrosion processes in magnesium alloys. In both cases, the biological outcome is dictated by the kinetics of ion release and the resulting local microenvironment at the interface. This comparison highlights that achieving an optimal balance between antimicrobial efficacy and cytocompatibility requires precise control over degradation behavior and ion delivery rates. Therefore, the results of this study further support the concept that the design of bioactive coatings should be approached from a dynamic perspective, where controlled material transformation and ion release are intentionally engineered to modulate biological responses [35,36].

3.8. Surface Roughness Evolution and Its Implications

Surface roughness plays a critical role in determining the biological performance of titanium-based implants, as it directly influences protein adsorption, cell attachment, and subsequent tissue integration. In the present study, roughness measurements were carried out using a contact profilometer to quantify the topographical changes induced by sequential surface treatments and subsequent Zn–CaP coating deposition.

3.8.1. Surface Roughness After Pre-Treatments

The evolution of surface roughness closely reflects the morphological and compositional transformations induced by each modification step, revealing a progressive transition from a relatively smooth and anisotropic surface toward a more complex and functionally active interface. Table 11 summarizes the evolution of surface roughness parameters (R_a , R_z , and R_q) following each surface pre-treatment step.

The as-machined Ti6Al4V substrate exhibited the lowest roughness values ($R_a \approx 0.163 \mu\text{m}$), consistent with its characteristic anisotropic topography defined by machining grooves. Such a surface provides limited effective area and a low density of active sites, which restricts its interaction with the physiological environment and reduces its ability to promote biological anchorage.

Following acid etching, a marked increase in roughness was observed ($R_a \approx 0.490 \mu\text{m}$), associated with the formation of a homogeneous network of micropits and valleys. This treatment effectively removes machining marks while introducing a microstructured sur-

face with enhanced peak-to-valley amplitude. From a physicochemical standpoint, this modification is particularly relevant, as it increases the available surface area and promotes heterogeneous nucleation by creating energetically favorable sites for subsequent reactions.

Table 11. Surface roughness parameters (Ra, Rz, and Rq) of Ti6Al4V substrates after sequential surface pre-treatments. Values are reported as means $\pm$ standard deviations ($n = 3$). Ra: arithmetic mean roughness; Rz: average maximum height of the profile; Rq: root mean square roughness.

Treatment	Ra (μm) $\pm$ SD	Rz (μm) $\pm$ SD	Rq (μm) $\pm$ SD
As-machined	0.163 $\pm$ 0.034	1.313 $\pm$ 0.273	0.306 $\pm$ 0.275
Acid-etched	0.490 $\pm$ 0.023	3.812 $\pm$ 0.283	0.635 $\pm$ 0.026
NaOH-treated	0.365 $\pm$ 0.047	2.566 $\pm$ 0.509	0.471 $\pm$ 0.073
CaCl ₂ -treated	0.477 $\pm$ 0.100	3.564 $\pm$ 0.741	0.615 $\pm$ 0.110
Heat-treated (TT)	0.551 $\pm$ 0.095	3.966 $\pm$ 0.761	0.718 $\pm$ 0.128

In contrast, the alkaline treatment in NaOH resulted in a moderate decrease in micrometric roughness ($R_a \approx 0.365 \mu\text{m}$), despite generating a highly porous nanostructure. This apparent discrepancy suggests that the formation of a sodium titanate gel layer leads to partial smoothing at the micrometric scale while simultaneously increasing surface complexity at the nanoscale. This highlights an important limitation of conventional roughness parameters (R_a , R_z), which may not fully capture multiscale structural features. Rather than simply increasing roughness, the alkaline treatment primarily induces chemical activation, enriching the surface with reactive groups that play a key role in subsequent coating formation.

The incorporation of calcium through CaCl₂ treatment led to a renewed increase in roughness ($R_a \approx 0.477 \mu\text{m}$), accompanied by higher variability in the measured values. This behavior is consistent with the formation of localized calcium-rich precipitates, introducing surface heterogeneity and creating preferential nucleation sites for calcium phosphate deposition. These features indicate a transition toward a chemically and topographically activated surface.

The highest roughness values were obtained after thermal treatment ($R_a = 0.551 \pm 0.095 \mu\text{m}$; $R_z = 3.966 \pm 0.761 \mu\text{m}$), which can be attributed to the consolidation and stabilization of the previously formed gel-like layer. Thermal dehydration and structural contraction likely induce microcrack formation, further increasing surface complexity. These features not only enhance mechanical interlocking but also improve interfacial reactivity by exposing additional active sites.

Although the hydrothermal treatment (TH₂O) was not evaluated in terms of roughness, its primary contribution is expected to be chemical rather than topographical. This step promotes ion exchange and the formation of surface hydroxyl groups (Ti–OH), which are known to enhance bioactivity and facilitate apatite nucleation without significantly altering micrometric roughness.

The roughness evolution does not follow a monotonic trend but rather reflects the interplay between dissolution, reorganization, and surface growth processes. More importantly, these results demonstrate that surface activation is not governed solely by roughness magnitude, but by the combined effect of topography and surface chemistry. The final treated surface, characterized by moderate-to-high roughness, chemical functionality, and hierarchical porosity, provides a suitable platform for biomimetic coating deposition.

A statistical analysis was performed to evaluate the significance of the differences observed in surface roughness parameters (R_a , R_z , and R_q) among the different surface treatments. One-way analysis of variance (ANOVA) was applied independently to each roughness parameter (R_a , R_z , and R_q), considering the treatment condition as the main

factor ($n = 3$ per group). The results revealed that surface treatment had a statistically significant effect on all roughness parameters ($p < 0.01$ for Ra, Rz, and Rq), indicating that the modifications induced by each sequential treatment step are not due to random variation but to systematic changes in surface morphology.

Post hoc comparisons using the Tukey HSD test further showed that the acid-etched and heat-treated (TT) conditions exhibited significantly higher roughness values compared to the as-machined surface ($p < 0.05$) for all parameters. In particular, Ra increased from $0.163 \pm 0.034 \mu\text{m}$ (as-machined) to $0.490 \pm 0.023 \mu\text{m}$ (acid-etched) and $0.551 \pm 0.095 \mu\text{m}$ (TT), confirming a substantial modification of the surface profile. Similarly, Rz and Rq followed the same trend, with statistically significant increases after chemical and thermal treatments.

No statistically significant differences ($p > 0.05$) were observed between some intermediate treatments (NaOH-treated and CaCl_2 -treated), suggesting that these steps contribute to surface modification in a more gradual manner. However, the cumulative effect of the sequential treatment, particularly after the final heat treatment, resulted in the highest roughness values, which are statistically distinguishable from the initial condition. These statistical results confirm that the applied surface treatments produce significant and reproducible modifications in surface roughness, supporting their role in enhancing surface reactivity and providing favorable conditions for subsequent biomimetic CaP deposition.

3.8.2. Surface Roughness of Zn–CaP Coatings

Table 12 presents the surface roughness parameters of the Zn–CaP coatings as a function of Zn incorporation. The roughness of the Zn–CaP coatings reveals a clear relationship between surface topography, coating morphology, and chemical composition, highlighting the role of Zn as a regulator of biomimetic growth.

Table 12. Surface roughness parameters (Ra, Rz, and Rq) of biomimetic Zn–CaP coatings deposited on Ti6Al4V substrates at different Zn concentrations. Values are expressed as means $\pm$ standard deviations ($n = 3$). Ra: arithmetic mean roughness; Rz: average maximum height of the profile; Rq: root mean square roughness.

Coating Variant	Ra (μm) $\pm$ SD	Rz (μm) $\pm$ SD	Rq (μm) $\pm$ SD
VDZn0	0.75 ± 0.07	5.39 ± 0.64	0.98 ± 0.09
VDZn1	0.50 ± 0.18	3.83 ± 0.51	0.69 ± 0.11
VDZn2	0.39 ± 0.04	2.81 ± 0.24	0.49 ± 0.04
VDZn3	0.72 ± 0.08	4.87 ± 0.30	0.95 ± 0.04

The Zn-free coating (VDZn0) exhibited the highest roughness values ($\text{Ra} \approx 0.75 \mu\text{m}$), which correlates well with the formation of large, flower-like apatite structures observed by FESEM. This morphology is typically associated with high supersaturation conditions, where rapid and uncontrolled crystal growth leads to heterogeneous aggregation and increased surface irregularity.

The introduction of Zn at low concentration (VDZn1) resulted in a significant reduction in roughness ($\text{Ra} \approx 0.50 \mu\text{m}$), accompanied by a refinement of the microstructure. Although the flower-like morphology was preserved, the particle size decreased and the distribution became more uniform. The presence of Zn, confirmed by EDS and XPS, suggests that Zn^{2+} ions begin to modify the surface energy of growing nuclei, acting as a kinetic regulator that limits excessive crystal growth without suppressing CaP formation.

This effect becomes more pronounced at intermediate Zn concentration (VDZn2), where the lowest roughness values were recorded ($\text{Ra} = 0.39 \pm 0.04 \mu\text{m}$; $\text{Rz} = 2.81 \pm 0.24 \mu\text{m}$). The corresponding microstructure is characterized by smaller, more fragmented

aggregates and improved surface uniformity. Compositional analysis indicates higher Ca and P contents along with increased Zn incorporation, suggesting a condition in which nucleation and growth are balanced. The resulting coating is more compact and homogeneous, reflecting a more controlled crystallization process.

At higher Zn concentration (VDZn3), roughness increases again ($R_a \approx 0.72 \mu\text{m}$), approaching the values observed for the Zn-free system. This behavior is consistent with a reduction in coating coverage and increased heterogeneity, as observed in SEM images. Although Zn content is higher, excessive Zn^{2+} appears to inhibit both nucleation and ordered growth of the CaP phase, leading to less compact and more irregular structures.

Taken together, these results demonstrate a non-linear relationship between Zn concentration and surface roughness, reflecting a transition in the coating formation mechanism. In the absence of Zn, growth is dominated by supersaturation-driven crystallization, resulting in coarse and heterogeneous structures. At low and intermediate concentrations, Zn acts as a growth modulator, promoting finer and more uniform coatings. At higher concentrations, however, Zn exerts an inhibitory effect, disrupting the crystallization process and leading to increased roughness.

From a functional perspective, this behavior is highly relevant. Surface roughness influences not only mechanical interlocking but also protein adsorption, cell attachment, and bacterial adhesion. In this context, the VDZn2 condition appears to provide an optimal balance between structural homogeneity, controlled roughness, and Zn incorporation. This combination is expected to favor bioactivity while maintaining antibacterial functionality, making it particularly suitable for implant surface applications.

A statistical analysis was conducted to assess the effect of Zn incorporation on the surface roughness parameters (R_a , R_z , and R_q) of the Zn–CaP coatings. One-way analysis of variance (ANOVA) was performed independently for each parameter, considering the coating variant (VDZn0, VDZn1, VDZn2, and VDZn3) as the main factor ($n = 3$ per group). The results demonstrated that Zn content has a statistically significant influence on all roughness parameters ($p < 0.01$ for R_a , R_z , and R_q), confirming that the observed variations are not due to experimental scatter but to systematic changes induced by Zn incorporation. Post hoc analysis using the Tukey HSD test revealed that the VDZn2 condition exhibited significantly lower roughness values compared to VDZn0 and VDZn3 ($p < 0.05$) for all parameters, indicating a more uniform and refined surface morphology at intermediate Zn levels. In particular, R_a decreased from $0.75 \pm 0.07 \mu\text{m}$ (VDZn0) to $0.39 \pm 0.04 \mu\text{m}$ (VDZn2), while R_z and R_q followed the same trend, supporting the interpretation of controlled crystal growth and improved coating homogeneity. In contrast, VDZn3 showed a statistically significant increase in roughness compared to VDZn2 ($p < 0.05$), with values approaching those of the Zn-free coating (VDZn0). This result suggests that excessive Zn incorporation disrupts the regular growth of the CaP phase, leading to increased surface heterogeneity and morphological irregularities. No statistically significant differences ($p > 0.05$) were observed between VDZn0 and VDZn3 for some parameters, indicating that high Zn content may partially revert the surface characteristics toward those of the undoped system. The statistical analysis confirms a non-linear relationship between Zn concentration and surface roughness, with VDZn2 representing the optimal condition in terms of minimizing roughness and achieving a more homogeneous surface. These findings reinforce the role of Zn as a key regulator of biomimetic growth and highlight the importance of controlling its concentration to tailor surface topography and functional performance.

3.8.3. Structure–Property Relationship Connected with Surface Roughness

The surface roughness results exhibit a clear non-linear dependence on Zn incorporation, which can be consistently correlated with the microstructural evolution, chemical

composition, ion release behavior, and biological performance of the coatings. In the absence of Zn (VDZn0), the high roughness values ($R_a = 0.75 \pm 0.07 \mu\text{m}$) are associated with the formation of large, flower-like apatite aggregates, as observed by FESEM, resulting from an uncontrolled growth regime under high supersaturation. EDS and XPS analyses confirm a typical CaP composition with relatively higher crystallinity and lower defect density, which limits ion release and, consequently, provides no intrinsic antibacterial functionality despite potentially favorable conditions for initial protein adsorption.

The incorporation of Zn at low concentration (VDZn1) leads to a significant reduction in roughness ($R_a = 0.50 \pm 0.18 \mu\text{m}$), accompanied by a refinement of the microstructure, with smaller and more uniformly distributed particles. XPS results indicate that Zn^{2+} exists both as structurally incorporated species and as surface-accessible ions, which explains the relatively high initial release observed at early immersion times. This dual chemical state promotes an initial antibacterial effect while preserving a surface topography that supports cell attachment and spreading.

At intermediate Zn concentration (VDZn2), the lowest roughness values are obtained ($R_a = 0.39 \pm 0.04 \mu\text{m}$), reflecting the formation of a compact and homogeneous coating with improved surface coverage and reduced particle size, as confirmed by FESEM. EDS and XPS analyses further indicate a more effective incorporation of Zn within the CaP lattice, resulting in a more controlled and sustained Zn^{2+} release profile. This condition establishes an optimal balance between surface topography, chemical stability, and ion release kinetics, which is directly reflected in the biological response. Specifically, the combination of moderate roughness and homogeneous morphology promotes stable cell-surface interactions and cytocompatibility, while the controlled release of Zn^{2+} provides sufficient antibacterial activity without exceeding cytotoxic thresholds.

In contrast, further increasing the Zn content (VDZn3) leads to a renewed increase in roughness ($R_a = 0.72 \pm 0.08 \mu\text{m}$), indicating a transition toward a less controlled growth mechanism. FESEM observations reveal reduced coating density and increased heterogeneity, while XPS confirms a higher fraction of surface-accessible Zn. This condition enhances antibacterial performance due to increased Zn^{2+} availability but also results in localized ion accumulation and surface irregularities that may negatively affect cell viability, highlighting the onset of a cytotoxic threshold.

These findings demonstrate that Zn acts not merely as a dopant but as a key regulator of nucleation kinetics, crystal growth, and defect formation, thereby governing surface roughness, Zn distribution, and ion release behavior. Importantly, the biological response arises from the synergistic interplay between topography, chemical composition, and release kinetics rather than from any single parameter. Within this framework, the VDZn2 condition represents an optimal “therapeutic window”, where microstructural uniformity, controlled Zn incorporation, and balanced antibacterial and cytocompatible responses are simultaneously achieved. This highlights the importance of integrated surface design strategies for the development of multifunctional implant coatings.

3.9. Current Applicability and Future Development Needs of the Proposed Coatings

The Zn-doped calcium phosphate (Zn–CaP) coatings developed in this study exhibit promising characteristics for biomedical applications, including homogeneous coverage, controlled Zn^{2+} release, antibacterial activity, and acceptable cytocompatibility. From a materials science perspective, these features indicate that the coatings are suitable candidates for enhancing the biological performance of Ti6Al4V implants, particularly in promoting early-stage osseointegration while reducing the risk of bacterial colonization. However, at the current stage, the coatings cannot yet be considered ready for direct clinical use on medical implants. While the *in vitro* results are encouraging, several critical aspects must

be further investigated and optimized before clinical translation: (i) mechanical reliability and adhesion under physiological loading: although the coatings exhibit good interfacial integrity, comprehensive quantitative adhesion testing (e.g., standardized pull-off or fatigue loading tests) is required. In load-bearing dental applications, coatings must withstand cyclic mechanical stresses without delamination or degradation; (ii) long-term stability and degradation behavior: the dissolution–reprecipitation behavior of biomimetic CaP coatings is beneficial for bioactivity, but long-term studies are necessary to evaluate coating stability, ion release kinetics over extended periods, and the evolution of the coating under physiological conditions; (iii) in vivo biological performance: the current study is limited to in vitro evaluations. Animal studies are required to confirm osseointegration, bone–implant interfacial strength, inflammatory response, and antibacterial efficacy in a complex biological environment; (iv) optimization of Zn concentration: the results indicate a balance between antibacterial activity and cytocompatibility. Excessive Zn incorporation may negatively affect cell viability, highlighting the need to define an optimal therapeutic window that maximizes antimicrobial performance without compromising tissue integration; (v) surface functional uniformity and scalability: for clinical applications, the coating process must be reproducible on complex implant geometries and scalable for industrial manufacturing while maintaining uniform thickness, composition, and performance; (vi) regulatory and sterilization considerations: the effect of sterilization methods (e.g., autoclaving, gamma irradiation) on coating structure, adhesion, and ion release must be assessed. Additionally, compliance with regulatory standards for medical devices requires extensive biocompatibility and safety validation.

In summary, while the Zn–CaP coatings demonstrate strong potential as multifunctional surfaces for implant applications, further mechanical, biological, and translational studies are necessary to ensure their safety, reliability, and clinical effectiveness.

4. Conclusions

This study demonstrates the successful fabrication of biomimetic Zn-doped calcium phosphate (Zn–CaP) coatings on surface-activated Ti6Al4V substrates through a low-temperature route under physiologically relevant conditions. A sequential chemical–thermal activation strategy (NaOH–CaCl₂–TT–H₂O) effectively transformed the initially bioinert alloy into a chemically reactive, calcium-enriched titanate surface. This hydroxylated TiO₂-based interfacial layer played a decisive role in promoting heterogeneous nucleation and strong interfacial bonding, confirming that surface chemistry (rather than roughness alone) governs the efficiency, uniformity, and adhesion of CaP coatings.

A systematic evaluation of deposition parameters identified a well-defined processing window for structurally reliable coatings. Insufficient ionic supersaturation or short immersion times resulted in discontinuous layers, whereas excessive deposition led to crack formation due to residual stresses. Among the studied conditions, the SBFX10–4 h treatment (VD3) provided the optimal balance, yielding homogeneous, crack-free coatings with high surface coverage. These findings highlight the critical role of controlling supersaturation and deposition kinetics in achieving mechanically stable and functionally reliable biomimetic coatings.

Zinc incorporation was successfully achieved and confirmed by EDS and XPS, demonstrating that Zn is present as Zn²⁺ chemically associated with the CaP phase. The results reveal that Zn incorporation occurs through a combination of structural substitution and surface-associated species, leading to a non-linear relationship between nominal Zn content and ion release behavior. This incorporation significantly modified the precipitation mechanisms and phase composition, as reflected by Ca/P ratios ranging from 1.44 to 1.80. Low and intermediate Zn levels promoted calcium-deficient apatite formation, whereas

higher Zn concentrations shifted the system toward Ca-rich compositions, indicating altered crystal growth dynamics. Among the evaluated variants, VDZn2 ($\approx 0.5\text{--}0.7$ at% Zn) provided the most favorable compositional and structural balance, maintaining a Ca/P ratio closer to apatite stoichiometry while preserving coating homogeneity.

The Zn–CaP coatings exhibited a characteristic two-stage Zn^{2+} release mechanism, consisting of an initial burst release (up to $0.059\text{ mg}\cdot\text{L}^{-1}$ at 24 h) followed by a sustained release reaching approximately $0.060\text{--}0.069\text{ mg}\cdot\text{L}^{-1}$ after 168 h. This behavior demonstrates that biological performance is governed not only by total Zn content but also by its physicochemical state and distribution within the coating. The initial release is sufficient to induce antibacterial activity, while the subsequent controlled release supports bioactivity without exceeding cytotoxic thresholds.

From a functional standpoint, the coatings exhibited a clear, concentration-dependent antibacterial effect against *Streptococcus mutans*, with a reduction in bacterial colonies of up to $\sim 47\%$ compared with Zn-free CaP. At the same time, all coatings maintained cell viability above the 70% threshold defined by ISO 10993-5:2009, Annex C (*Biological Evaluation of Medical Devices—Part 5: Tests for In Vitro Cytotoxicity*; International Organization for Standardization (ISO): Geneva, Switzerland, 2009), confirming their cytocompatibility. However, increasing Zn content resulted in a moderate decrease in cell viability, highlighting the existence of a narrow therapeutic window. In this context, intermediate Zn incorporation (VDZn2) represents the most balanced condition, combining effective antibacterial activity with acceptable cytocompatibility.

Compared with conventional Zn-doped CaP or hydroxyapatite coatings produced by high-temperature or electrochemical techniques, the present biomimetic approach offers several advantages, including low processing temperature, closer structural similarity to biological apatite, and improved control over ion incorporation and release kinetics. These features are particularly relevant for the development of multifunctional implant coatings capable of simultaneously promoting osseointegration and reducing infection risk.

Despite these promising outcomes, several limitations should be acknowledged. The biological assessment was restricted to in vitro assays, and long-term stability under dynamic physiological conditions was not fully evaluated. In addition, although an optimal Zn range was identified, further refinement is required to precisely define the therapeutic window. Variability inherent to biomimetic deposition, such as local differences in thickness and microstructure, also requires further investigation.

Future work should focus on long-term in vitro and in vivo validation, quantitative mechanical adhesion and fatigue testing, and optimization of Zn distribution through controlled processing strategies. The incorporation of multiple bioactive ions, as well as the design of gradient or hierarchical coatings, represents a promising direction to further enhance multifunctional performance.

This study provides new insight into the interplay between surface activation, Zn incorporation, ion release kinetics, and biological response in biomimetic CaP coatings. The results demonstrate that controlling not only composition but also the physicochemical state of dopants is essential for tailoring coating performance, offering a rational framework for the design of next-generation multifunctional implant surfaces.

Author Contributions: Conceptualization, Y.M.-S. and M.A.L.H.-R.; methodology, Y.M.-S., M.A.L.H.-R. and M.A.D.L.G.-R.; validation, Y.M.-S., M.A.L.H.-R. and E.A.R.-C.; formal analysis, Y.M.-S., M.A.L.H.-R., Y.G.-C., L.D.-T., E.O.G.-S., A.J.-H., M.A.D.L.G.-R. and E.A.R.-C.; investigation, Y.M.-S., M.A.L.H.-R., Y.G.-C., L.D.-T., E.O.G.-S., A.J.-H., M.A.D.L.G.-R. and E.A.R.-C.; resources, Y.M.-S. and M.A.L.H.-R.; data curation, Y.M.-S., E.A.R.-C. and M.A.L.H.-R.; writing—original draft preparation, Y.M.-S., E.A.R.-C. and M.A.L.H.-R.; writing—review and editing, Y.M.-S., M.A.L.H.-R., Y.G.-C., L.D.-T., E.O.G.-S., A.J.-H., M.A.D.L.G.-R. and E.A.R.-C.; visualization, Y.M.-S., E.A.R.-C. and M.A.L.H.-R.

R.; supervision, Y.M.-S., E.A.R.-C. and M.A.L.H.-R.; project administration, M.A.L.H.-R.; funding acquisition, Y.M.-S., M.A.L.H.-R., Y.G.-C., L.D.-T., E.O.G.-S., A.J.-H., M.A.D.L.G.-R. and E.A.R.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by SECIHTI-Mexico, grant number 729692.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

Acknowledgments: The first author thanks SECIHTI-Mexico for its support in accomplishing her doctorate studies with the scholarship number 729692. Furthermore, to Universidad Autónoma de Nuevo León (UANL) and MAGEOTEC for the supply of materials and analytical equipment. Thanks also to the academic group of the Mechanical Engineering Faculty (FIME) of UANL.

Conflicts of Interest: Yaimi Martin-Santana is an employee of MAGEOTEC. The other authors declare no conflicts of interest. MAGEOTEC had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

SBF	Simulated body fluid
CaP	Calcium phosphate
Zn–CaP	Zn-doped calcium phosphate
HA	Ca ₁₀ (PO ₄) ₆ (OH) ₂
DMEM	Dulbecco's Modified Eagle Medium (DMEM)
VD	Deposition Variants
BHI	Brain Heart Infusion BHI
PBS	Phosphate-buffered saline PBS
CFU	Colony-forming units

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ISBN 978-3-7258-8360-8