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**Innovative Applications of Collagen-Based Biomaterials in the
Dental Field: From *in vitro* and *in vivo* Studies to the Potential Use
as a Coating for Novel Implant Surfaces**

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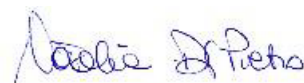
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1. ABSTRACT

Collagen, the most abundant structural protein in the human body, plays a pivotal role in tissue integrity and regeneration, making it an essential biomaterial for various medical applications. This doctoral research explored innovative applications of collagen-based biomaterials in the dental field, focusing on their use for soft and hard tissue regeneration, oral wound healing, as well as enhancement of implant integration and vascularization. The project addressed the critical challenges associated with peri-implant and periodontal tissue repair by leveraging the unique properties of type I collagen, including biocompatibility and bioactivity. The research was conducted through a series of *in vitro*, *ex vivo*, and *in vivo* experiments, systematically investigating the biological responses of collagen-based biomaterials. Key areas of study included (i) the evaluation of a liquid collagen formulation for oral wound healing, (ii) the design of collagen coatings for innovative dental implant surfaces aimed at improving osseointegration, and (iii) the assessment of tissue regeneration and angiogenic properties of a collagenated xenograft.

Overall, the findings demonstrated that collagen-based biomaterials promoted cellular adhesion, proliferation, and differentiation while facilitating vascularization and mechanotransduction. Moreover, a novel implant design, together with a collagen coating of the screw, was developed and tested both on soft and hard tissue cells and an *in vivo* animal model, showcasing enhanced cell-implant interactions, which are critical for maintaining peri-implant tissue health. Subsequently, several *in vitro* assessments and numerical simulations of the biomechanical behavior of the novel collagen-coated implant were conducted. Furthermore, the collagenated xenograft demonstrated significant potential in promoting tissue regeneration and angiogenic properties. The tested biomaterials were shown to support the integration of both soft and hard tissues, emphasizing their dual functionality in preventing biofilm adhesion and ensuring mechanical stability.

Despite these promising outcomes, challenges remain in optimizing the physical and biochemical properties of collagen-based biomaterials to match the hierarchical structure of the natural extracellular matrix. Future directions include exploring a better functionalization of the coating on the implant surfaces, incorporating bioactive molecules to enhance regenerative and antimicrobial capacities, and investigating the potential of the collagen-coated surface in improving tissue integration in further cellular, animal, and human studies.

This research highlighted the regenerative potential of collagen-based biomaterials in the dental field, offering new paradigms for improving the clinical outcomes of implant therapies and regenerative procedures. The findings set the stage for further advancements in biomaterial science and regenerative medicine, aiming to enhance both functional and aesthetic results for patients.

2. INTRODUCTION

2.1. COLLAGEN IN PERIODONTAL AND PERI-IMPLANT TISSUES

Collagen constitutes approximately 30% of the total protein content within the human body, rendering it the most prevalent protein across various biological systems. As the principal structural protein of the extracellular matrix (ECM), collagen plays a vital role in the integrity and functionality of a wide range of connective tissues, including cartilage, bone, blood vessels, skin, and the cornea. Among the 28 known collagen subtypes, types I, II, and III collectively account for approximately 80–90% of the total collagen present in the body, underscoring their significance in numerous structural roles. Although "minor collagens" exist in smaller quantities, they fulfill essential functional roles crucial for the proper functioning of complex tissues, thereby illustrating the diverse functionalities of collagen within the human body [1]. The variations among different collagen types are intricately associated with their unique structural and biological functions at various levels: submolecular, molecular, and supramolecular. These variations enable collagens to provide specialized support that meets the specific mechanical and biochemical requirements of different tissues [2]. Among the various collagen types, type I collagen is particularly noteworthy as it is the most abundant, comprising approximately 70% of the entire collagen family. This collagen subtype is extensively studied and acknowledged for its fundamental role as the primary fibrous component of connective tissues throughout the body. Type I collagen is crucial for maintaining the structural architecture, shape, tensile strength, and elasticity of a diverse array of biological tissues, including skin, bone, ligaments, and tendons [2].

As an essential component of the ECM, collagen significantly influences cellular behavior by regulating vital processes such as cell adhesion, proliferation, differentiation, and wound healing. Its unique hierarchical structure creates an intrinsic scaffold that supports cell attachment and growth, fostering an optimal environment for cellular activities. This abundant protein serves not only as a structural support framework but also actively engages in the biochemical signaling pathways that are critical for cellular function and tissue regeneration [3]. Over the past decade, collagen has evolved from being recognized merely as a fundamental structural protein to a pivotal element in the intricate architecture of multiple tissues within the stomatognathic system [4]. Collagen interacts dynamically with several key oral cell types, including human gingival fibroblasts

(hGFs), osteoblasts, and endothelial cells, thus facilitating essential biological activities such as tissue healing, osseointegration, and adequate vascularization. For instance, the interaction between collagen and fibroblasts is critical for the formation of new fibrous tissue. In addition, collagen's presence within the bone matrix is indispensable for the attachment and functionality of osteoblasts, which are responsible for bone formation. Moreover, endothelial cells, which line blood vessels, depend on collagen for proper adhesion, signaling, and the formation of new blood vessels, a process referred to as angiogenesis [5]. Furthermore, the role of collagen in tissue repair and regeneration is paramount. In response to injury or inflammation, collagen is crucial in mediating the healing process by promoting the recruitment of various cell types necessary for tissue repair. The organization of collagen fibers significantly influences the development of granulation tissue and subsequent remodeling, which are essential for restoring both function and aesthetics in the oral cavity. Therefore, the dynamic interplay among collagen, cells, and the surrounding microenvironment is indispensable for maintaining the overall health and longevity of both natural teeth and dental implants [6].

Recent research has significantly highlighted the essential role of the ECM, particularly collagen, in regulating various biological processes that are critical for the success of implants and the health of surrounding tissues. The interaction between collagen and various cellular entities is vital for the maintenance of both soft and hard tissues surrounding teeth and dental implants. This communication is particularly important in the context of dental implants, where the successful integration of the implant with adjacent bone and soft tissue is fundamental for long-term stability and functionality. Any disruption in this interaction may lead to complications such as implant failure or peri-implantitis, thereby emphasizing the necessity for a healthy collagen architecture in these tissues [7,8]. Moreover, this interaction affects not only the biological integration of the implant but also its mechanical stability, as well-organized collagen fibers can effectively distribute loads and reduce stress concentrations at the implant-bone interface [9]. Furthermore, the quality and spatial distribution of collagen surrounding the implant site have been demonstrated to significantly impact the overall success of dental procedures, emphasizing the need for ongoing research and the exploration of innovative approaches aimed at enhancing collagen structure and function in the context of dental implants. Research indicates that the success of dental implants is contingent not only upon the effective management of the bone-implant interface but also upon the abundance and spatial arrangement of collagen fibers surrounding the implant. This distribution is

essential for ensuring adherence between periodontal and peri-implant tissues and the tooth or implant, which is crucial for preventing bacterial infiltration—a precursor to conditions such as gingivitis and chronic inflammatory diseases, including periodontitis and peri-implantitis. These infections can result in severe complications, including significant bone and implant loss [10,11]. Notably, multiple studies have documented a distinct distribution pattern of collagen fibers within periodontal and peri-implant tissues, revealing a greater prevalence of longitudinally oriented peri-implant fibers compared to transverse fibers. This suggests that such orientations may confer different mechanical properties and functional advantages [7,12-14].

Osseointegration and infection prevention are critical factors that significantly impact the long-term sustainability of the peri-implant region, which plays a central role in the overall success of dental implants. Consequently, it is essential for the peri-implant area to maintain a delicate balance of biological and mechanical properties to ensure the stability and functionality of the implant. Complications in these domains can lead to significant failures in implant therapy, highlighting the necessity for effective strategies to address challenges such as microbial infiltration and inadequate tissue integration [15]. Recently, there has been an increased emphasis on the integration of soft oral tissues with implants. This harmonious relationship between the implant and the surrounding peri-implant tissues is vital, as it aids in preventing microbial invasion and preserving crestal bone levels. Furthermore, successful soft tissue integration enhances support for the entire peri-implant area, which is crucial not only for functional success but also for achieving optimal aesthetic outcomes. For instance, a well-integrated soft tissue profile can improve the visual appeal of the dental implant, thereby contributing to a more natural appearance [16,17]. As research continues to explore the complexities of soft tissue interactions with implants, the focus remains on developing innovative solutions that promote tissue health, regeneration, and integration to ensure sustained success in dental implant therapies. To address these concerns, numerous studies have explored novel biomaterials and advanced implant surface coatings designed to enhance the biological response and integrative capabilities of dental implants [18,19].

2.2. COLLAGEN IN HARD AND SOFT TISSUE REGENERATION

The remarkable capacity of type I collagen to support tissue regeneration

constitutes a fundamental factor contributing to its widespread use as a biomaterial across numerous therapeutic domains. Its intrinsic biocompatibility and biodegradability, combined with low immunogenicity and minimal antigenicity, significantly enhance its suitability for medical applications. Furthermore, the inherent bioactivity of type I collagen facilitates cellular communication and bolsters essential cellular functions required for effective tissue repair and regeneration. As a result, collagen is widely acknowledged as a highly attractive biomaterial for tissue engineering approaches. Its biocompatibility—indicative of its ability to coexist with living tissues without eliciting an immune response—along with its bioactivity and natural cell-binding sites, renders it an ideal candidate for a multitude of regenerative medicine strategies [20-22].

Research efforts increasingly focus on harnessing the properties of collagen to develop innovative scaffolds that foster tissue repair and regeneration in diverse clinical scenarios, including orthopedic applications, skin grafting, and dental implants [23]. Moreover, the array of processing techniques available for collagen enables the creation of a broad spectrum of scaffolds, such as hydrogels, sponges, and fibrous mats, which can be tailored to meet specific biomedical requirements [21,24]. However, the effectiveness of collagen-based scaffolds in promoting cellular interactions is markedly influenced by their capability to replicate the natural ECM structure across various length scales. Achieving an optimized scaffold design that accurately represents the hierarchical organization of native collagen is essential for facilitating appropriate cellular responses, including adherence, proliferation, and differentiation. This consideration is paramount for the success of tissue engineering strategies, as it greatly impacts the overall functionality and durability of the engineered tissue [25,26]. Therefore, current research endeavors are focused on the development of ideal scaffolding materials that not only mimic the natural architecture of collagen but also improve the biomechanical and biological performance of engineered tissues.

Collagen-based materials are extensively employed in the regeneration of both bone and soft tissue, owing to their ability to support cellular processes such as osteogenesis, chondrogenesis, and wound healing [5]. In the field of dentistry, collagen scaffolds provide the requisite architecture for the regeneration of the periodontal ligament, cementum, gingiva, and alveolar bone, promoting cell attachment, proliferation, migration, and differentiation into fibroblasts and osteoblasts. Additionally, collagen facilitates the recruitment of stem cells and other progenitor cells to injury sites, thereby enhancing the regenerative process [5,26]. Notably, collagen regulates several signaling pathways crucial

for regeneration; it activates integrin receptors on the surface of periodontal ligament cells (PDLs), triggering signaling cascades that promote cell survival, proliferation, and differentiation. For example, the activation of integrin $\alpha 2 \beta 1$ leads to downstream signaling via the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, which are associated with cellular growth and matrix production. Furthermore, collagen promotes angiogenesis, a vital process in peri-implant regeneration. The presence of blood vessels is essential for delivering nutrients and oxygen to regenerating tissues, which is critical for cell function and metabolic activities. Collagen fibrils can stimulate the release of angiogenic factors, such as vascular endothelial growth factor (VEGF), further enhancing blood vessel formation in the area surrounding implants [3,28,29].

A comprehensive understanding of the multifaceted roles of type I collagen, in conjunction with advancements in scaffold design, holds significant potential for advancing regenerative medicine and improving patient outcomes. Ongoing research continues to investigate collagen's functional properties, revealing its potential to enhance therapeutic outcomes and the quality of life for patients engaged in tissue repair and regeneration processes. The synthesis and organization of collagen are integral to the overall mechanical characteristics of these tissues, positioning it as a central focus of research in the realms of tissue engineering and regenerative medicine [30].

2.3. COLLAGEN-BASED BIOMATERIALS

Collagen-based biomaterials have emerged as pivotal components in the field of regenerative medicine, specifically in the form of allografts and xenografts. Both types of grafts present a range of advantages and limitations within clinical practice. Thus, a comprehensive understanding of their characteristics is essential for effective tissue repair and regeneration. The application of xenografts and alloplastic bone substitutes serves as a valuable and safe technique, offering high manageability while obviating the necessity for donor site harvesting associated with autologous graft retrieval [31]. Allografts are derived from donors of the same species as the recipient, typically involving human tissue obtained from cadaveric sources. Collagen-based allografts demonstrate excellent biocompatibility and are extensively employed due to their inherent properties that promote cellular adhesion, integration, and proliferation. These allografts find application across various domains, including orthopedic surgeries (such as bone repair), periodontal regeneration,

and soft tissue reconstruction. Despite the well-documented low immunogenicity of type I collagen, some patients may still elicit an immune response due to residual cellular components or antigens, particularly in cases involving partially processed grafts. Consequently, meticulous donor selection and processing methods are indispensable in minimizing immunogenic risks. Conversely, xenografts are sourced from different species and primarily utilized to reduce donor site morbidity and enhance graft availability. Collagen-based xenografts possess properties akin to those of allografts, providing structural support while facilitating the cellular activities necessary for tissue regeneration. Their advantages include greater abundance and reduced cost relative to allografts, thus extending the availability of collagen-derived matrices in clinical applications, especially in high-demand surgical interventions [32-34]. The scope of xenograft applications is wide-ranging, encompassing bone augmentation, periodontal treatments, and dermal substitutes for skin grafting. These grafts have demonstrated effective integration within diverse tissue types, leading to successful healing outcomes. However, the use of xenografts is accompanied by significant safety concerns, primarily related to the potential transmission of zoonotic diseases and immunogenicity associated with foreign proteins. While rigorous donor animal screening and advanced preparation techniques can mitigate these risks, they continue to incite ethical debate within the medical community [35,36].

Within the context of xenografts, collagen can be derived from various materials, including animal-derived products (e.g., bovine and porcine sources) as well as marine sources (e.g., fish skin). The investigation of sustainable collagen sources, such as recombinant collagen, is also progressing [32]. The selection of the collagen source for extraction is a critical initial step in the production of collagen-based scaffolds, as this decision significantly impacts subsequent processing and influences the physical, chemical, mechanical, and biological properties of the resulting scaffolds [25,37]. Medical-grade collagen utilized in scaffold manufacturing is traditionally acquired from mammalian tissues. Only recently have recombinant production systems begun to be implemented. Notably, recombinant human collagen can be expressed by both prokaryotic and eukaryotic hosts, presenting the opportunity for consistent large-scale production. However, it is important to note that recombinant collagen does not fully replicate native human collagen due to incomplete posttranslational modifications. While mammalian cells can partially address this concern, their employment poses challenges such as elevated production costs and extended production timelines. Despite the promise of recombinant collagens, unresolved issues render them less appealing and more costly, thus establishing

animal-derived collagen as the current gold standard in the field [38,39]. Mammalian-derived collagen is preferred due to its high sequence homology with human collagen. Nonetheless, apprehensions regarding immune responses, zoonotic risks, and religious considerations restrict the application of mammal-derived collagen. For example, bovine collagen, which constitutes a significant industrial source, poses allergic risks for approximately 3% of the population and entails additional risks related to prion contamination and the transmission of bovine spongiform encephalopathy (BSE). Additionally, collagen obtained from porcine skin and bone is widely utilized in industry but is not permissible within Judaism and Islam. Although the incidence of allergic reactions to porcine collagen is lower in comparison to bovine collagen, the potential for zoonotic transmission remains a concern [40,41]. In contrast, collagens derived from avian and aquatic sources present fewer religious dilemmas and demonstrate relative safety; however, they differ from land animal collagens in several aspects, including amino acid composition, glycosaminoglycan content, and thermal behavior. Collagen isolated from marine organisms exhibits unique properties, such as lower gelling and melting temperatures and reduced extraction yields in comparison to mammalian-derived collagen [42]. Equine collagen represents a compelling alternative in this discussion due to its elimination of zoonotic risks, absence of religious restrictions, and minimal reported immune reactions, distinguishing it from other animal sources [43,44].

Tendons and skin are frequently selected for collagen extraction due to their substantial collagen content. For example, the Achilles tendon may contain up to 85% collagen in its dry mass, while skin typically consists of approximately 70% collagen. The yield of collagen from these tissues can vary considerably based on the extraction method and the specific characteristics of the animal source, with yields potentially exceeding 70%. Collagens isolated from the skin and tendons of mammalian sources demonstrate distinct properties. Tendon collagen fibers are characterized by dense packing and alignment, which contribute to a high degree of hierarchical organization and increased stability. In contrast, skin collagen fibers are arranged in a loose network, rendering them more susceptible to enzymatic cleavage. Moreover, aging is associated with an increase in collagen cross-links, which results in reduced flexibility and increased acid-insolubility of the collagen. Although the isolation of type I collagen from skin, articular cartilage, and flexor tendons has been reported, tendons are regarded as the optimal source for extraction due to their highest concentration of type I collagen (approximately 90%) and the minimal presence of protein contaminants [44,45].

Recent investigations have focused on the development of mineralized collagen scaffolds that improve mechanical properties, thereby enhancing their applicability for load-bearing functions in bone repair [46]. Techniques such as the incorporation of hydroxyapatite into collagen matrices have demonstrated promising outcomes in terms of osteogenic capacity and biocompatibility [47]. Furthermore, collagen membranes utilized around dental implants serve a dual role: they act as barriers to preclude soft tissue encroachment while simultaneously promoting bone regeneration. This selective permeability ensures that only osteogenic cells are permitted access to the area, which is crucial for the successful integration of implants. The capability of collagen membranes to inhibit the migration of non-osteogenic cells preserves the space necessary for new bone formation [48]. Moreover, collagen-based hydrogels have proven effective in the repair of cartilage and soft tissue by providing a hydrated environment that fosters the growth of chondrocytes and fibroblasts, as well as the deposition of the ECM. These hydrogels can also be modified to incorporate growth factors or other bioactive molecules, thereby enhancing tissue integration and reparative processes [49]. In the arena of periodontal regeneration, collagen membranes are frequently employed in guided tissue regeneration (GTR) techniques, serving as physical barriers that prevent the migration of epithelial cells into wound sites. This characteristic allows for the uninterrupted repopulation of the area by progenitor cells, facilitating the formation of new periodontal tissue. The collagen membranes create a conducive microenvironment that supports the growth of essential bone and soft tissues necessary for periodontal health [50].

In this context, the use of innovative double-phase collagenated biomaterials (OsteoBiol[®], TecnoSS[®] s.r.l., Giaveno, Italy) has seen over 20 years of application in ensuring successful tissue regeneration and implant integration [51]. These materials are porcine collagenated xenografts that exhibit exceptional biocompatibility and osteoconductive properties, which currently stand as the gold standard for applications in regenerative medicine. A specialized product has been developed for each clinical indication, aiming to provide optimal handling, granulometry, and consistency to achieve ideal regenerative outcomes. Notably, the dual-phase heterologous bone matrix granules consist of a mineral phase and a xenogenic collagen phase, which contributes to superior biocompatibility, a chemical composition akin to autogenous bone, controlled resorption of the bone matrix with progressive replacement by newly formed bone at the time of re-entry, and significant angiogenic potential. These factors are crucial for the success of bone regeneration procedures, which may be further enhanced through the utilization of some of

these xenografts [51].

The various processing techniques employed in the extraction and modification of collagen facilitate the customization of essential properties, including mechanical strength, degradation rate, and porosity. This versatility renders collagen-based biomaterials appropriate for a wide range of clinical applications [52]. However, the processing steps involved in collagen isolation and scaffold fabrication can significantly influence the inherent assembly of collagen, potentially affecting cellular responses and overall biological functionality [53]. Previous research on collagens derived from diverse sources has established a correlation between varying production treatments and collagen's structural features at both submolecular and supramolecular levels, which considerably impacts the mechanical and biological characteristics of collagen-based medical devices [54,55].

Addressing these research gaps presents substantial potential for the enhancement of implant therapies and the improvement of patient outcomes in the future. Ultimately, a deeper understanding of collagen's multifaceted role within the ECM may pave the way for the development of advanced biomaterials and therapeutic strategies that promote optimal tissue integration and extend the longevity of dental implants.

3. AIMS OF THE PRESENT THESIS

Based on the aforementioned Introduction, this doctoral research project was designed to conduct a thorough evaluation of cell adhesion mechanisms and the expression of various biomarkers associated with critical biological processes, such as mechanotransduction, osteogenic differentiation, and neoangiogenesis, in response to collagen application. The investigation aimed to explore the implications of these processes on gingival tissue health, osseointegration, and the promotion of vascularization. Additionally, acknowledging the significant impact of implant surface composition and morphology on tissue interactions, a primary objective was to design and develop an innovative collagen-coated implant specifically engineered to enhance tissue integration and improve overall clinical outcomes.

To systematically address these objectives, this research was organized around three principal macro-objectives, each dedicated to elucidating the multifaceted role of type I collagen concerning various aspects of tissue health and regeneration. The first objective focused on the preservation of soft tissue health, underscoring the vital functions that collagen serves in maintaining the structural integrity and resilience of gingival tissues. The second objective primarily investigated the enhancement of osseointegration, detailing the interactions between collagen and osteogenic cells that facilitate effective bonding between implants and surrounding osseous structures. The third objective mainly examined the promotion of vascularization, emphasizing collagen's capacity to stimulate neoangiogenesis, thereby ensuring an adequate blood supply that is essential for tissue regeneration and healing.

To pursue these aims, the research employed a rigorous evaluation and testing of various collagen-based biomaterials through a series of *in vitro*, *ex vivo*, and *in vivo* experiments designed to assess cellular responses and tissue interactions with collagenated biomaterials and implant surfaces. This comprehensive approach sought to provide valuable insights into the intricate dynamics of collagen's role in periodontal health, implant success, and the enhancement of soft tissue and bone regeneration outcomes. Ultimately, the findings may hold significant implications for the advancement of clinical practices within the fields of dental implantology and regenerative medicine.

4. COLLAGEN-BASED BIOMATERIALS TESTED

4.1. A LIQUID COLLAGEN FOR ORAL WOUND HEALING [56]

The absence of adequate keratinized mucosa and insufficient buccal depth may significantly contribute to the onset and progression of periodontal and peri-implant inflammation [57]. Such complications necessitate the prompt initiation of healing procedures to facilitate the restoration of both the structure and function of the soft tissues. This specific healing response, akin to that observed in wound healing, is characterized by three temporally overlapping but distinct phases: (i) hemostasis and inflammation, (ii) formation of new tissue, and (iii) tissue remodeling. Generally, the healing of connective tissues is contingent upon the involvement of various factors and resident cell populations capable of synthesizing an appropriately structured ECM, which, in turn, supports cellular activation, proliferation, and differentiation [58].

Regarding the initial section of this project, it has become increasingly evident that type I collagen plays a crucial role in the final stages of the oral wound healing process. During this critical phase, dense collagen fibers are synthesized and organized into an aligned ECM fibrillar network, which is essential for facilitating the remodeling and maturation of newly formed soft tissues. This alignment not only confers structural integrity but also enhances the mechanical properties of the tissue, thereby promoting optimal healing conditions [59]. Notably, hGFs are instrumental in the formation and remodeling of periodontal and peri-implant tissues. They synthesize and organize the ECM, ensuring structural stability and creating a network of collagen fibers that enables the re-establishment of the mucosal seal. Furthermore, fibroblasts can communicate with adjacent cells by secreting growth factors and cytokines or releasing them in the ECM, thus creating intricate cellular communication networks and transmitting mechanosensory signals [60].

Recent research has underscored the significance of the ECM, particularly type I collagen, on the morpho-functional properties of cells and mechanotransduction—the intracellular molecular mechanism whereby a physical stimulus is converted into a biological response. It has been demonstrated that, in cultured fibroblasts, focal adhesions represent one of the most extensively studied adhesive structures. These structures enable cells to perceive the mechanical characteristics of the ECM, serving as a critical determinant of tissue homeostasis. Following the integrin-mediated binding of cells to the

ECM, focal adhesion kinase (FAK) is among the first molecules activated and recruited to the adhesions, where it undergoes autophosphorylation. FAK plays a pivotal role in controlling cellular processes linked to adherence-dependent signaling, thus regulating cell migration, proliferation, and survival [61]. Additionally, various mechanotransduction mechanisms may contribute, including regulation of the Hippo pathway, which ultimately aims to restrict the transcriptional activity of anti-Phospho-WW domain-containing transcription regulator protein 1 (WWTR1, TAZ) and anti-Yes-associated protein (YAP) by preventing their entry into the nucleus. Consequently, YAP and TAZ are recognized as key mechanotransducers capable of sensing and transducing mechanical cues, such as the stiffness of the extracellular environment. Research has shown that when a single cell stretches on the ECM, cytoskeletal adaptation to cell morphology activates YAP/TAZ and promotes their nuclear accumulation, which fosters cell proliferation while inhibiting differentiation. Conversely, manipulation of cell morphology into a rounded, compact shape—thereby limiting adhesion to a restricted ECM adhesive area—or exposure to a stiff matrix result in the phosphorylation and exclusion of YAP/TAZ from the nucleus, thereby disabling their transcriptional functions and initiating differentiation while halting proliferation [62]. Accordingly, various upstream stimuli—including mechanical cues from the cytoskeleton, cellular stress, intercellular adhesion, cell polarity, and extracellular signaling—lead to the activation of the Hippo pathway. Given that the primary function of fibroblasts is the synthesis of critical ECM components, such as collagen, which interact dynamically with the newly formed collagen matrix to provide structural support to the tissue, it is evident that collagen itself can serve as a stimulus for cellular proliferation, differentiation, and activation [63].

A range of sources and formats of collagen has been proposed for the regeneration of periodontal and peri-implant tissues. For instance, collagen barrier membranes and collagen matrices are typically employed for soft tissue regeneration, while collagen sponges and cones are utilized for the stabilization of oral wounds and extraction sockets. Additionally, with advances in aesthetic medicine, collagen is increasingly utilized in its injectable form as a tissue stimulator [64].

One of the objectives of regenerative dentistry is to promote the endogenous repair of tissues through the manipulation of stem cells or by optimizing the intrinsic characteristics and plasticity of differentiated cells within adult tissues. In this context, fibroblasts represent a viable alternative to stem cells, as they exhibit both phenotypic and regenerative properties. Notably, hGFs have demonstrated enhanced regenerative capacity

when compared to other fibroblast populations. Moreover, the accessibility of hGFs through minimally invasive procedures, which do not compromise aesthetic outcomes, coupled with their rapid *in vitro* expansion and robust responsiveness to extrinsic factors, establishes them as a valuable resource for regenerative medicine [65].

Despite these advancements, there remains a scarcity of studies that elucidate the specific effects of physical stimuli on the efficacy of soft tissue regeneration procedures. Therefore, the first macro-aim of this doctoral project was to investigate the influence of a liquid collagenated coating on hGFs viability, proliferation, and migration in the context of wound healing, as well as its effects on the expression and activation of certain mechanotransduction markers [56].

4.1.1. MATERIALS AND METHODS

The Collagen-Based Biomaterial

The primary biomaterial examined in the initial experimentation of this doctoral project was a liquid medical device comprised predominantly of porcine type I collagen, alongside ancillary components including ascorbic acid, magnesium gluconate, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, sodium chloride, and water for injection (Dental SKIN BioRegulation, Guna S.p.a., Milan, Italy). This collagen formulation was employed as a coating for primary cultures of hGFs and was subsequently assessed through a series of comprehensive *in vitro* experiments. Notably, this product has been engineered to counteract the physiological aging of connective tissues and is intended to function as a mechanical bio-scaffold, offering both physical and chemical support to the collagen fibers present in the soft tissues surrounding teeth. Given that the biological effects induced by this collagen formulation in hGFs have not been previously explored, the findings of this study may illuminate the mechanisms underlying the potential beneficial impacts of its application in the dental domain and could represent a novel approach to facilitating collagen maturation and promoting soft tissue healing around natural teeth or dental implants [66].

The Collagen Coating

Based on the findings reported by Randelli et al. [66] concerning human tenocytes, this study posited that cultivating hGFs on a collagen-based coating could serve as an effective substrate for cell cultures, potentially enhancing their mechanical

stimulation and influencing their proliferation and migratory capabilities. Dental SKIN BioRegulation (100 µg/ 2 mL) was employed to create a thin coating on multi-well plates or Petri dishes, with the addition of 500 µL per 24-well plate and 5 mL per 100 mm diameter Petri dish. To ensure proper adhesion of the collagen to the plastic surfaces, the coated multi-well plates and Petri dishes were incubated at room temperature (RT) for 4 h prior to the removal of the excess solution. Subsequently, the coated surfaces were allowed to dry under a laminar flux hood. These coated plastics were utilized immediately or stored at 4 °C, in accordance with recommendations from previous research [66]. The hGFs cultured on collagen-coated cell culture plastic substrates were designated as test cultures (COL) and were subjected to comparison with hGFs seeded on uncoated cell culture plastic substrates, which served as untreated controls (CTRL).

Cell Cultures

The hGFs utilized for the two-dimensional (2D) culture experiments were isolated from human gingival biopsies obtained from nine patients undergoing partial gingivectomy procedures at the dental clinic of the University G. D'Annunzio Chieti-Pescara (CE, N° 1968-24/07/2020). The gingival samples collected were immediately rinsed with a physiological solution containing 1% Amphotericin B (cat. A2942, Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin-Streptomycin (P/S; cat. P4333, Sigma-Aldrich, St. Louis, MO, USA) prior to being subjected to enzymatic digestion using a collagenase/dispase solution (cat. 10269638001, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. Subsequently, the digested specimens were placed in a 60 mm diameter Petri dish containing a culture medium composed of Dulbecco's modified Eagle medium (DMEM) low glucose (cat. D6046, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 20% fetal bovine serum (FBS; cat. 41A0045K, Thermo Fisher Scientific, Waltham, MA, USA), 100 mM L-glutamine (cat. G7513, Sigma-Aldrich, St. Louis, MO, USA), and 1% P/S, allowing the cells to migrate spontaneously from the tissue. The isolated hGFs were then cultured in a controlled atmosphere of 5% CO₂ at 37 °C until they reached confluence, at which point they were employed for various experiments.

Furthermore, the characterization of specific markers expressed by hGFs was conducted to assess their profile, including the analysis of CD105 (FITC-conjugated antibody; cat. 326-040, BD Biosciences, San Jose, CA, USA), CD73 (PE-conjugated antibody; cat. 550257, BD Biosciences, San Jose, CA, USA), CD90 (FITC-conjugated antibody; cat. 555595, BD Biosciences, San Jose, CA, USA), CD326 (PerCP-Cy5.5-

conjugated antibody; cat. 347199, BD Biosciences, San Jose, CA, USA), and CD45 (FITC-conjugated antibody; cat. 196-040, BD Biosciences, San Jose, CA, USA). For cytometric analysis, the FACSVerse, FACSDiva version 6.1.3, and IDEAS software (BD Biosciences, Milan, Italy) were employed.

Additionally, the morphology of COL samples was compared to that of CTRL samples using scanning electron microscope (SEM) analysis (Phenom XL; Alfatest, Cernusco sul Naviglio, Italy). In summary, the cells were cultured on 12 mm diameter round coverslips, either uncoated or coated with Dental SKIN BioRegulation, within 24-well culture plates for a duration of 24 h. The samples were fixed with 3.5% glutaraldehyde (cat. 49630, Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M sodium cacodylate (NaCaCO; cat. C0250, Sigma-Aldrich, St. Louis, MO, USA) buffer for 24 h and subsequently stored at 4 °C, followed by post-fixation in 2% osmium tetroxide (OsO₄; cat. 75632, Sigma-Aldrich, St. Louis, MO, USA) prepared in the same buffer. The specimens were washed in distilled water, dehydrated through successive incubation in increasing concentrations of ethyl alcohol (50%, 70%, and 100%; cat. 1009832511, Sigma-Aldrich, St. Louis, MO, USA), and then treated with hexamethyldisilazane (HMDS; cat. 804324, Sigma-Aldrich, St. Louis, MO, USA) before air-drying overnight at RT. The dried specimens were mounted on SEM stubs using double-sided carbon tape and sputter-coated with gold (150 Å) prior to imaging acquisition with the Phenom XL microscope.

Cryopreserved hGFs from passages three to six were utilized to conduct morphological, functional, and molecular evaluations. The cells were cultured in 24-well plates with a culture medium (DMEM low glucose, 10% FBS, 100 mM L-glutamine, and 1% P/S) for both short- and long-term viability assessments, as well as wound healing assay. Additionally, 100 mm diameter Petri dishes were utilized for flow cytometric analysis after 48 h of culture. **Figure 1** provides a summary of the experimental design for this study.

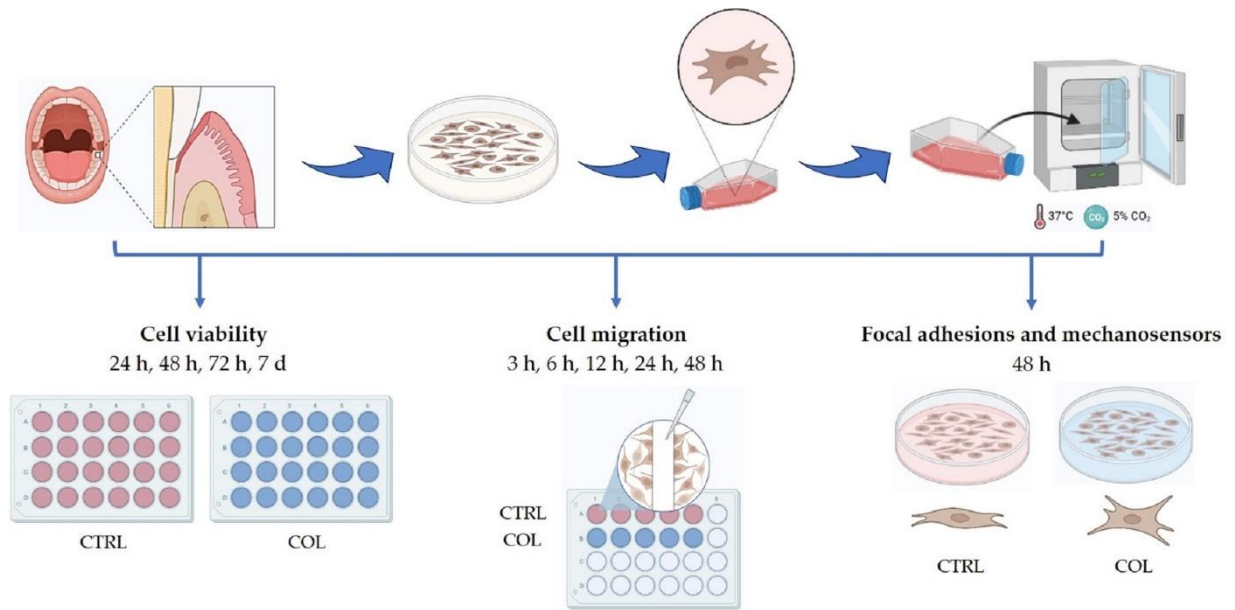


Figure 1. The study design delineating the experimental conditions and treatments utilized. The control group (CTRL) consists of human gingival fibroblasts (hGFs) cultured under standard conditions with the appropriate culture medium. The experimental group (COL) includes hGFs cultured under specific test conditions on a collagen-coated surface, accompanied by the designated culture medium. h: hours, d: days.

Calcein AM Cell Viability Assay

For viability assessments, CTRL and COL hGFs were cultured in 24-well culture plates at varying cell densities tailored for each experimental time (7×10^4 , 6×10^4 , 4×10^4 , 2.5×10^4 , and 2×10^4 cells/well for 24 h, 48 h, 72 h, and 7 days, respectively). At each designated time point, cells were washed twice with phosphate buffer saline (PBS 1 $\times$; cat. D8662, Sigma-Aldrich, St. Louis, MO, USA) prior to being trypsinized with a 5 mM trypsin/EDTA solution (cat. 59418C, Sigma-Aldrich, St. Louis, MO, USA) and subsequently harvested in PBS 1 $\times$ within flow cytometry tubes. Following centrifugation at 1500 rpm for 5' at RT, the supernatant was discarded. All samples, with the exception of the negative controls, were incubated for 20' at RT in the dark with calcein AM solution (calcein AM powder dissolved in dimethyl sulfoxide–DMSO—at a concentration of 1 mM; cat. C3099, Thermo Fisher Scientific, Waltham, MA, USA), achieving a final concentration of 0.1 μ M in PBS 1 $\times$. Subsequently, the fluorescence of the samples (λ_{ex} 488 nm, λ_{em} 516 nm) was quantified using a FACS Canto II flow cytometer (BD Biosciences, Milan, Italy), and the data were analyzed employing FACSDiva version 6.1.3 and IDEAS software. Results were presented as a mean fluorescence intensity (MFI) ratio, calculated by dividing the MFI of positive events by the MFI of negative events (MFI of secondary antibody).

Scratch Assay

The scratch assay is a well-established *in vitro* model utilized for investigating the wound-healing properties of cells, specifically in terms of cell activation and migration in response to injury [67]. Cell migration for both CTRL and COL hGFs was analyzed using 24-well culture plates. Cells were seeded at a density of 7×10^4 per well and subsequently subjected to a 24-h starvation period in DMEM with low glucose and 0.1% FBS. A scratch was then created on the monolayers using a 200 μ L plastic tip. Following the creation of the wound, the monolayers were washed with DMEM low glucose to eliminate cell debris. Cell migration occurred in 1 mL of culture medium supplemented with 10% FBS. Observations were made, and images were captured immediately following the scratching (T0) as well as at various time points—3 h, 6 h, 12 h, 24 h, and 48 h (T3-T48)—utilizing a Personal AUtomed Lab Assistant (PAULA) Smart Cell Imager (Leica Microsystems Srl, Buccinasco, Italy). The percentage of wound closure (%) from T0 to T48 was calculated using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA), employing the following formula [68]:

$$\text{wound closure \%} = \frac{T_0 - T_{24}}{T_0} \times 100.$$

Flow Cytometry Analysis

The influence of collagen coating on the expression of FAK, YAP/TAZ, and their phosphorylated forms in hGFs was assessed through flow cytometry analysis. Following a 48-h culture period, the cells were detached using a trypsin/EDTA solution (5 mM), collected in PBS 1 $\times$, and centrifuged at 1500 rpm for 5' at 4 °C. The cells were then resuspended in a fixation solution (BD™ FACST™ Lysing Solution; cat. 349202, BD Biosciences, San Jose, CA, USA) at a dilution of 1:10, followed by permeabilization (BD FACST™ Permeabilizing Solution; cat.340973, BD Biosciences, San Jose, CA, USA) also at a dilution of 1:10 for 5' in darkness. Subsequently, all samples, with the exception of negative controls, were incubated with primary antibodies, specifically anti-FAK (1:100; cat. A32309, Anti-bodies.com Europe AB, Stockholm, Sweden), anti-YAP (1:50; cat. A3237, Anti-bodies.com Europe AB, Stockholm, Sweden), anti-TAZ (1:50; cat. E5P2N, Anti-bodies.com Europe AB, Stockholm, Sweden), anti-pYAP (1:200; cat. BK130085, Cell Signaling Technology, Milan, Italy), and anti-pTAZ (1:500; cat. SAB5701813, Thermo Fisher Scientific, Waltham, MA, USA) for 30' at 4 °C in the dark. The same incubation parameters were applied to Alexa Fluor 488- (cat. 11034, Thermo Fisher

Scientific, Waltham, MA, USA) and 647- (cat. A-21235, Thermo Fisher Scientific, Waltham, MA, USA) conjugated secondary antibodies prior to the collection of samples in PBS 1× for analysis using the FACS Canto II flow cytometer. The resulting data were evaluated using FACSDiva version 6.1.3 and IDEAS software, with outcomes expressed as MFI ratios, calculated by dividing the MFI of positive events by that of negative events (specifically the MFI of the secondary antibody).

Statistical Analysis

Data were presented as the mean $\pm$ standard error of the mean derived from a minimum of three independent experiments utilizing isolated cell strains obtained from distinct hGF biopsies. Each experiment was carried out in technical triplicates. Statistical analysis was conducted employing GraphPad Prism Software Analysis version 9 (GraphPad Software, San Diego, CA, USA), utilizing the Kruskal–Wallis test for comparisons involving more than two groups, or the Kolmogorov–Smirnov test for comparing the effects of the COL and CTRL groups. Statistical significance was established at a p -value ≤ 0.05 .

4.1.2. RESULTS

Evaluation and Characterization of hGFs and Cellular Morphology

Before assessing the collagen coating effect on this cellular model, the phenotype of hGFs was first characterized. The cells were obtained from gingival biopsies through a combined isolation technique that employed enzymatic digestion followed by spontaneous migration [69]. These cells demonstrated a distinct fibroblast-like morphology (**Figure 2a**) and exhibited the expression of typical markers, specifically CD105, CD73, and CD90, while lacking expression of CD45 and the epithelial marker CD326 (**Figure 2b**).

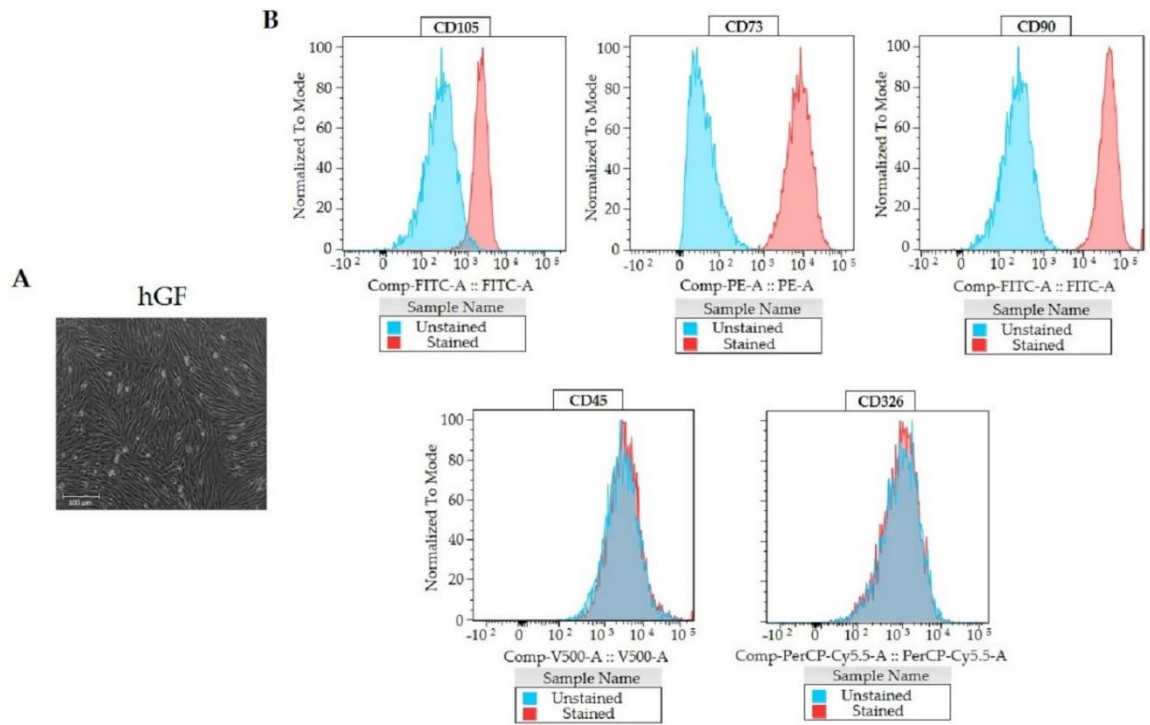


Figure 2. (a) Representative phase-contrast micrograph of isolated hGFs obtained using the PAULA Smart Cell Imager; (b) Characterization of hGFs. Representative graphs depicting the expression of CD105, CD73, CD90, CD45, and CD326, assessed through cytometric analysis.

Upon examination through SEM of CTRL and COL hGFs after 24 h (**Figure 3**), both groups exhibited typical fibroblastoid morphology characterized by a spindle shape.

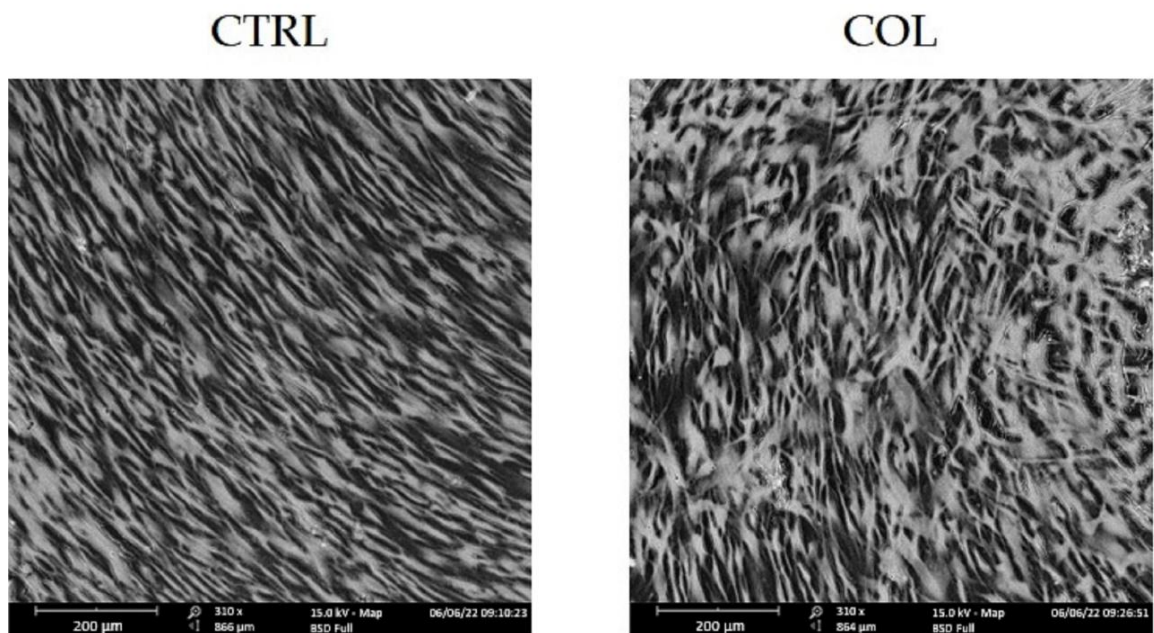


Figure 3. Representative scanning electron microscopy (SEM) images of CTRL and COL hGFs cultivated on glass coverslips. Magnification: 310 $\times$; scale bar: 200 μ m.

However, the cells cultivated on the collagen coating displayed a greater degree of interconnection, with prominent radial filopodia and lamellipodia-like structures. Conversely, the CTRL cultures appeared more elongated and were arranged in a more regular manner on the glass substrate, indicating a weaker attachment to the surface.

Impact of Dental SKIN BioRegulation on Cell Viability

According to the experimental protocol outlined in **Figure 1**, the cell viability of CTRL and COL hGFs was evaluated using cytometric analysis. As illustrated in **Figure 4**, the COL specimen demonstrated significantly higher viability values in comparison to the CTRL group at each assessed time point, achieving statistical significance specifically at the 48-h mark.

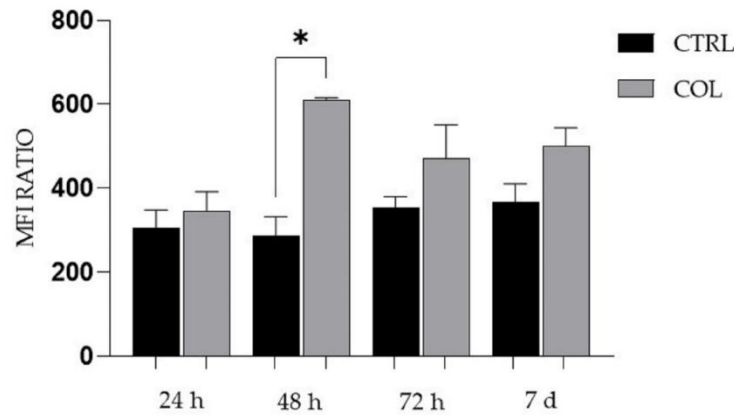


Figure 4. Impact of collagen coating on the short- and long-term viability of hGFs at intervals of 24 h, 48 h, 72 h, 7 days, and 14 days, in comparison to CTRL hGFs. The results are presented as the mean fluorescence intensity (MFI) ratio $\pm$ standard error of the mean ($n \geq 3$), calculated by dividing the MFI of positive events by the MFI of negative events (MFI corresponding to the secondary antibody). * $p < 0.05$.

Wound Healing Assay

The migratory capacity of hGFs on collagen-coated surfaces was evaluated to investigate the wound-healing properties of these cells *in vitro*, utilizing a scratch assay. The results indicated that at every assessed time point, wound closure occurred at a significantly faster rate in the COL hGFs compared to the CTRL group. Statistical significance was achieved after 24 h, at which point the closure rate appeared nearly complete. By 48 h, a continuous monolayer was observed with no gaps between cells, signifying complete wound closure (**Figure 5**). Additionally, a morphological assessment at 48 h revealed a more aligned and organized orientation of hGFs in the direction of

wound closure when cultured on the collagen coating.

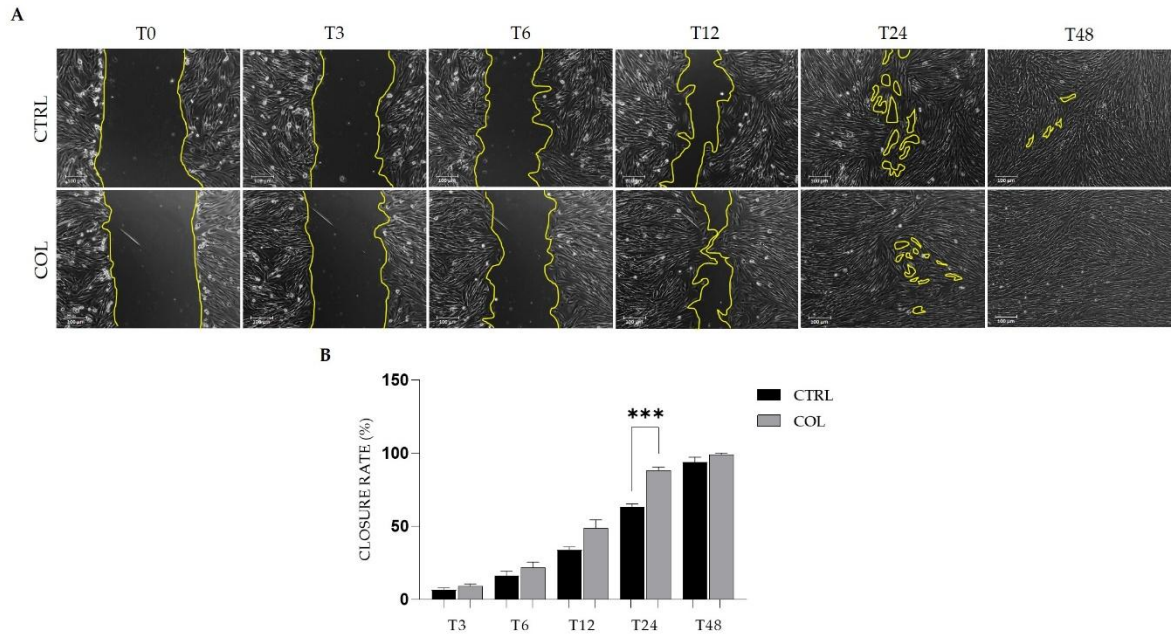


Figure 5. (a) Representative phase-contrast micrographs illustrating the migration properties of both CTRL and COL hGFs, captured from the initiation of the scratch (T0) to 48 h post-injury (T48). The imaging was conducted using the PAULA Smart Cell Imager. Scale bar: 100 μ m; (b) Histograms depicting the percentage of closure rates under varying experimental conditions. The results are presented as the mean $\pm$ standard error of the mean ($n \geq 3$). Data were acquired by analyzing a minimum of three distinct fields for each image using ImageJ software. *** $p < 0.001$.

Effect of Dental SKIN BioRegulation on FAK and YAP/TAZ Activation

Upon achieving promising results in wound healing, we sought to elucidate whether the collagen bio-scaffold exerts an influence on the migration of hGFs through mechanical stimulation. To this end, the protein expression of key mechanosensors implicated in cell migration was analyzed. One of the primary adhesion molecules that undergoes recruitment by cells following ECM mechanical stimuli and is critical for mechanosensory signaling is FAK. Notably, it was observed that FAK was significantly upregulated in the presence of the collagen coating when compared to control conditions devoid of mechanical stimulus (**Figure 6a**).

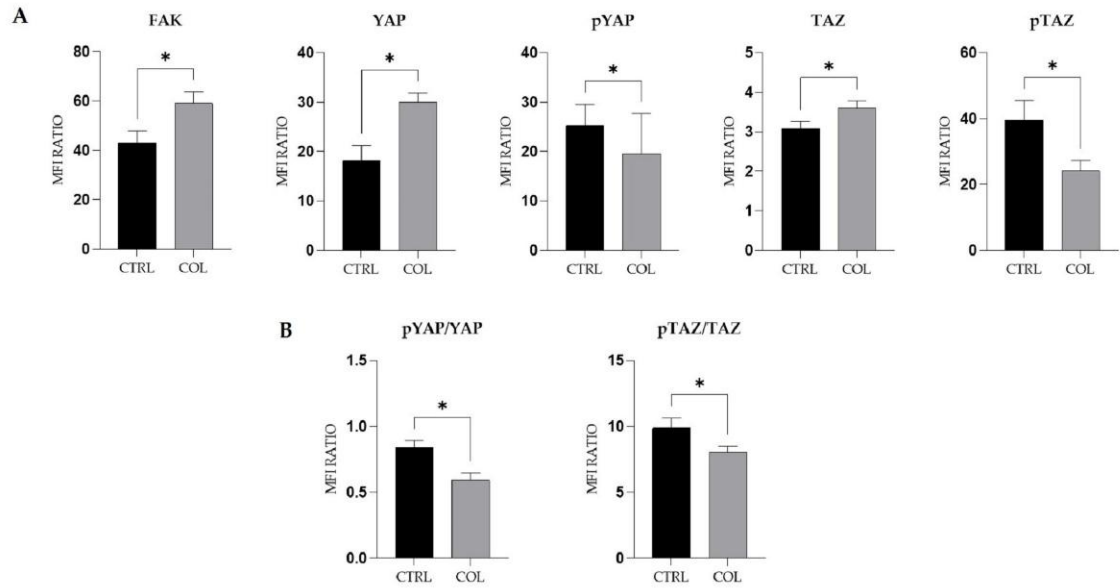


Figure 6. (a) Bar graphs depicting the activated and inactivated (phosphorylated) forms of anti-Yes-associated protein (YAP) and anti-Phospho-WW domain-containing transcription regulator protein 1 (WWTR1, TAZ), and (b) the ratios of pYAP/YAP and pTAZ/TAZ as measured in hGFs cultured on collagen-coated or uncoated cell culture plastic substrates after a 48-h incubation period. Data are presented as the mean $\pm$ standard error of the mean ($n \geq 3$). * $p < 0.05$.

In addition, another mechanism pertinent to cellular mechanotransduction was evaluated, specifically the Hippo pathway, characterized by the activity of the transcription factors YAP and TAZ. The activity of these transcription factors is modulated by phosphorylation, leading to their nuclear-cytoplasmic translocation. Phosphorylation serves to inactivate YAP and TAZ, resulting in their relocation to the cytoplasm. Conversely, the stiffness of the ECM influences their activation, promoting dephosphorylation and co-translocation to the nucleus. Interestingly, the cytometric analysis revealed a significant enhancement in the protein expression levels of both YAP and TAZ after culturing on the collagen coating. This enhancement was also evident in their phosphorylated forms during culturing of hGFs on uncoated cell culture substrates in the absence of mechanical stimulus (**Figure 6a**). A similar trend was noted when assessing the ratios of pYAP/YAP and pTAZ/TAZ, thereby confirming the role of the collagen coating in activating YAP and TAZ compared to control conditions (**Figure 6b**).

4.1.3. DISCUSSION

Collagen plays a pivotal role in maintaining the structural integrity of the ECM by regulating molecular and cellular interactions, thereby defining the shape, thickness, and

mechanical resistance of tissues. Notably, the maxillofacial region comprises both hard and soft tissues, where collagen serves as a critical component [4]. Consequently, collagen is exceptionally well-suited for development as a biomedical material. In recent decades, a diverse array of collagen-based medical products, derived from various sources and available in multiple formats, have demonstrated safety and efficacy across numerous biomedical applications [70].

In the current study, the *in vitro* effects of Dental SKIN BioRegulation, an injectable medical device primarily composed of porcine-derived type I collagen, were investigated on primary cultures of hGFs. The focus of this investigation was on assessing cell viability, proliferation, and migration effects. Furthermore, given that Dental SKIN BioRegulation is designed to function as a mechanical bio-scaffold for both skin and oral soft tissues, the expression and activation of specific mechanotransduction markers were also examined.

The results obtained from these experiments provided compelling evidence of the efficacy of the collagen biomaterial. Notably, a significant enhancement in cell viability was recorded after 48 h ($p < 0.05$), along with a distinct alteration in cell morphology compared to the CTRL group after a shorter duration time (24 h). Specifically, the COL hGFs exhibited a more intertwined appearance, characterized by numerous large radial filopodia and lamellipodia-like structures, compared to the CTRL group, which displayed a more elongated and regularly arranged configuration. This observation suggested that the application of this collagen coating likely resulted in enhanced adhesion to the substrate. These findings are consistent with previously published research that demonstrated increased viability and substrate attachment in human tenocytes, which are specialized fibroblasts of tendons, when subjected to the same collagen-based coating [66,71]. In contrast, some authors have reported no advantages associated with the usage of a collagen coating, indicating that human skin fibroblasts grown on collagen-coated dishes exhibited a rounder shape and a shorter perimeter compared to those cultivated on uncoated polystyrene or glass dishes [72]. Furthermore, the number and density of cells at confluence were reported to be unaffected by this specific collagen coating; however, it is important to consider that the origin of the fibroblasts and the type of collagen utilized in that study differed from those in the current investigation as well as in the research conducted by Randelli et al. [71]. Despite this discrepancy, a substantial body of literature exists that employs more complex collagen matrices than mere coatings, corroborating the favorable impact of this structural protein on the growth, viability, and migration of

fibroblasts [73-76].

In relation to the latter aspect, the *in vitro* migration assay conducted in this study demonstrated that the Dental SKIN BioRegulation coating significantly enhanced wound healing within 24 h and achieved complete reconstitution of the cell monolayer by 48 h, in comparison to the CTRL group. This effect promoted a more aligned and regular orientation of hGFs toward the direction of wound closure. Supporting this observation, extensive literature exists regarding the positive effects of collagen on wound healing across various tissue types, including oral tissues [76,77].

It is essential to emphasize that, alongside exogenous sources such as injectable collagen and more complex collagen matrices, diverse populations of multifunctional fibroblasts residing in the connective tissue of the gingiva and periodontal ligament are capable of synthesizing collagen and mediating the remodeling of the developing collagen matrix. This capacity is crucial for the restoration of a functional periodontium. Interestingly, during the remodeling phase, the ECM is influenced by mechanical stimuli, which, in turn, affect the population of fibroblasts. Mechanoresponsive cells play a vital role in detecting mechanical signals and subsequently converting them into biological responses [78,79]. In this context, the data obtained from this study, which demonstrated accelerated and directional migration of hGFs cultured on the collagen coating, supported the hypothesis that the regenerative activity of this medical device may be initiated through a mechanotransduction system, as previously suggested [71]. To further validate this concept, the protein expression and activation of specific key markers implicated in mechanotransduction processes, such as FAK and the YAP/TAZ proteins from the Hippo pathway, were examined. Notably, COL cultures demonstrated a significant upregulation of FAK, a principal regulator of cell adhesion and migration, alongside a significant decrease in the phosphorylation levels of YAP/TAZ. This reduction was associated with their co-translocation into the nucleus and subsequent activation. More explicitly, the activation of YAP/TAZ typically fosters a proliferative cell phenotype by inhibiting differentiation. These findings align with those reported in previous research conducted by Randelli et al. [71], which represents the only study assessing the identical collagen product utilized in the present work, albeit without revealing significant differences in the expression and phosphorylation of YAP/TAZ in tenocytes cultivated on coated versus uncoated substrates. It is essential to acknowledge that the cellular types were distinct, and various factors might concurrently influence the intricate mechanisms of YAP/TAZ phosphorylation and translocation [62]. Nevertheless, immunofluorescence analysis

corroborated a more pronounced nuclear localization of YAP/TAZ in tenocytes grown on the collagen coating, as well as an upregulation of FAK mRNA levels and a significant enhancement in wound closure at 24 h under similar conditions. The findings in this study are supported by numerous other investigations that emphasized the pivotal roles of YAP, TAZ, and FAK in the regulation of mechanosensing and mechanotransduction in response to cell-substrate rigidity and tensile stimuli from the ECM [62,80,81]. Recently, there has been increased attention on the functionality of the Hippo signaling pathway within the oral field, particularly concerning craniofacial development and pathological processes. It has been postulated that exploring the Hippo pathway could unveil new strategies for periodontal tissue regeneration, representing a promising avenue for future research [82].

Within the limitations of the present study, a noticeable gap in research aimed at further characterizing the Hippo pathway in relation to the expression of other markers and the investigation of COL-1 production induced by collagen coating has been identified. Although this research provided preliminary evidence of the morpho-functional properties associated with Dental SKIN BioRegulation, the significant enhancement observed in wound closure and the protein expression of critical markers involved in cellular mechanoresponsiveness to ECM biomechanical changes suggested the efficacy of this medical device in promoting hGF focal adhesion and migratory capacity for regenerative objectives.

Overall, these results tend to support the hypothesis that Dental SKIN BioRegulation could serve as an effective approach for the regeneration of oral and skin tissues, acting as a mechanical bio-scaffold, as initially conceived. This medical device is designed to mechanically facilitate cell migration while mitigating the physiological deterioration of connective tissues, thereby offering both physical and chemical support to the collagen fibers present within the oral soft tissues and the subcutaneous connective tissue. Although the effects of this particular type of collagen on dermal fibroblasts have yet to be extensively studied, it is noteworthy that these cells demonstrate 95% homology with hGFs [83], which may imply that analogous mechanisms of action could be established in the skin. In line with the current study design, prior research has demonstrated that this medical device, although referred to by an alternate commercial name and tested on human tenocytes, not only induced type I collagen secretion but also increased levels of tissue inhibitor of matrix metalloproteinases (TIMP-1), indicating an anti-collagenolytic effect. Furthermore, it upregulated the expression of focal adhesions and mechanosensors (FAK, YAP, and TAZ), signifying a mechanical input for cell

migration [66,71]. Clinical studies have also corroborated these findings, establishing that the collagen-based medical compound was effective in connective tissue repair, even in cases of osteoarticular pathologies [84,85].

In conclusion, the data compellingly indicated that this collagen-based biomaterial functions effectively as a bio-scaffold, mechanically promoting the proliferation, migration, and adhesion of oral soft tissue cells. This innovation represents a promising strategy for enhancing gingival tissue regeneration and emphasizes the potential applications of this collagen in clinical environments. The results of this study not only deepened the understanding of collagen's role in soft tissue healing but also underscored its influence in the domains of tissue engineering and regenerative medicine. Furthermore, the investigation and assessment of other potential effects of Dental SKIN BioRegulation on osteoblasts derived from jawbone may serve as a valuable method for advancing the long-term integration of bony and soft tissues in dental implants.

4.2. A COLLAGEN COATING FOR A NEW DENTAL IMPLANT SURFACE

Achieving clinical success with dental implants requires not only optimal osseointegration—the process by which the implant integrates with the surrounding bone—but also the establishment of a robust biological seal around the transmucosal portion of the implant. This biological seal serves as a protective barrier, effectively preventing bacterial infiltration and reducing the risk of infection and subsequent peri-implant diseases. The interaction between osseointegration and the formation of a biological seal is crucial. While osseointegration provides the necessary mechanical stability for the implant, healthy peri-implant soft tissue supports immune responses and facilitates tissue regeneration [86,87].

The domain of dental implants has undergone notable advancements in recent years, largely attributable to innovative surface modifications designed to enhance both aesthetic qualities and functional efficacy [19]. Dental implants are regarded as a fundamental aspect of restorative dentistry, with an increasing emphasis on customization to address the specific biological and mechanical needs of patients. Nevertheless, ensuring the long-term success of these implants poses several challenges, particularly in relation to achieving effective osteogenesis, facilitating tissue integration, and reducing bacterial adhesion [16,88]. These challenges have prompted researchers to investigate various techniques and materials to optimize dental implant surfaces, thereby improving biocompatibility and overall performance.

To this end, the second macro-objective of this doctoral project focused on the design and development of innovative dental implant surfaces, emphasizing not only the identification of the most effective micro- and macro-design features for both the abutment and screw components but also exploring the potential use of a collagen-based biomaterial to coat the screw. The ultimate goal of these investigations was to promote comprehensive peri-implant regeneration.

4.2.1. ABUTMENT AND INTEGRATION OF SOFT TISSUES

[89]

It is well established that the health of periodontal and peri-implant soft tissues is integral to maintaining the long-term health and functionality of both natural teeth and dental implants. The condition of these soft tissues directly impacts the overall success of dental restorations, contributing to essential biological and mechanical functions [90]. The biological seal formed by keratinized mucosa plays a pivotal role in this relationship, possessing unique properties that enhance the resilience of the implant environment against microbial colonization. Research has indicated that the presence of adequate keratinized tissue surrounding implants is associated with enhanced plaque control, decreased incidence of peri-implantitis, and improved patient comfort. In addition to aesthetic considerations, a well-structured connective tissue forms a protective seal that safeguards both the mechanical and biological integrity of the underlying bone. This protective function stands in contrast to the migration of junctional epithelium toward the implant apex, thereby playing a significant role in the mitigation of marginal bone loss (MBL). Furthermore, the quality and quantity of soft tissue surrounding dental implants significantly influence the long-term outcomes of implant therapy [7]. Thus, a comprehensive understanding of the biological mechanisms underlying soft tissue health is essential for optimizing implant placement and ensuring favorable conditions for healing and functional stability. For instance, the presence of healthy keratinized tissue can enhance blood supply to the area, thereby improving tissue regeneration and bolstering defenses against infection [91].

In summary, integrating effective soft tissue management strategies with implant therapy is critical for achieving long-term success. By promoting a healthy interface between periodontal tissues and dental implants, clinicians can significantly enhance implant stability and mitigate the risk of complications associated with inadequate biological sealing. The development of a biological seal around dental implants necessitates a multifaceted approach that combines advanced materials, effective surgical techniques, and comprehensive patient management strategies. By enhancing the interaction between implants and the surrounding soft tissues, clinicians can markedly improve clinical outcomes and the longevity of implant therapies [90].

In the literature, numerous factors have been identified that influence the quality and quantity of connective tissue attachment and healing around dental implants. Various

surface treatments, including plasma or argon activation, air abrasion, acid etching, laser treatment, micro-grooving, and electrochemical oxidation, have been employed to achieve specific abutment micro-geometry and surface bio-activation, thereby affecting soft tissue morphogenesis [92]. Additionally, various materials have been utilized for these purposes, including titanium, zirconium oxide, gold alloy, aluminum oxide, ceramics, titanium nitride (TiN), and hydroxyapatite. Notably, titanium and zirconia have demonstrated favorable responses in soft tissue, whereas gold alloy has been found inadequate in establishing an appropriate peri-implant soft tissue response [93]. Overall, substrates with rougher surfaces exhibit improved characteristics concerning peri-implant soft tissues, and it has been observed that epithelial cells adhere more effectively to metallic surfaces than ceramic surfaces [94].

Furthermore, the macro-design of the abutment has also been shown to impact the biological response of peri-implant soft tissues. Although collagen fibers are not predetermined, their formation depends on the local environment. The geometry and behavior of the pre-existing ECM can be influenced through mechanical and geometrical cues. The design features of the implant prosthesis–abutment complex have been established as critical for fostering healthy and stable peri-implant soft tissues [95,96]. Over the years, various abutment shapes, including scalloped, parallel-walled, platform-switching, and concave designs, have been proposed [97,98]. The latter design presents an inward narrowed profile that facilitates the creation of a macroscopic concave shape just above the implant platform. Recently, several researchers have posited that a concave design provides greater space for stable blood clot formation, subsequently promoting fibroblast proliferation and migration, ECM deposition and remodeling, protein adsorption, granulation tissue formation, and an increased contact area between soft tissues and the abutment, all contributing to enhanced connective tissue stability and mechanical properties [99-101]. Moreover, a concave transmucosal design has resulted in improved soft tissue stability, denser and better-organized collagen fibers with higher connective tissue attachment, as well as significantly reduced peri-implant bone resorption compared to divergent or straight transmucosal abutments [101-103].

In light of the considerations mentioned above, the most effective micro-topography of a novel abutment surface was initially assessed through *in vitro* tests on hGF adhesion and evaluations of the induced mechanotransduction. Subsequently, it was hypothesized that a concave macro-geometry of the implant neck might spontaneously align the collagenous network, thereby influencing cell polarization, migration, and the

orientation and arrangement of fiber growth. To validate this hypothesis, a proof-of-principle animal study was designed to investigate the structure and distribution of collagen fibers and bundles in the peri-implant soft tissues by comparing two implants with identical bodies but differing healing abutment geometries: a concave abutment with a surface selected based on favorable *in vitro* results, and a parallel-walled abutment serving as a control.

***In vitro* Experimentation**

4.2.1.1. MATERIALS AND METHODS

Implant Surfaces

This investigation employed implant discs (5×2 mm; RESISTA[®] Company, Ing. Carlo Alberto Issoglio and C. S.r.l., Omegna, Italy) constructed from grade 4 commercially pure titanium, which are coated with TiN via physical vapor deposition (PVD) techniques. The TiN coating serves a dual purpose: it provides an aesthetic barrier, as its gold hue remains concealed when illuminated from behind by mucosal tissue, and it offers functional benefits by repelling negatively charged bacteria. Additionally, this coating isolates the implant while preserving its intrinsic properties and biomechanical functionality, thereby enhancing hardness and lubricity and yielding a low coefficient of friction [104]. The discs were categorized into six groups based on their surface treatments and micro-morphologies, denoted as machined (M); with 50×5 µm threading (M1); with 75×7.5 µm threading (M2); and their corresponding treated surfaces, which underwent a double acid etching (DAE) treatment (T, T1, and T2).

A schematic illustration of the surfaces being analyzed is reported in **Figure 7**.



Figure 7. Schematic illustration of the characteristics of the surfaces under analysis. The discs were divided into six groups based on their surface treatments and micro-morphologies: machined (M); with 50×5 µm threading (M1); with 75×7.5 µm threading (M2); and their corresponding treated surfaces undergoing a double acid etching (DAE)

treatment (T, T1, and T2). The surfaces identified as M, M1, and M2 were classified as control (CTRL), whereas the surfaces designated as T, T1, and T2, which received the DAE treatment, were classified as test (TEST).

Surface Characterization

A comprehensive microscale evaluation of surface characterization was conducted by using SEM analysis (Phenom XL). Surface topography was studied only on untreated discs using a magnification of 420×. Images were taken using an accelerating voltage of 15 kV with the backscattered electron detector (BSD) and TOPO A program. The first program is used to generate images based on the atomic number contrast of the sampled materials. It is ideal for distinguishing different materials based on their chemical composition, thanks to its sensitivity to heavier elements (which appear brighter) and lighter elements (which appear darker). The "Full" mode maximizes the potential of the BSD detector, providing detailed and high-quality images for advanced applications. The TOPO A program is designed to acquire information about the sample's topography, such as its surface characteristics, including roughness, edges, and structural details. It uses the secondary electron (SE) signal or a combination of signals to highlight topographical variations with high spatial resolution. It is particularly useful for morphological analyses and for examining fine surface details.

Wettability Analysis

The sessile drop method was utilized to assess the surface wettability of all surfaces. Specifically, 5 µl of colored distilled water was deposited onto each disc, followed by immediate imaging with a Nikon D90 DSLR camera (Nikon Corporation, Tokyo, Japan). Contact angle measurements were subsequently obtained using the drop snake method in ImageJ software. To ensure precision and consistency, each sample underwent six separate tests.

Cell Cultures

The hGFs used for the study were sourced from human gingival biopsies collected from seven patients undergoing partial gingivectomy at the dental clinic of the University G. D'Annunzio Chieti-Pescara (CE, N° 1968-24/07/2020). The gingival tissues obtained were promptly rinsed with a physiological solution containing 1% Amphotericin B and 1% P/S before undergoing enzymatic digestion with a collagenase/dispase solution for 1 h at 37 °C. After digestion, the specimens were placed in a 60 mm diameter Petri dish with a culture medium consisting of DMEM low glucose, enriched with 20% FBS, 100 mM L-

glutamine, and 1% P/S, enabling spontaneous cell migration from the tissue. The isolated hGFs were subsequently cultured in an environment controlled for 5% CO₂ at 37 °C until they became confluent, after which they were utilized in various experiments.

Additionally, to evaluate their profile, specific markers expressed by hGFs were characterized, including the examination of CD105 (FITC-conjugated antibody), CD73 (PE-conjugated antibody), CD90 (FITC-conjugated antibody), CD326 (PerCP-Cy5.5-conjugated antibody), and CD45 (FITC-conjugated antibody). The cytometric analysis was conducted using FACSVerse, FACSDiva version 6.1.3, and IDEAS software.

Cell Viability

The impact of discs on the metabolic activity of hGFs was evaluated utilizing the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (cat. 475989, Sigma-Aldrich, St. Louis, MO, USA), in accordance with the manufacturer's protocol. The cells were plated in a 96-well plate at a density of 5×10^3 cells/well (2×10^4 cells/mL) and incubated with the culture medium in the presence of discs for durations of 24 h, 72 h, and 7 days. On the day of the selected time point, 20 μ L of MTT solution (5 mg/mL) was introduced to each well, and the incubation continued for 3 h at 37 °C in the dark. Subsequently, 200 μ L of DMSO (cat. D2438, Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for an additional 30' at 37 °C. Absorbance measurements were performed at a wavelength of 540 nm using a microplate spectrophotometer (SpectraMAX 190, Molecular Devices, San Jose, CA, USA).

Scanning Electron Microscopy (SEM) for Cell Adhesion

The impact of surface topography on the adhesion and morphology of cells was examined using SEM, with hGFs being cultivated on the discs for periods of 24 h, 72 h, and 7 days. Cells were seeded at a density of 1×10^4 cells/disc in 96-well plates. The cultured cells were incubated at specified time points at 37°C in a 5% CO₂ atmosphere. Loosely attached cells were eliminated from the experimental wells by rinsing twice with PBS 1 $\times$, while the adherent cells were fixed with 3.5% glutaraldehyde in 0.1 M NaCaCO buffer for 24 h and then stored at 4 °C, followed by post-fixation in 2% OsO₄ made in the same buffer. The specimens were rinsed in distilled water, dehydrated through successive incubation in increasing concentrations of ethyl alcohol (50%, 70%, and 100%), and then prepared with HMDS before air-drying overnight at RT. The dried specimens were affixed to SEM stubs with double-sided carbon tape and sputter-coated with gold (150 Å) prior to imaging using the Phenom XL microscope. Adherent cells on the surfaces were analyzed

at a magnification of 420 $\times$, and the percentage of the covered area was determined by examining a minimum of three distinct fields for each image utilizing ImageJ software. Images were captured with an accelerating voltage of 15 kV using the BSD Full detector.

Immunofluorescence Analysis for Cell Adhesion

Immunofluorescence analysis was further employed to identify the most effective surface for enhancing cell adhesion over a period of 72 h. The immunofluorescence experiments were conducted using hGFs seeded on titanium discs within the Nunc[®] Lab-Tek[®] Chamber Slide[™] system (cat. C7182, Sigma-Aldrich, St. Louis, MO, USA) at a density of 1×10^4 cells/disc. Following a 72-h culture period, the cells were fixed in 4% paraformaldehyde (cat. J61899.AK, Thermo Fisher Scientific, Waltham, MA, USA) for 10' at RT. They were then permeabilized using a solution comprising HEPES (cat. H3375, Sigma-Aldrich, St. Louis, MO, USA; 1:1000 for 15' at RT 20 mM, pH 7.4), saccharose (300 μ M), NaCl (50 mM), MgCl₂ (3 mM), and Triton X-100 (0.5%; cat. X100, Sigma-Aldrich, St. Louis, MO, USA) for 10' at RT. Subsequently, the specimens were incubated with Alexa Fluor[®] 488 Phalloidin (cat. 8878, Cell Signaling Technology, Milan, Italy; 1:50 for 1 h at RT) to visualize F-actin within the cytoskeleton. The nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; cat. D9542, Sigma-Aldrich, St. Louis, MO, USA; 1:1000 for 15' at RT) before observation under a confocal microscope (Zeiss LSM-800, Carl Zeiss Meditec AG, Oberkochen, Germany) at a magnification of 20 $\times$. The cell count was determined by analyzing a minimum of three distinct fields for each image using ImageJ software.

Expression of Mechanotransduction Markers

The impact of surface micro-topography on the expression levels of FAK, YAP, and TAZ, along with their phosphorylated forms in hGFs, was evaluated through flow cytometry analysis. Following culture periods of 24 h, 72 h, and 7 days, the cells were detached using a trypsin/EDTA solution (5 mM), collected in PBS 1 $\times$, and subjected to centrifugation at 1500 rpm for 5' at 4 $^{\circ}$ C. The cells were subsequently resuspended in a fixation solution (BD[™] FACST[™] Lysing Solution) at a dilution of 1:10, followed by permeabilization with BD FACST[™] Permeabilizing Solution, also at a dilution of 1:10, for 5' in darkness. All sample groups, excluding negative controls, underwent incubation with primary antibodies—specifically, anti-FAK (1:100), anti-YAP (1:50), anti-TAZ (1:50), anti-pYAP (1:200), and anti-pTAZ (1:500)—for 30' at 4 $^{\circ}$ C in darkness. Subsequent to this, analogous incubation parameters were utilized for Alexa Fluor 488- and 647-

conjugated secondary antibodies before collecting the samples in PBS 1× for subsequent analysis using the FACS Canto II flow cytometer. The data obtained were analyzed using FACSDiva version 6.1.3 and IDEAS software, with the results expressed as MFI ratios, calculated by dividing the MFI of positive events by that of negative events, specifically the MFI of the secondary antibody.

Statistical Analysis

All experimental data are presented as the mean $\pm$ standard deviations. Each experiment was conducted in both technical and biological triplicates, utilizing a minimum of three distinct primary culture strains of hGFs. Statistical analyses were performed using a two-way analysis of variance (ANOVA), followed by Tukey's test for post hoc comparisons. *P*-values ≤ 0.05 were considered to be statistically significant. Data analysis and graphing were executed using GraphPad Prism Software Analysis version 9.

4.2.1.2. RESULTS

Surface Topography

The SEM images provided a comprehensive macroscopic view of the surfaces of the discs (**Figure 8**).

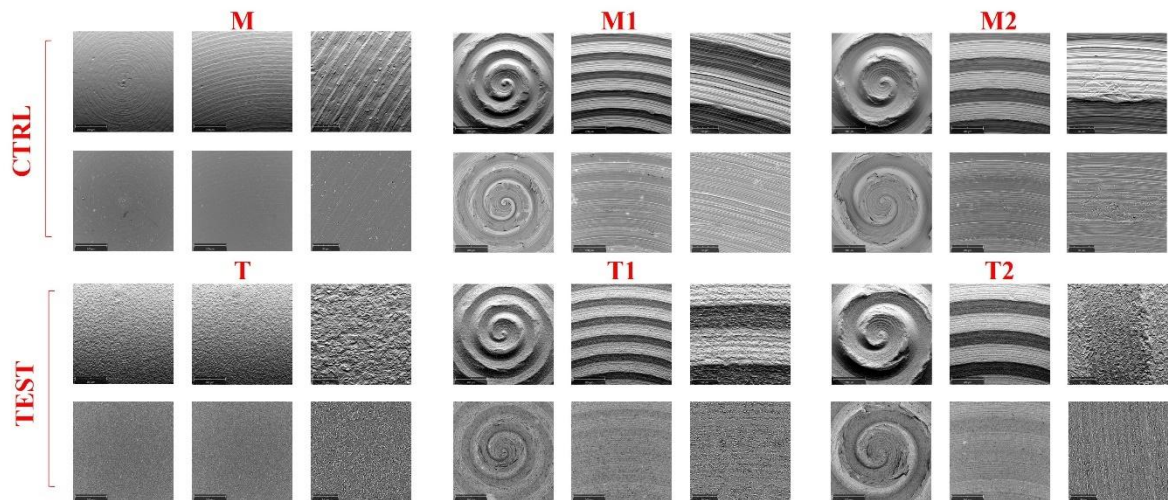


Figure 8. Representative SEM images of the six distinct surface types examined in this study. The upper panels of both CTRL and TEST groups were captured using the TOPO A program, while the lower panels utilized the backscattered electron detector (BSD) Full program. Magnification 420×; scale bar: 200 μ m.

Images captured at a magnification of 420× revealed that the machined surfaces

were characterized by circular micro-threads and exhibited a mild roughness. The treated surfaces presented noticeable topographic changes as a result of the double acid treatment, which prominently enhanced the superficial roughness, as previously established in the literature [105]. Additionally, analysis of the SEM images allowed for the identification of distinct macro-structural features resulting from variations in micro-thread pitch and thread depth among M1, T1, M2, and T2.

Surface Wettability

The evaluation of the wetting properties demonstrated an overall hydrophilicity of the surfaces, as indicated by their contact angles measuring less than 90°. The M and T surfaces displayed the lowest and highest contact angles, respectively, with values of $61.11^\circ \pm 2.69$ and $83.24^\circ \pm 1.13$ ($p < 0.0001$). The intermediate contact angles were observed for M1 ($73.02^\circ \pm 2.67$), T2 ($74.15^\circ \pm 2.93$), T1 ($75.62^\circ \pm 2.55$), and M2 ($75.55^\circ \pm 0.91$), which exhibited increasing contact angles; however, no significant differences were noted among these surfaces. Conversely, T1 and T2 presented statistically significant differences in contact angle when compared to T ($p < 0.05$ and $p < 0.01$, respectively), as did M1 and M2 in relation to M ($p < 0.001$ and $p < 0.0001$, respectively) (**Figure 9**).

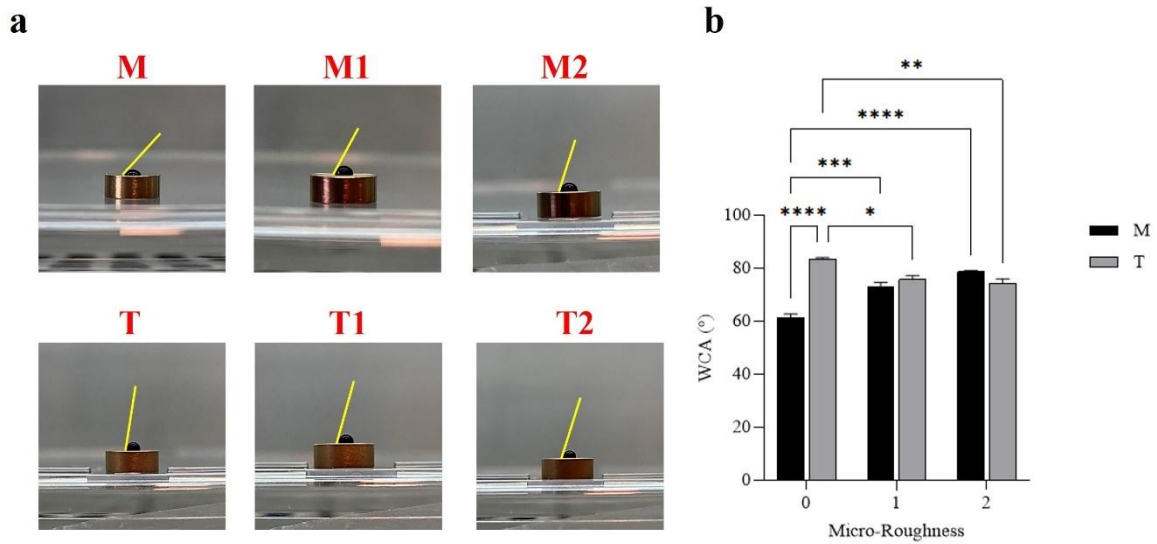


Figure 9. Measurements of the water contact angle (WCA) on the surfaces using the sessile drop method. (a) Representative images of the measurements utilizing ImageJ software; (b) The average WCA values for the examined surfaces. Data are presented as the mean $\pm$ standard error of the mean ($n \geq 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Cell Viability on Surfaces

Before the initiation of cell viability experiments, isolated hGFs were

characterized for their phenotypic properties. As detailed in **Table 1**, the cells exhibited expression of typical fibroblast markers, specifically CD105, CD73, and CD90, while lacking expression of CD45 (the leukocyte common antigen) and CD326 (epithelial cell adhesion molecule).

Table 1. Flow cytometry analysis conducted on the hGF phenotype. “+”: positive expression; “-”: negative expression.

Markers	Expression Levels
CD 105	+
CD 73	+
CD 90	+
CD 45	-
CD 326	-

The MTT analysis indicated that all surfaces exhibited complete biocompatibility and did not have any negative effects on the viability of hGFs at each evaluated time point. Notably, the T1 surface provided the most favorable conditions for cell viability and proliferation at the 72-h mark ($p < 0.05$ versus T; $p < 0.0001$ versus T2), although outcomes similar to those of M1 were also recorded. Furthermore, at the same time point, M1 demonstrated statistically significant higher values compared to M ($p < 0.05$) and M2 ($p < 0.001$). A significant difference was also identified between T and T2, with T exhibiting significantly higher values ($p < 0.01$). No significant differences were observed among the surfaces at the 24-h and 7-day time intervals (**Figure 10**).

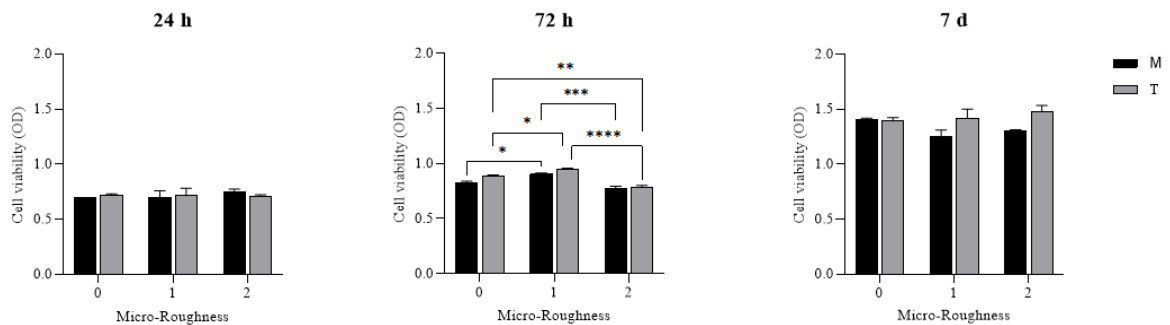


Figure 10. Evaluation of hGF viability on the surfaces examined after 24 h, 72 h, and 7 days of culture. The optical density (OD) was determined at 540 nm utilizing a microplate spectrophotometer (SpectraMAX 190). The data are expressed as the mean $\pm$ standard error of the mean ($n \geq 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Cell Adhesion through SEM and Immunofluorescence

The SEM analysis performed on hGFs seeded on the surfaces after 24 h, 72 h, and 7 days showed the effect of surface topography on morphology and adhesion of hGFs

(Figure 11).

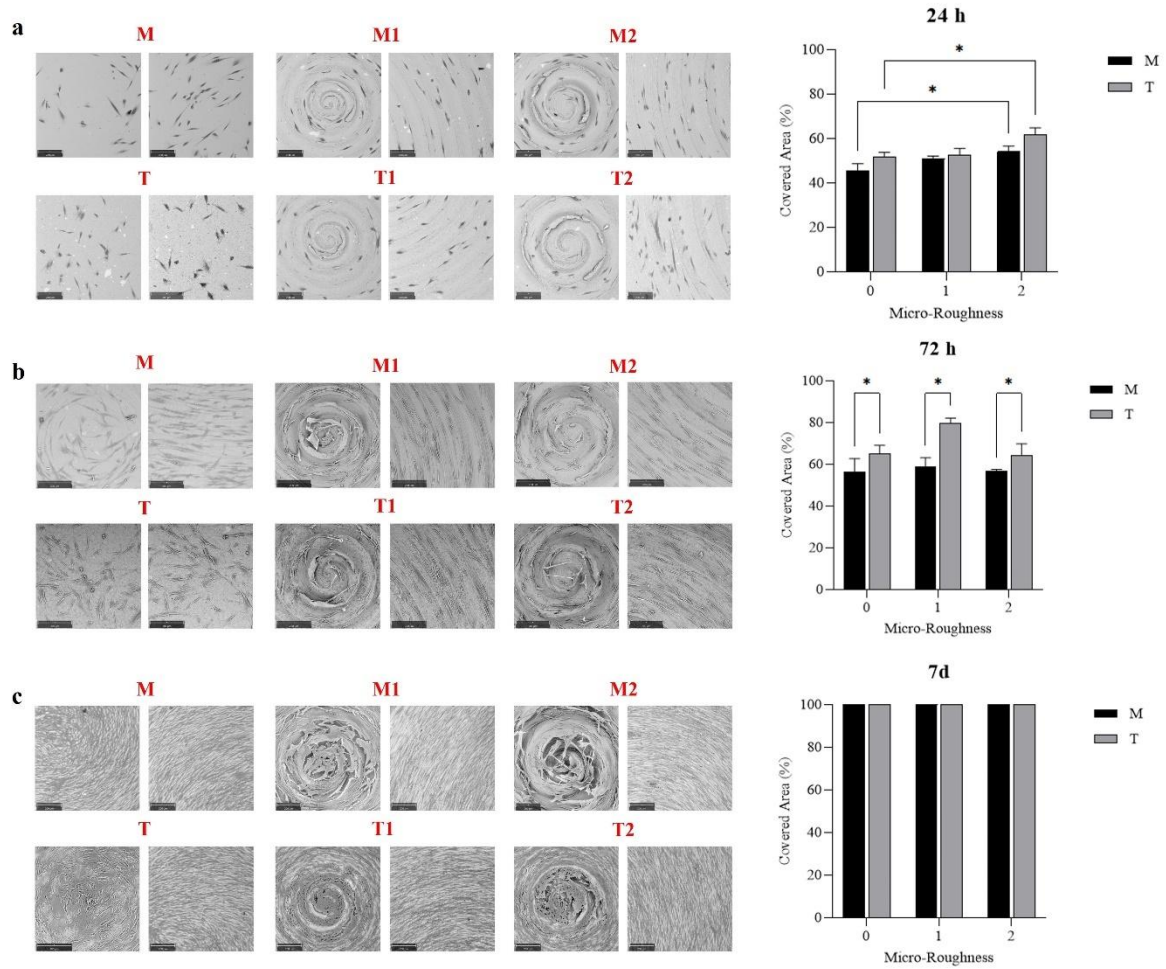


Figure 11. Representative central and peripheral views of the disc surfaces and adherent hGFs following incubation periods of (a) 24 h, (b) 72 h, and (c) 7 days of culture. Additionally, a quantitative evaluation of the percentage of covered area was conducted using ImageJ software. The images were captured using the BSD Full detector. Data were obtained by evaluating a minimum of three distinct fields for each image with ImageJ software. The results are presented as the mean $\pm$ standard error of the mean ($n \geq 3$). * $p < 0.05$. Magnification 420 $\times$; scale bar: 200 μ m.

The SEM analysis conducted after 24 h (**Figure 11a**) demonstrated that hGFs exhibited significantly elevated adhesion on M2 ($p < 0.05$ versus M) and T2 ($p < 0.05$ versus T) surfaces, along with all T surfaces at 72 h when compared to their respective M counterparts ($p < 0.05$). It is noteworthy that T1 displayed superior adhesion among the TEST surfaces (**Figure 11b**). Moreover, after 7 days, a uniform cell monolayer was consistently observed across all surfaces (**Figure 11c**).

In terms of morphological characteristics, hGFs assumed a polygonal spindle-shaped appearance and effectively spread over all surfaces with greater micro-roughness. In contrast, the cells appeared more rounded on the machined surfaces (M and T).

Furthermore, an improvement in cell adhesion was consistently observed on TEST surfaces subjected to DAE treatment, accompanied by the formation of filopodia and lamellipodia structures.

Due to the inability to clearly distinguish which surface enhanced hGF adhesion more effectively than the others through SEM analysis, the data collected at the 72-h interval were subjected to further examination via immunofluorescence analysis. This additional evaluation aimed to identify the surface among the TEST variants that optimally improve cell adhesion. The results indicated that surface T1 emerged as the superior performer among the various TEST surfaces, as determined by the assessment of the number of stained cells present on the surface. This finding demonstrated statistical significance ($p < 0.05$) in comparison to surface M1, as illustrated in **Figure 12**.

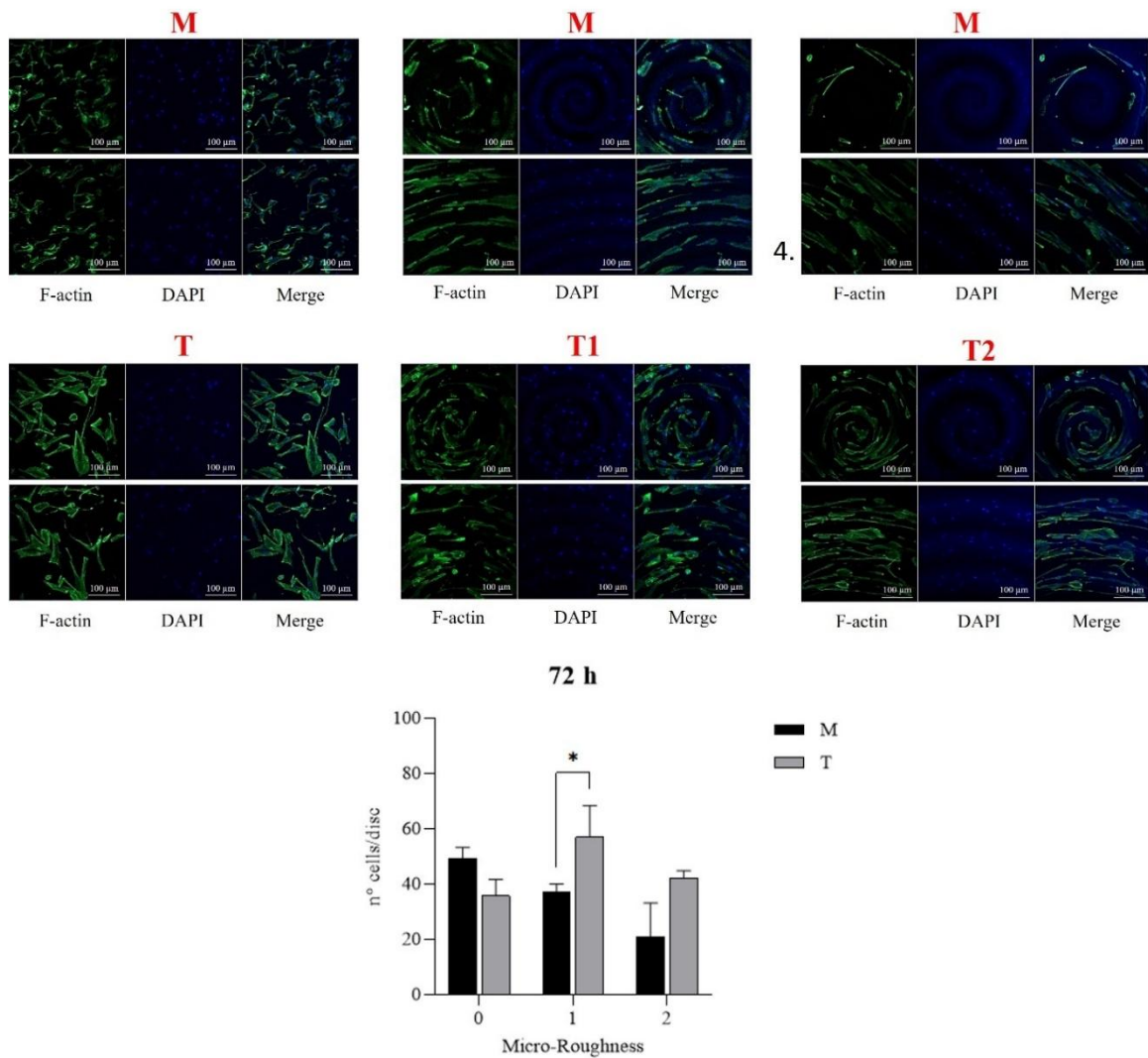


Figure 12. Representative immunofluorescence images and corresponding graphical representation illustrating the adherence of hGFs on the examined surfaces. F-actin has been visualized using Alexa Fluor® 488 Phalloidin (green), while nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). The data are expressed as the mean $\pm$

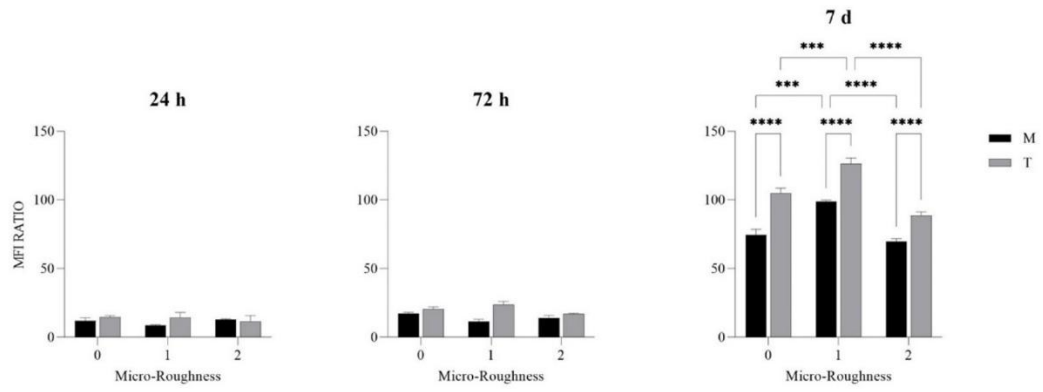
standard error of the mean ($n \geq 3$), with a minimum of three distinct fields analyzed per image. $*p < 0.05$. Magnification 20 $\times$; scale bar: 100 μm .

Activation of Mechanotransduction Markers on Surfaces

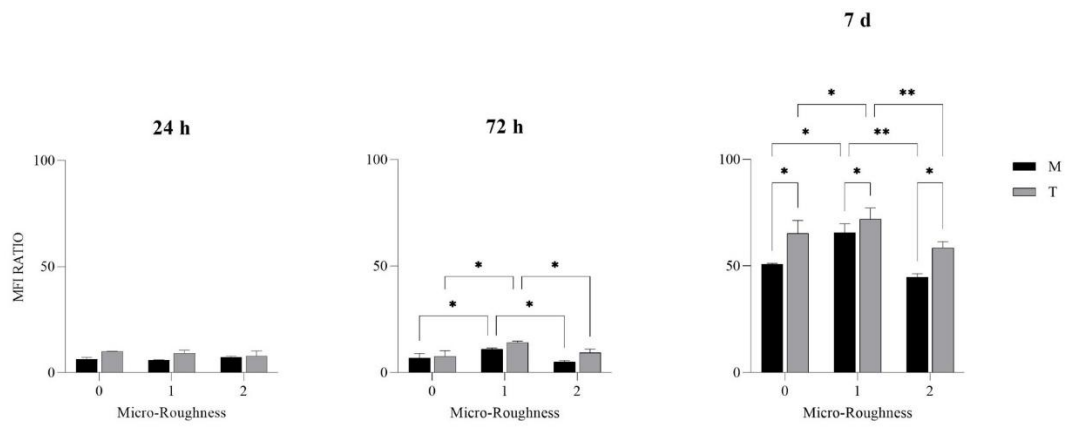
The previous findings were further corroborated by the activation of the Hippo pathway, as indicated by the significantly higher protein expression levels of FAK, YAP, and TAZ (**Figure 13a**) at various time points on the T1 surface when compared to the corresponding untreated surface (M1). Specifically, significant differences were observed at 7 days for FAK ($p < 0.0001$) and YAP ($p < 0.05$), as well as at 24 h and 72 h for TAZ ($p < 0.05$ and $p < 0.01$, respectively). Conversely, reduced levels of pTAZ were recorded at each time point, along with significantly lower levels of pYAP observed at 72 h and 7 days ($p < 0.05$ and $p < 0.01$, respectively) (**Figure 13b**). Additionally, the ratios of pYAP/YAP at 24 h and 72 h exhibited significantly lower values ($p < 0.05$), as did the ratios of pTAZ/TAZ at the same time intervals ($p < 0.01$ and $p < 0.05$, respectively) when compared to M1 (**Figure 13c**). While not achieving statistical significance, the T1 surface was shown to positively activate the pathway, even at all other measured time points.

a

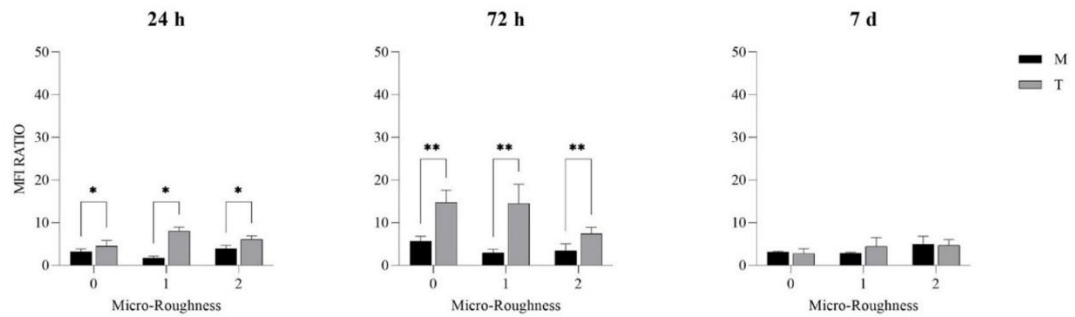
FAK



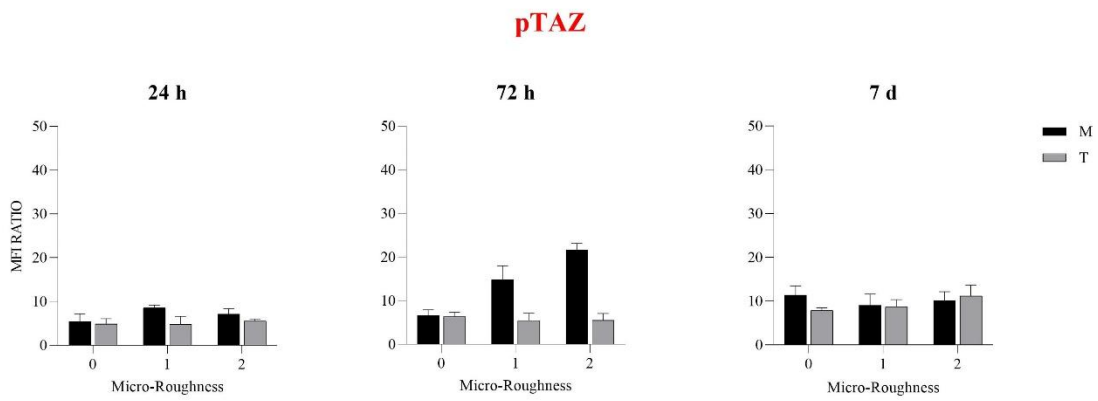
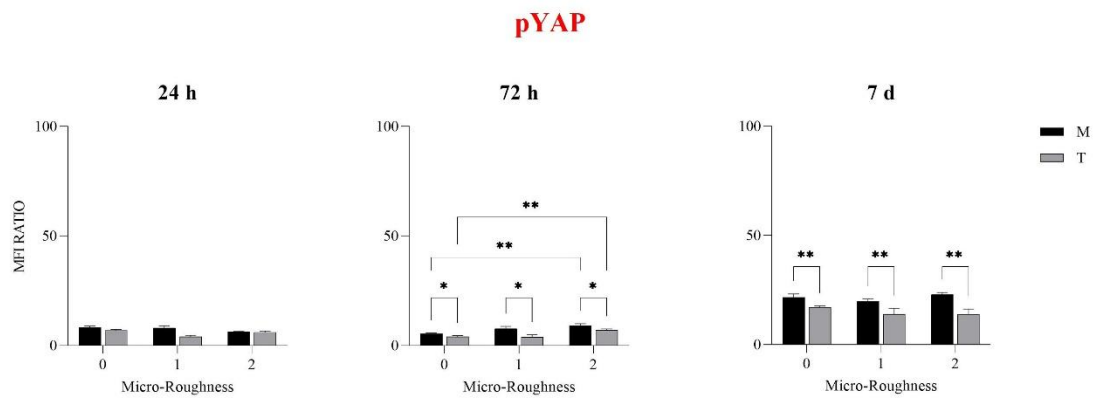
YAP



TAZ



b



c

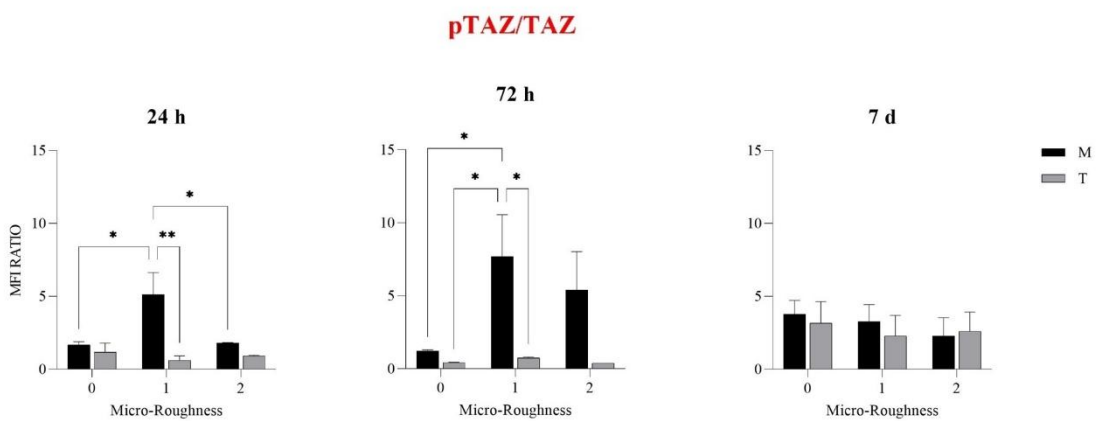
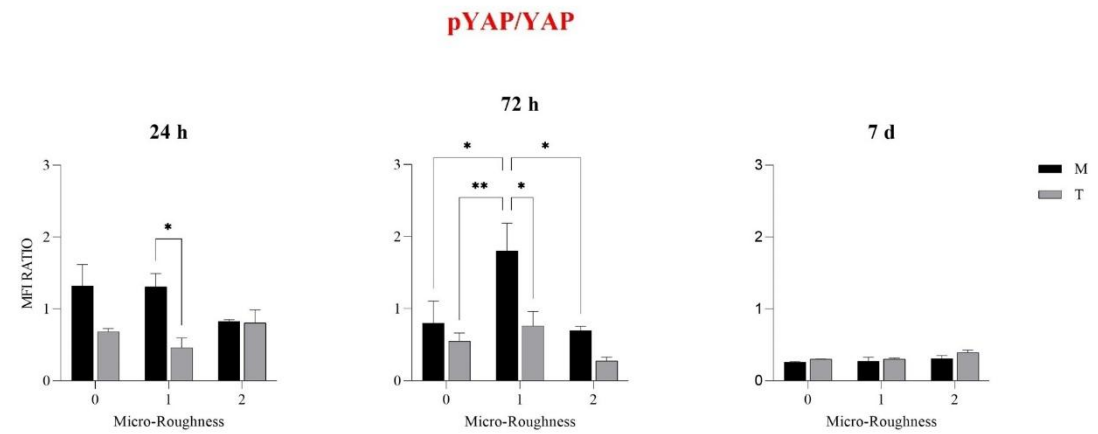


Figure 13. Bar graphs illustrating the protein expression levels of (a) activated and (b) inactivated (phosphorylated) forms of YAP and TAZ, along with (c) the ratios of pYAP/YAP and pTAZ/TAZ. These measurements were conducted in hGFs cultured on varying implant surfaces after 24 h, 72 h, and 7 days. The data are presented as the mean $\pm$ standard error of the mean ($n \geq 3$) and expressed as MFI ratios. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In addition, the T1 surface demonstrated a significantly elevated expression of FAK in comparison to the T ($p < 0.001$) and T2 ($p < 0.0001$) surfaces at the 7-day mark. Furthermore, YAP expression was significantly increased at 72 h ($p < 0.05$ versus T and T2) and at 7 days ($p < 0.05$ versus T and $p < 0.01$ versus T2) relative to the surfaces mentioned above.

It is pertinent to note that FAK expression in hGFs was also significantly upregulated at 7 days when comparing the other TEST surfaces to their CTRL surfaces ($p < 0.0001$ with T versus M and T2 versus M2). At this same time point, additional statistical differences were observed, with M1 exhibiting higher values than M ($p < 0.001$) and M2 ($p < 0.0001$). A similar pattern was noted for YAP expression, as all TEST surfaces showed significantly elevated levels compared to CTRL surfaces ($p < 0.05$). Additionally, M1 exhibited significantly higher values when compared to M ($p < 0.05$) and M2 ($p < 0.01$) after 7 days. Moreover, YAP expression at 72 h indicated a significant upregulation on the M1 surface when compared to M and M2 ($p < 0.05$).

Furthermore, all TEST surfaces illustrated a significant downregulation of pYAP expression at both 72 h ($p < 0.05$) and 7 days ($p < 0.01$) compared to their respective CTRL groups. Moreover, at 72 h, the M2 and T2 groups showed statistically significant higher values than the M and T surfaces ($p < 0.01$). The ratio of phosphorylated YAP and TAZ on their total protein content did not reveal other statistical differences between the TEST and CTRL groups, except for the T1 surface. However, the M1 surface demonstrated significantly higher pYAP/YAP and pTAZ/TAZ expression levels than M and M2 ($p < 0.05$) at 72 h. Notably, pTAZ/TAZ also exhibited a significant upregulation at 24 h, when comparing the M1 surface to M and M2 ($p < 0.05$).

4.2.1.3. DISCUSSION

The interaction between surface topography and cellular behavior is crucial in fields such as tissue engineering and regenerative medicine, particularly concerning the development of implantable devices. The physical and chemical properties of implant surfaces significantly influence cellular responses, including adhesion, proliferation, and

differentiation, which are essential for successful integration into biological systems [19,106]. Recent advancements in dental implant surfaces have underscored the importance of optimizing surface roughness and wettability to enhance cellular activities and overall biocompatibility. Such optimization promotes effective healing and regeneration of soft tissue, thereby ensuring a biological seal against microbial adhesion and infiltration and facilitating the adequate integration and maintenance of oral soft tissue around the implant abutment over time [19,92]. HGFs are integral to establishing and maintaining an efficient soft tissue seal around dental implants, as they contribute numerous ECM components and are involved in the inflammatory response [60].

This study aimed to investigate the effects of various surface modifications on hGFs, specifically focusing on how distinct topographic features influence cellular behavior and mechanotransduction. Surface characterization was conducted utilizing SEM to reveal changes in micro-architecture, while cellular assays were employed to evaluate fibroblast adhesion and viability across six different surface treatments. Specifically, the response of hGFs to DAE-treated surfaces with varying roughness (TEST) was compared to machined surfaces with analogous roughness (CTRL). The findings provided a deeper understanding of how engineered surfaces can be optimized to enhance cell function, thus offering valuable insights into the design of more effective abutment surface topographies for clinical applications.

The SEM images of the CTRL and TEST surfaces (**Figure 8**) indicated that the machined substrates exhibit circular micro-threads with minimal roughness, whereas the surfaces subjected to DAE demonstrate significant topographic alterations characterized by increased roughness. This alteration aligned with existing literature that highlights the importance of surface topology in mediating biological responses, particularly in the context of dental and orthopedic implants [107]. It is noteworthy that the enhanced roughness observed on TEST surfaces was likely to promote increased protein adsorption and cellular adherence, both of which are critical factors in tissue integration and overall healing [108]. However, it is critical to consider that excessively rough surfaces may be undesirable, as they have been shown to promote an increased likelihood of biofilm formation [109]. In contrast, a relatively smooth surface in the neck area has been documented in some studies to create an optimal interface for the tight fit of the mucosa, thereby mitigating the risk of infections [110].

The evaluation of surface wettability indicated distinct hydrophilic properties among the analyzed surfaces, as evidenced by water contact angles measuring below 90°

(**Figure 9**). The measured contact angles suggested that the M and T surfaces demonstrate the lowest and highest wettability, respectively ($61.11^\circ \pm 2.69$ and $83.24^\circ \pm 1.13$; $p < 0.0001$), while the intermediate values recorded for the M1, T2, T1, and M2 surfaces indicate a potential for enhanced interaction with fibroblasts. The established correlation between surface hydrophilicity and improved fibroblast adhesion and proliferation has been well-documented, affirming that surfaces with lower contact angles typically enhance cell attachment [111].

The results of the viability assays, performed using the MTT method, indicated that all surfaces were biocompatible and supported hGF viability (**Figure 10**). Particularly notable was the T1 surface, which provided superior conditions for cell viability at the 72-h assessment ($p < 0.05$ versus T; $p < 0.0001$ versus T2). This observation is supported by evidence indicating that surface characteristics, including optimal roughness and chemical composition, can significantly influence not only initial cell attachment but also subsequent cellular proliferation [112].

Furthermore, the cell adhesion data obtained from SEM and immunofluorescence analyses (**Figure 11** and **Figure 12**) corroborated that the enhanced surface roughness and hydrophilicity of the TEST surfaces, particularly T1, significantly foster superior hGF adhesion. The observation of distinct cellular morphology, characterized by increased spreading and the development of cytoplasmic extensions such as filopodia and lamellipodia on these surfaces, further suggested an optimal environment conducive to cell growth. These morphological alterations emphasized the critical role of surface nano-architecture in supporting cellular dynamics [105,113]. The literature supports these findings, demonstrating that TiN surfaces with comparable roughness exhibited increased rates of proliferation and adhesion of hGFs, especially after 72 h, thereby establishing TiN abutments as favorable options in aesthetically challenging areas [114]. Additionally, it is important to highlight that the TiN coating also provides functional advantages by repelling negatively charged bacteria, thus isolating the implant while preserving its intrinsic properties and biomechanical functionality [104].

Moreover, there was a marked activation of mechanotransduction markers, such as FAK and the Hippo pathway components YAP and TAZ, on the T1 surface relative to its CTRL surface (**Figure 13**). The upregulated protein expression levels of FAK indicated enhanced mechanotransduction signaling, which is vital for cell adhesion, proliferation, and differentiation [80]. The significant activation of YAP and TAZ suggested that the T1 surface creates a favorable environment that not only promotes cell viability but also

enhances signaling pathways essential for tissue regeneration [115]. The observed reduction in the levels of phosphorylated YAP and TAZ across all TEST surfaces in comparison to the CTRL surfaces indicated a transition towards an active YAP/TAZ signaling state that promotes gene expression essential for cellular growth and survival, particularly regarding fibroblastic activity. This phenomenon is crucial, as YAP/TAZ signaling has been implicated in the regulation of cell proliferation and tissue growth [62]. The significant increases in the ratios of pYAP/YAP and pTAZ/TAZ observed at 24 h and 72 h further corroborated the hypothesis that surface modifications on the T1 surface can influence cellular signaling pathways that regulate tissue homeostasis and repair mechanisms.

In conclusion, the results collectively demonstrated that surface topography and wettability play pivotal roles in mediating hGF adhesion, proliferation, and the activation of mechanotransduction pathways [116]. The information derived from SEM and immunofluorescence analysis provided a detailed understanding of the relationship between surface characteristics and the biological responses of hGFs seeded on these surfaces. The findings revealed significant differences in the macroscopic and microscopic properties of the six surface types examined in this study, thereby contributing valuable insights regarding how these variations impact cell behavior and viability. The T1 surface, characterized by adequate roughness and favorable wetting properties, emerged as an optimal substrate for promoting fibroblast interactions, thus supporting its potential application in tissue engineering and regenerative oral therapies.

This *in vitro* investigation identified the T1 surface as the optimal condition for enhancing hGF viability, adhesion, and mechanotransduction compared to other surfaces. Consequently, it was proposed as the candidate for the abutment surface to be evaluated *in vivo* with the aim of determining the optimal abutment macro-morphology that ensures superior soft tissue attachment and integration with the implant, thereby facilitating desirable healing outcomes. Additionally, it was hypothesized that a concave implant neck could induce spontaneous alignment of the collagenous network, thereby influencing fibroblast polarization, migration, and the direction and arrangement of fiber growth. To validate this hypothesis, a proof-of-principle animal study was designed to investigate the structure and distribution of collagen fibers and bundles in the peri-implant soft tissues. This study compared two implants with identical bodies but varied in their healing abutment geometries. The experimental abutment was designed with a concave area above

the implant platform, selected based on positive findings reported in the previous *in vitro* study as well as in animal and clinical studies involving similar concave designs [99,101]. In contrast, the control healing abutment utilized a parallel-walled configuration.

In vivo and ex vivo Experimentation [89]

4.2.1.4. MATERIALS AND METHODS

Implant Characteristics

All implants utilized in this study were of a tapered shape (IK Internal Hexagon, RESISTA[®] Company, Ing. Carlo Alberto Issoglio and C. S.r.l., Omegna, Italy). The test abutments (TEST) were designed with a concave portion measuring 2 mm in height and featured a DAE surface, corresponding to the T1 surface used in the *in vitro* assessment. In comparison, the control abutments (CTRL) exhibited a parallel-walled design, also with a DAE surface. Both implants possessed a switching platform with a diameter of 4 mm and a total length of 10 mm (**Figure 17**).

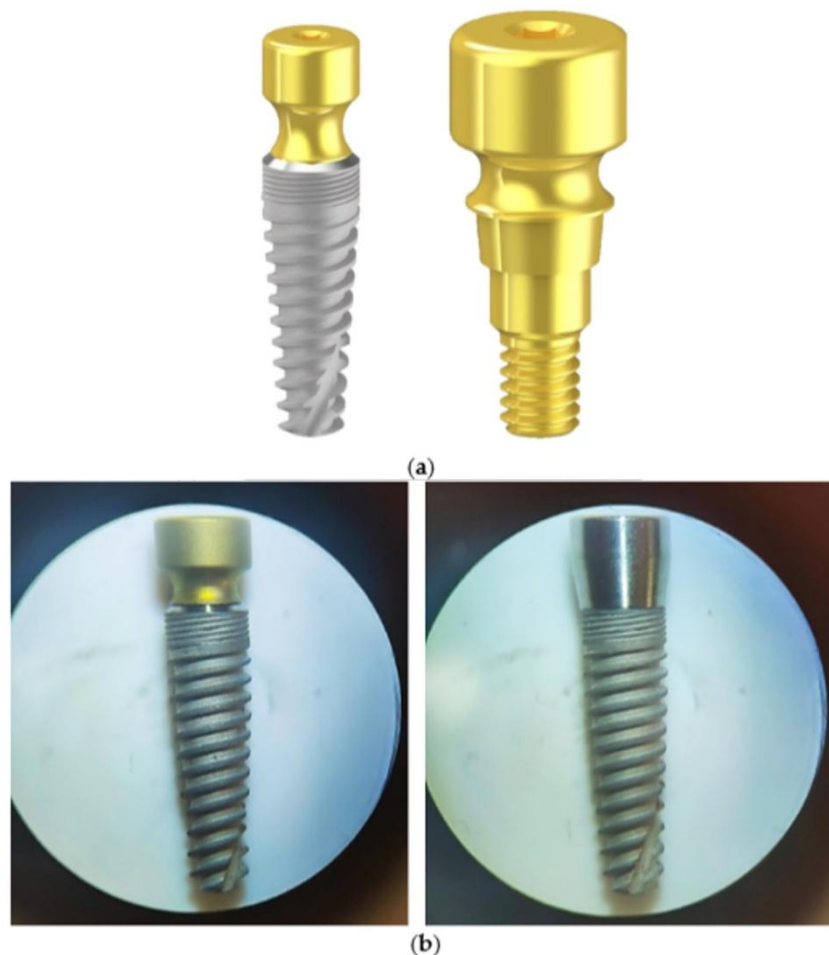


Figure 17. (a) Schematic representation of the test implant and abutment (TEST); (b) Optical microscopy images depicting the TEST implant (on the left) and the control implant (CTRL; on the right).

Ethical Statement

This animal study received approval from the Animal Ethical Committee of the

Junta de Andalucía, Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible on 14 December 2021 (n° 29/11/2021/184). The animal study and associated procedures were conducted in compliance with the animal protection laws of Spain and adhered to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines [117], utilizing a randomized prospective design.

Experimental Animals and Housing

Two swine (*Sus scrofa*), with an average age of three years, were acclimatized for a duration of three weeks prior to the commencement of the study. The animals were identified through the use of ear tags. They were provided with an antibiotic-free diet that was softened by soaking in water and had continuous access to water *ad libitum*. The individual responsible for animal welfare managed the aeration and administration of food and water, as well as monitoring the animals' behavioral and health conditions throughout the study period. The entire study was conducted under the supervision of a veterinarian, and all surgical procedures were performed by surgeons with extensive experience in the field.

Experimental Design

Animals underwent the placement of implants within the left or right mandibular alveolar ridges. These implants were strategically positioned either in the physiologically mature bone between the lower canine and first premolar or within the mandibular premolar region at tooth extraction sites. Each implant received a healing abutment at the time of placement, followed by a designated healing period of 12 weeks. Each subject animal had a total of six implants inserted, comprising three per hemimandible. The CTRL and TEST implants were arranged symmetrically across the jaw in a controlled manner.

At the end of the 12-week healing period after implant placement, all animals were euthanized, resulting in a cumulative total of 12 implants placed. However, two CTRL implants and two TEST implants were excluded from subsequent analysis due to early implant failure. Ultimately, a total of eight implants, consisting of four CTRL implants and four TEST implants, were analyzed.

Surgical and Terminal Procedures

Prior to surgical intervention, all animals were subjected to an overnight fasting period and subsequently weighed.

On the day of surgery, each animal was anesthetized with intramuscular

administration of medetomidine at a dosage of 0.05 mg/kg in conjunction with Zoletil (a combination of zolazepam and tiletamine) at 3 mg/kg. Subsequently, inhalation of a mixture of 2–5% isoflurane and oxygen was initiated via a mask. The animals were then transferred to the surgical area and intubated using an endotracheal tube, after which general anesthesia was maintained with 2–5% isoflurane. Throughout the surgical procedures and the postoperative period, monitoring of heart rate, blood oxygen saturation, and blood pressure was conducted continuously. All surgical operations were carried out under aseptic conditions within a designated animal operating theater while animals remained under general anesthesia. The tooth extraction procedure was performed meticulously; gentle pressure was applied to the gingival sulcus utilizing a small periosteal elevator. Subsequently, the mandibular premolars and molars were sectioned in a buccolingual orientation at the furcation of the mesial and distal roots, employing a rotary instrument for this task. A straight elevator was then utilized to verify the sectioning, followed by the elevation and removal of both the mesial and distal roots using dental forceps. In mature sites, a No. 15c blade was employed to create a midcrestal incision situated between the canine and the first premolar. A full-thickness mucoperiosteal flap was elevated, and dental implants were positioned with a minimum spacing of 1.5 mm from adjacent teeth, adhering strictly to the manufacturer's drilling protocol. Healing abutments were placed, and the surgical site was sutured closed with 4–0 silk sutures, ensuring that all implants were positioned equicrestally without the application of bone graft material.

During the initial days following the surgery, routine monitoring of the animals was conducted, with additional analgesia administered as deemed necessary. Postoperative surgical pain was managed with buprenorphine hydrochloric acid (HCl), dosed at 0.12–0.24 mg/kg and administered subcutaneously. The animals were euthanized 12 weeks post-surgery. After sedation with the aforementioned agents, cardiac arrest was induced through intravenous administration of 110 mg/kg pentobarbital at each designated time point.

Sample Preparation

Block sections were obtained utilizing an oscillating autopsy saw to preserve the integrity of the soft tissue. Samples of peri-implant soft tissues designated for histological analysis were fixed through immersion in 10% buffered formalin (HT501128, Sigma-Aldrich, St. Louis, MO, USA), followed by dehydration with escalating alcoholic rinses. The specimens were ultimately embedded in glycol-methacrylate resin (Technovit 7200

VLC, Wehrheim, Germany). The processing of these specimens adhered to the methodology established in a prior study conducted by Iezzi and collaborators [7]. In summary, the tissues were sectioned along their longitudinal axis to yield histological longitudinal sections of the peri-implant tissues. The histological examination was performed under a light microscope (Laborlux S, Wetzlar, Germany) equipped with a high-resolution video camera (3CCD, JVCKY-F55B, JVC, Yokohama, Japan) and connected to a computer.

Conversely, samples of peri-implant soft tissues intended for transmission electron microscopy (TEM) analysis were sectioned along their longitudinal axis and subsequently preserved in a solution of 3.5% glutaraldehyde in a 0.1 M NaCaCO buffer.

Transmission Electron Microscopy (TEM)

All implants and associated adjacent peri-implant soft tissues were meticulously excised from the mandible of each swine specimen. Eight specimens were retrieved, with four specimens surrounding CTRL abutments and four specimens surrounding TEST abutments. These specimens were fixed at RT with 3.5% glutaraldehyde in a 0.1 M NaCaCO buffer (pH 7.2) and subsequently stored at 4 °C in the fixative until shipment. Careful dissection was performed to obtain small portions of the fixed soft tissues from the vicinity, which were subsequently rinsed in a 0.1 M NaCaCO buffer. This was followed by post-fixation with 2% OsO₄ in the same buffer for 1 h, block-staining using saturated uranyl acetate, and rapid dehydration through graded ethanol and acetone. The specimens were then embedded in epoxy resin (Epon 812; cat. 45345, Sigma-Aldrich, St. Louis, MO, USA) [118]. For TEM examination, ultrathin sections, approximately 40 nm in thickness, were cut utilizing a Leica Ultracut R microtome (Leica Microsystem, Wetzlar, Germany) with a Diatome diamond knife (Diatome Ltd., Biel, Switzerland). Following double staining with uranyl acetate and lead citrate, these sections were analyzed at 60 kV using an FP 505 Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic), which was equipped with a Megaview III digital camera (Olympus, Tokyo, Japan) and Soft Imaging System (GmbH, Munster, Germany).

For the qualitative and quantitative analysis employing TEM, small samples were procured from a ring of tissue meticulously dissected surrounding the abutment area. The ultrastructural examination of collagen bundles within the peri-implant soft tissues primarily focused on images illustrating the cross-sectional configuration of the collagen fibers. Observations were limited to sample regions adjacent to the abutment surface,

specifically the CTRL group (n = 4) and the TEST group (n = 4).

For the quantitative analysis, 15 micrographs/group were randomly selected from non-overlapping regions at a magnification of 7100 $\times$. These micrographs were utilized for the following quantitative assessments: (i) in each micrograph, the total area covered by collagen bundles was determined by outlining each bundle using the Soft Imaging System. The values measured for all bundles were subsequently summarized mathematically. Only cross-sectioned bundles with a minimum size of 0.5 μm^2 , wherein collagen fibers were clearly distinguishable and not oriented longitudinally, were included in the analysis. Given that each micrograph at 7100 $\times$ magnification encompasses an area of 142.6 μm^2 , the relative presence of collagen bundles (%) within each sample was calculated by dividing the aggregate area of outlined collagen fibers (in μm^2) by the total area of analyzed samples (i.e., 142.6 μm^2 multiplied by 15 micrographs). (ii) Additionally, in each micrograph, the number of longitudinally oriented collagen bundles of varying sizes was quantified and reported as the mean $\pm$ standard error of the mean within a 100 μm^2 area.

Statistical Analysis

In all comparisons conducted between the CTRL and TEST conditions, the data were found to be not normally distributed and were thus analyzed utilizing a non-parametric t-test (Mann–Whitney test). The experimental values were processed using the statistical software package GraphPad Prism Software Analysis version 9. The statistical significance of the differences between the groups was established at a threshold of $p < 0.05$. Data are presented as the mean $\pm$ standard error of the mean or as standard deviation.

4.2.1.5. RESULTS

Radiographic Analysis

At the conclusion of the 12-week observation period, proper histologic healing was noted around both the CTRL and TEST implants (**Figure 18**). Additionally, the radiographic assessments indicated good osseointegration of the implants.

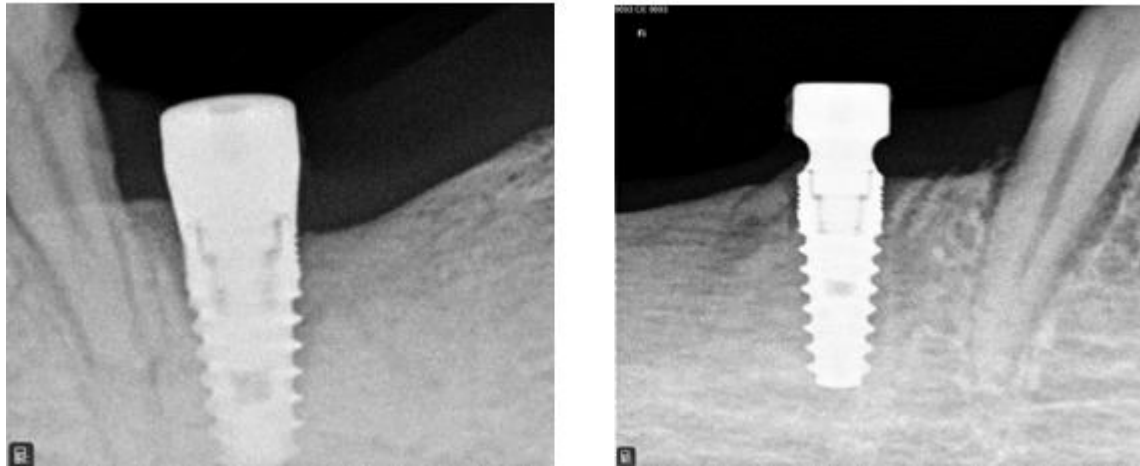


Figure 18. Representative intraoral radiographs from a 3-month follow-up after the placement of implants in the extraction sites of two premolars. On the left, a CTRL implant with a parallel-walled abutment, while on the right, a TEST implant with a concave abutment. Both implants demonstrated complete osseointegration.

Histological Analysis

For the purpose of histological analysis, longitudinal undecalcified sections of the connective tissue were obtained. The histological results (**Figure 19**) indicated the presence of peri-implant soft tissues in close association with both the TEST and CTRL abutments. Notably, although this observation is primarily qualitative, the soft tissue appeared to demonstrate a greater adherence to the concave abutment in comparison to the straight abutment.

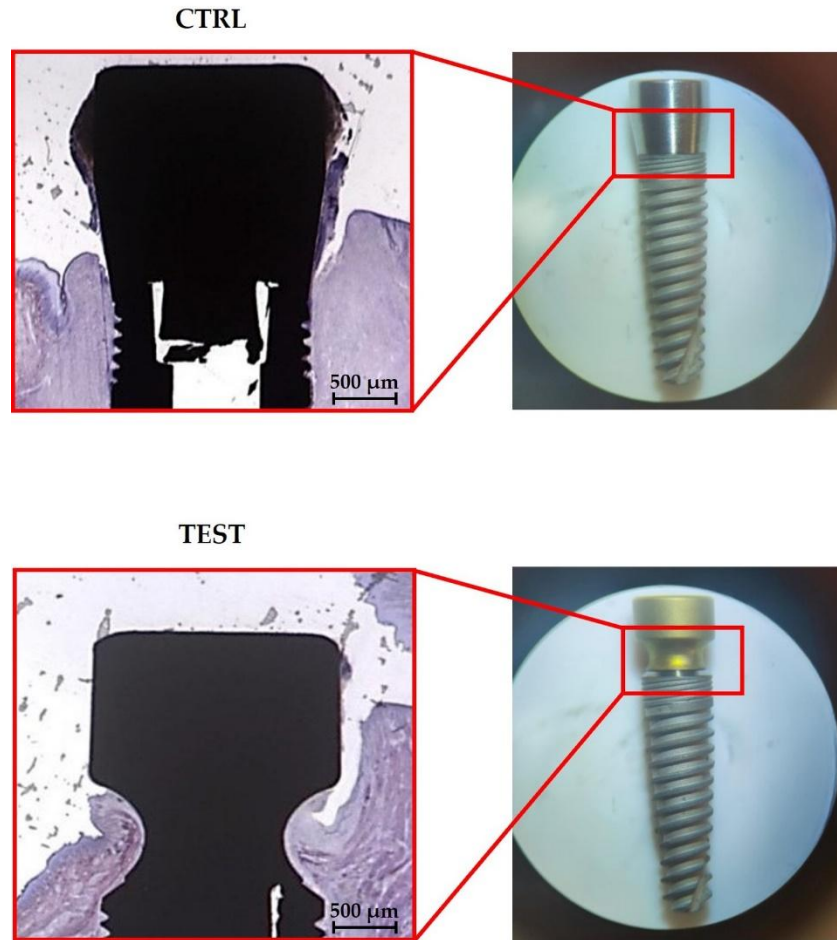


Figure 19. Histological longitudinal sections of the implant–abutment units. The upper line illustrates the soft tissues encircling the CTRL abutment, while the lower line depicts the soft tissues surrounding the TEST abutment. Staining: acid fuchsin-toluidine blue; magnification 20×; scale bar: 500 µm.

TEM Ultrastructural Qualitative Analysis of Collagen Fibers in Peri-Implant Soft Tissue

Ultrastructural analysis of the peri-implant soft tissue collected from sites adjacent to the two distinct implants (CTRL and TEST) was initially conducted under blinded conditions. During the TEM analysis, the composition of the peri-implant soft tissues was predominantly characterized by collagen fiber bundles and cellular components, specifically fibroblasts (refer to “f” in **Figure 20a**), which exhibited a stellate morphology. Notably, the processes of these fibroblasts were observed to segregate individual collagen bundles (**Figure 20**, black arrows).

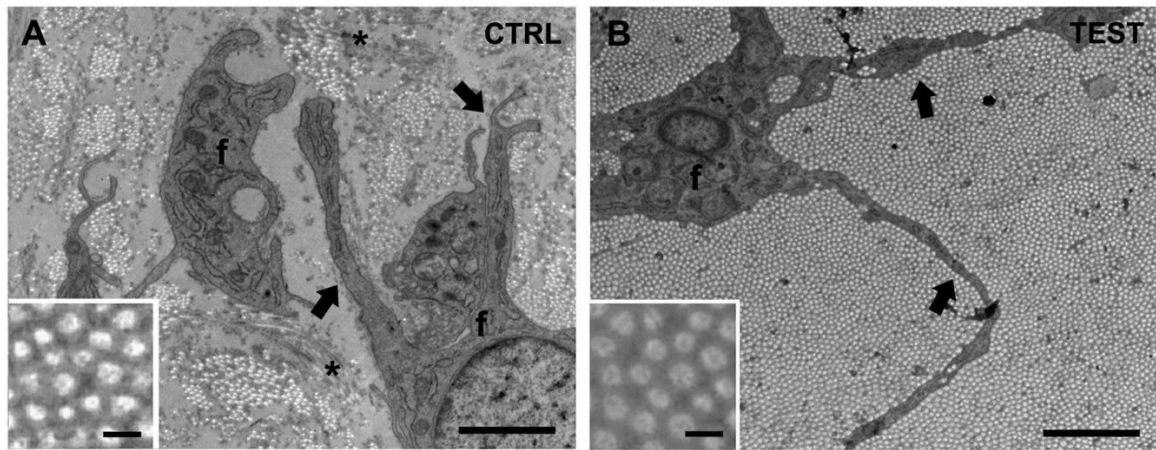


Figure 20. Representative transmission electron microscopy (TEM) images of peri-implant soft tissues surrounding the two abutments. The composition of the peri-implant soft tissue primarily consisted of collagen fiber bundles and fibroblasts (f). **(a)** The CTRL samples exhibited only a limited assembly of collagen fibers, which form small, scattered bundles, compared to **(b)** the TEST samples, where collagen bundles were notably thick and dense, frequently covering a large area of the analyzed section. NOTE: Within the analyzed longitudinal region, the collagen fibers were predominantly observed in cross-sections, appearing as small circles (insets) that clustered into bundles of varying sizes. Asterisks in panel **(a)** indicate longitudinally oriented collagen fiber bundles. Black arrows denote fibroblast processes (f). Magnification: 7100 $\times$; scale bars: 2 μm (insets: 0.1 μm).

The collagen fibers exhibited an arrangement of elongated, parallel, and straight structures. When sectioned transversally (i.e., cross-sectioned), these fibers manifested as “bunches of circular spots” (**Figure 20**, insets), indicative of bundles with heterogeneous sizes. In the cross-sectional images, the collagen fibers displayed a relatively uniform diameter (0.125 nm) in both CTRL and TEST samples (**Figure 20**, insets).

During the TEM analysis of the cross-sectioned collagen bundles at low magnifications (7100 $\times$), a marked difference in the structural arrangements of collagen fibers between the samples was evident. This observation allowed for the classification of samples into two distinct groups: CTRL specimens, where extensive aggregation of thick collagen bundles was rare or absent (**Figure 20a**), and TEST samples, which exhibited a high density of tightly packed and organized collagen fiber bundles (**Figure 20b**).

A detailed examination revealed that in CTRL samples, collagen fibers formed only a few small, scattered bundles with significant variability in their distribution within the extracellular space. In contrast, TEST samples displayed notably thick and dense collagen bundles that frequently encompassed the entirety of the analyzed section. Specifically, CTRL samples (**Figure 20a**) demonstrated a collagen matrix characterized by randomly distributed bundles at variable distances from one another. Conversely, TEST

samples (**Figure 20b**) featured extensive areas filled with thick and densely packed collagen bundles, significantly reducing the available space for extracellular components. These findings suggested, for the first time, a distinct difference in the growth and assembly of the collagen matrix surrounding TEST abutments when compared to CTRL abutments, with the concave shape seemingly influencing an increase in the size of collagen fiber bundles.

TEM Ultrastructural Quantitative Analysis of Collagen Fiber Bundles

To validate the qualitative findings, a quantitative TEM analysis of images was conducted. Specifically, from the cross-sectional images of the CTRL (**Figure 21a,b**) and TEST groups (**Figure 21c,d**) within the area of interest, the following parameters were assessed: (i) the percentage of the total analyzed surface area covered by cross-sectioned collagen fibers (**Figure 21e**); and (ii) the average dimensions of these collagen bundles (**Figure 21f**).

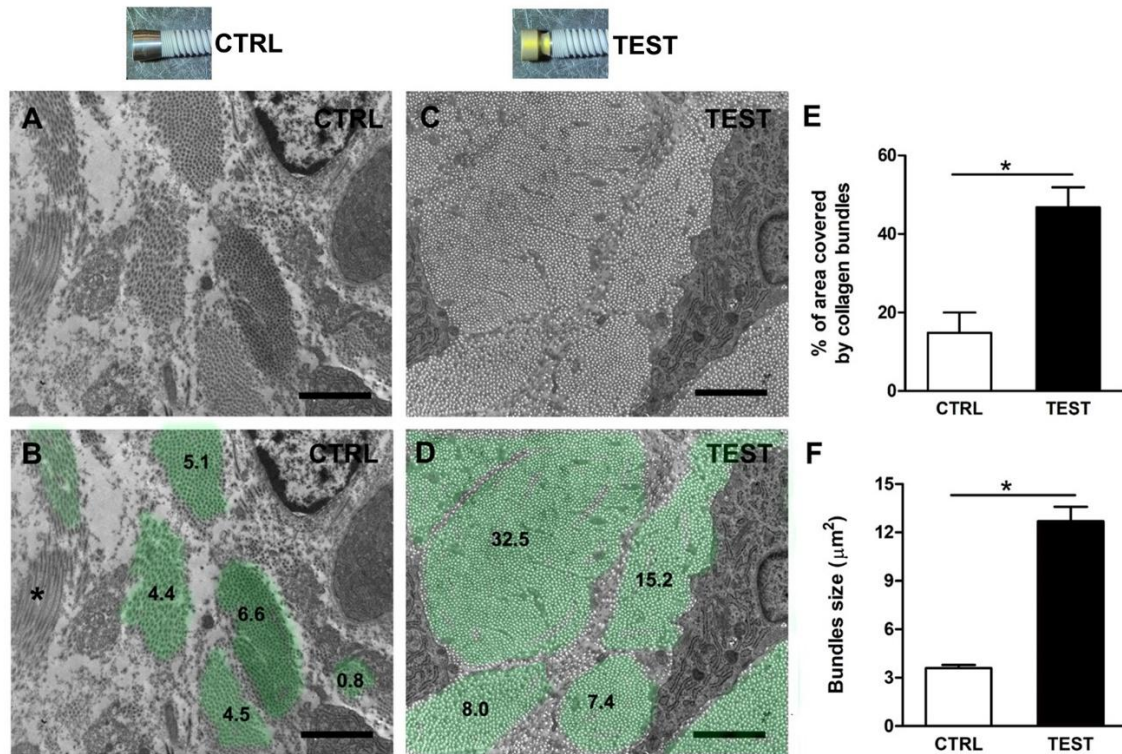


Figure 21. Quantitative analysis of the cross-sectioned collagen fiber bundles from CTRL and TEST samples using TEM. Representative TEM images of the cross-sectional area of collagen fiber bundles for (a) CTRL and (c) TEST samples with (b,d) the corresponding surfaces of the collagen bundles, highlighted in light green. The numerical values indicate the surface areas of the bundles in μm^2 . The asterisk in panel (b) denotes a longitudinal collagen fiber bundle. (e) Bar graph representing the quantitative analysis of the percentage of the analyzed area that is occupied by collagen fibers, and (f) the average size of the

collagen bundles (μm^2). Scale bars: 2 μm . * $p < 0.05$.

To facilitate the visualization of collagen bundle size, the surfaces of these bundles have been highlighted in light green within the cross-sectional images (**Figure 21b,d**). The mathematical summation of each value provided a representative percentage of the surface area occupied by collagen, with the numerical results documented in **Figure 21b,d**. The quantitative analysis demonstrated that the total surface area covered by collagen fibers was significantly enhanced with the use of TEST implants compared to CTRL implants. Specifically, the percentage of total surface area occupied by collagen was significantly greater in the TEST samples (approximately 47%) in contrast to the CTRL samples (about 18%) (**Figure 21e** and **Table 2**).

Table 2. Percentages of the analyzed area covered by collagen bundles. The data are shown as the mean $\pm$ standard deviations (n = 15 per sample). * $p < 0.05$ versus CTRL.

	Sample 1	Sample 2	Sample 3	Sample 4
CTRL	18 $\pm$ 5	28 $\pm$ 3	8 $\pm$ 3	5 $\pm$ 1
TEST	47 $\pm$ 11 *	46 $\pm$ 6 *	63 $\pm$ 10 *	43 $\pm$ 19 *

Furthermore, the utilization of TEST abutments demonstrated a significant enhancement in the average size of the collagen bundles, increasing from 4 μm^2 in the CTRL samples to 13 μm^2 (**Figure 21f**, **Table 3**, and **Figure 22**).

Table 3. Bundle size (μm^2). The data are shown as the mean $\pm$ standard deviation (n = 15 per sample). * $p < 0.05$ versus CTRL.

	Sample 1	Sample 2	Sample 3	Sample 4
CTRL	4.2 $\pm$ 5.5	4.7 $\pm$ 4.4	2.6 $\pm$ 3.8	1.5 $\pm$ 1.3
TEST	20.7 $\pm$ 27.7 *	7.5 $\pm$ 9.1	23.7 $\pm$ 12.6 *	12.6 $\pm$ 16.6 *

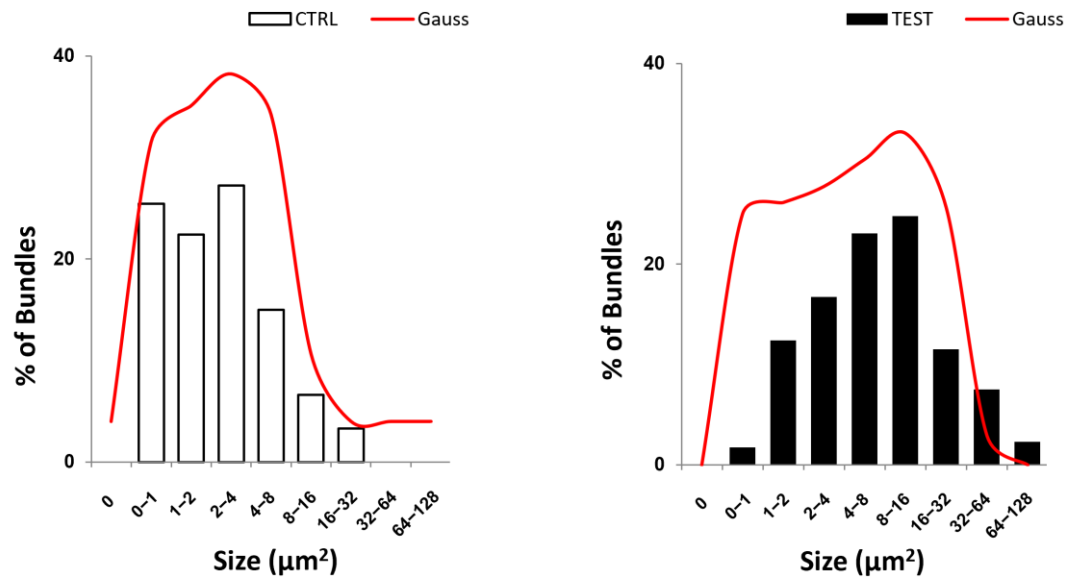
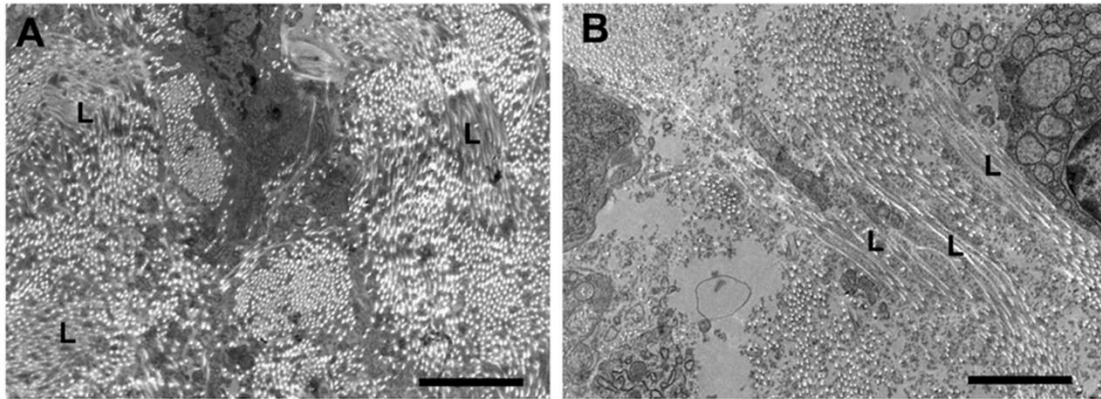


Figure 22. Representative bar and curve plots illustrating the distribution frequency of bundle sizes for both the CTRL and TEST groups. The analysis of the distribution frequency concerning the cross-sectional area, i.e., the size of bundles from the CTRL and TEST groups, revealed that the CTRL group exhibits an average bundle size of 3.4 ± 4.0 . In contrast, the TEST samples demonstrate a significantly increased average bundle size of 12.8 ± 16.0 . This finding was further evidenced by the leftward shift of the TEST frequency distribution curve in comparison to that of the CTRL group.

TEM Ultrastructural Quantitative Analysis of Longitudinal Collagen Fiber Bundles

In addition to the previously described qualitative and quantitative differences, further distinctions have been identified between CTRL and TEST specimens. Notably, areas exhibiting abrupt changes in the direction of collagen bundles have been observed. In the cross-sectional images of peri-implant soft tissues, collagen fibers typically appeared as small circles densely grouped in bundles of varying sizes (**Figures 20 and 21**). However, instances of collagen bundles featuring longitudinally oriented fibers were also occasionally noted (**Figure 23**).

CTRL



TEST

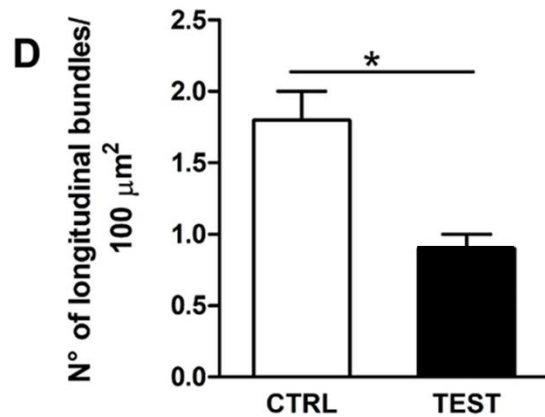
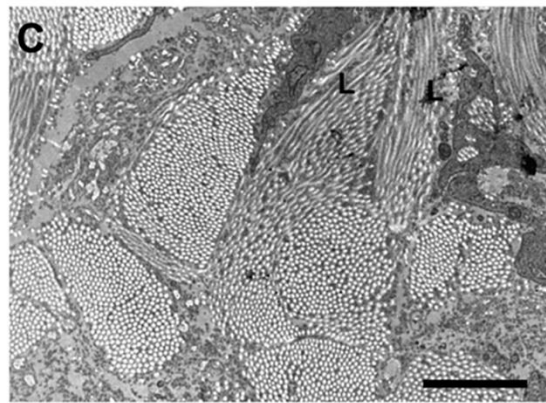


Figure 23. Representative TEM images illustrating the orientation of various collagen fibers, along with a relative quantification for (a,b) CTRL samples and for (c) TEST samples. In the longitudinally sectioned soft tissue surrounding the implant within the analyzed regions, collagen fibers predominantly manifested as circumscribed circular spots of varying sizes. Notably, a longitudinal alignment of collagen fibers was observed (L); this alignment occurred more frequently in CTRL samples than in TEST samples. (d) Bar plot presenting the average number of longitudinal fiber bundles per 100 μm^2 in both CTRL and TEST samples. Scale bars: 2 μm . * $p < 0.05$.

Following a thorough examination of the specimens, a notable difference in the longitudinally oriented fibers was observed between the CTRL and TEST samples (**Figure 23**). Specifically, in the CTRL specimens (**Figure 23a,b**), the longitudinally oriented collagen fibers (L) typically formed small-sized bundles, exhibiting a relatively random orientation among the fibers. Contrarily, the TEST specimens displayed longitudinally oriented collagen fibers (L) that were organized into larger bundles containing several straight and parallel-oriented fibers (**Figure 23c**). Quantification of the number of longitudinally oriented collagen bundles per 100 μm^2 revealed a significantly higher incidence in the CTRL samples compared to the TEST samples, as presented in **Table 4**.

Table 4. Number of longitudinal bundles per 100 μm^2 . The data are shown as the mean $\pm$ standard error of the mean (n = 15 per sample). * $p < 0.05$ versus CTRL.

	Sample 1	Sample 2	Sample 3	Sample 4
CTRL	1.2 $\pm$ 0.3	0.9 $\pm$ 0.2	3.4 $\pm$ 0.4	1.7 $\pm$ 0.2
TEST	1.1 $\pm$ 0.2 *	0.8 $\pm$ 0.2	1.1 $\pm$ 0.3 *	0.5 $\pm$ 0.2 *

4.2.1.6. DISCUSSION

In a study conducted by Berglundh and Lindhe in 1996 utilizing an animal model [119], it was established that a specific level of mucosal thickness is critical for the development of the supracrestal tissue attachment surrounding dental implants. In instances of insufficient mucosal thickness, crestal bone resorption occurs until adequate space is created to accommodate both connective tissue and junctional epithelium. The structure of connective tissue, indeed, has been identified as crucial in preventing epithelial down-growth and providing mechanical protection to the osseointegrated portion of the implant [7]. Despite their analogous composition and structure, research has demonstrated that this attachment apparatus is extended around dental implants compared to natural dentition, requiring a greater amount of soft tissue height surrounding implant fixtures [120,121]. Moreover, the dense three-dimensional (3D) framework formed by the connective tissue bundles contributes significantly to the mechanical resistance of soft tissues under the forces generated during mastication [122].

The present investigation was undertaken to compare the peri-implant soft tissue healing process surrounding non-submerged implants equipped with either a parallel-walled abutment or a concave abutment, employing a swine model as the experimental framework. TEM was utilized to quantify the 3D topographical and morphological properties of collagen bundles with nanoscale resolution. The hypothesis proposed that a concave implant neck may promote the spontaneous alignment of the collagenous network, thereby influencing the direction and arrangement of fiber growth.

In recent years, numerous studies involving both animal models and human subjects have elucidated the characteristics, arrangement, and structural properties of peri-implant soft tissues utilizing various techniques, including light microscopy, polarized light microscopy, SEM, TEM, and high-resolution X-ray phase-contrast micro-topography (XPCT) [7,97,123]. For instance, an animal study conducted over three decades ago involving monkeys revealed that substantial collagen fiber bundles encircled the implant collar in a parallel orientation, exhibiting a tangential circular arrangement that converged

to form a “circular ring” [123]. Observations made using TEM indicated that these circular fibers comprised bundles of parallel collagen fibrils with a mean diameter of 90 nm. However, the inner bundles adjacent to the metal surface displayed a less organized arrangement, characterized by a random orientation and a mean diameter of 45 nm. Conversely, Iezzi et al. [7], utilizing high-resolution XPCT to study peri-implant tissues around retrieved human implants, reported both transverse and longitudinally intertwined collagen bundles. Analysis of longitudinal sections indicated that fiber bundles closer to the metal surface exhibited a more symmetrical and regular directional alignment. In contrast, examination of transverse collagen fiber bundles demonstrated a semicircular orientation, aligning around the abutment, thus conforming to its circular profile. Comparable findings were corroborated by other researchers. For example, Bolle et al. [124], in an animal study, found that collagen fibers oriented medially toward the healing abutment perpendicularly, and the connective tissue appeared dense, abundant in fibroblasts, and collagen fibers aligned parallel to the implant surface. Other studies involving swine noted varying degrees of connective tissue organization, with some areas displaying well-organized fibers, while others exhibited a disorganized structure with indistinct orientations [125]. Furthermore, human studies have reported the presence of a 3D network of collagen fibers surrounding Morse cone implant connections [7]. Similar observations were made by Mangano et al. [126] through polarized light and SEM investigations, which indicated that collagen fibers were oriented perpendicularly up to a distance of 100 μm from the implant surface, subsequently transforming into a dense, chaotic 3D network of parallel fibers oriented in multiple directions. An intimate association between the fibrous matrix and the implant surface was also noted. Following the maturation process, the peri-implant connective tissue exhibited sparse cellularity and blood vessels yet was characterized by an abundance of collagen fibers with a limited number of scattered fibroblasts [123].

The present findings indicated that incorporating a concave profile in the abutment facilitates the formation of a robust, wire-shaped connective tissue cuff, approximately 0.5 mm in thickness, surrounding the implant platform. This cuff exhibited a high degree of anisotropy, characterized by the arrangement of cells, fibrils, and the ECM. These results illustrated the potential to modulate both the dimensions and quality of collagen fibers, as well as to influence the morphogenesis of a highly organized capillary-like network by regulating the spatial configuration of the neo-formed ECM. In summary, these data suggested that during the maturation of ECM around the abutment interface, the

local microenvironment may be affected by the macroscale geometry of the tissue. This, in turn, may trigger long-range signaling by producing internal gradients of mechanical cues, as documented in the literature [96] and demonstrated in our previous *in vitro* study. Thus, tissue geometry serves as both a template and an instructive signal for further morphogenetic processes. In the current study, the CTRL group featuring a parallel-walled neck displayed a significantly higher ratio of randomly oriented fibers. It is well established that moderate crosslinking enhances the mechanical properties of collagen fibers, whereas excessive crosslinking can result in increased fragility of the collagen network [127]. Conversely, in the TEST group with a concave design, collagen fibers appeared to be predominantly organized into parallel bundles, oriented circumferentially around the implant when viewed from axial planes. This outcome aligned with prior studies that have described similar fiber orientations around implants with a switching platform interface, which is considered an additional mechanical retention factor for periodontal fiber alignment [128,129]. Moreover, investigations conducted in other animal models, including monkeys and dogs, have demonstrated a supracrestal circular collagen fiber network comparable to gingival ligaments [123,128].

Overall, it can be argued that the mechanical environment is posited to play a significant role in the orientation of collagen fibers. This phenomenon is thought to arise from an uneven surface shear, the effects of which diminish with increasing distance [130]. The configuration of the artificial substrate may facilitate contact guidance, potentially resulting in the formation of a highly polarized capillary-like network. This observation suggests promising clinical applications, particularly in stimulating rapid angiogenesis and enhancing perfusion in damaged tissues surrounding the implant [26]. Moreover, collagen fibers are capable of remodeling into aligned, anisotropic structures in response to mechanical stimuli, leading to fiber orientation in the direction of the highest applied strain. It is important to note that the self-assembly of collagen is an entropy-driven process, which occurs due to the dehydration of water between monomers [131]. The objective of the experiments concerning mechanics-mediated fiber orientation is not to recombine collagen monomers by overcoming their intermolecular interactions. Instead, the focus is on applying external forces to the interacting monomers, thereby encouraging bonding along the direction of the applied force. It can be inferred that when the distal ends of the fibers encounter the curved periphery of the abutment concavity, cellular contraction may generate sufficient force to instigate the aggregation of fibers into bundles.

Other researchers have posited that the organization of collagen fibers is primarily

influenced by functional factors, particularly the loading associated with implants [10]. This perspective suggests the interpretation of radial fibers as forming a circular ligament encircling implants. Additionally, these same researchers have provided evidence that this collagen cuff is connected to the periosteum through oblique bundles. Nevertheless, there is a lack of temporal studies to validate this assumption, as well as a deficiency in research examining the arrangement of collagen in various rehabilitation designs. Furthermore, it is important to note that one hypothesis may not necessarily exclude the other and vice versa.

It is important to note that collagen is a meticulously structured molecule characterized by inherent weaknesses that serve as binding sites for metalloproteinases (MMPs) and bacterial collagenases. This mechanism facilitates the regulation of collagen remodeling in response to specific stimuli. Research has demonstrated that applying strain and external loading to collagen fibrils can enhance their strength in the direction of the load while simultaneously inhibiting the spontaneous formation of entry points for MMPs. This limitation serves to restrict their accessibility and reduce collagen degradation [132]. Consequently, the directionality and immediate tension applied to early wounds surrounding implants may influence collagen assembly and maturation. Human tissues often exhibit distinct alignment patterns within the ECM of interstitial connective tissue, stromal cells, and the vascular network [133]. Collagen is organized into bundles of aligned fibers, which not only dictate the mechanical properties of tissues but also affect various biological processes. The density and alignment of collagen fibers influence key phenomena such as cell migration, morphogenesis, vascularization, innervation, tissue regeneration, and wound healing [134]. Nonetheless, achieving control over the alignment of an ECM network synthesized by fibroblasts continues to pose a significant challenge within the field of dentistry.

In conclusion, this study presents certain limitations arising from the use of a small sample size of animals and implants, which may introduce uncertainty and risk to the research outcomes. Nevertheless, the investigation into the peri-implant connective tissue structure, assessed through histological and TEM analysis, indicated that a concave transmucosal design may enhance the deposition and growth of connective tissue. This concave configuration resulted in a notable increase in connective tissue during the early healing phase, contributing to the thickening of the circular peri-implant network and facilitating the convergence of collagen fibers toward the abutment collar, ultimately leading to the development of an extensive circular collagen structure over the implant platform. Therefore, building on the findings from this initial proof-of-concept animal

study, it is imperative that future research expands the sample size and incorporates a greater variety of implants. Additionally, employing alternative mechanical detection methods alongside histological and TEM analysis will be essential to validate and reinforce the results of this study.

4.2.2. SCREW AND OSSEOINTEGRATION

Surface modification of implants is performed to achieve a biocompatible and bioactive surface that promotes the osseointegration process. While commercially pure titanium and its alloys remain the standard materials for endosseous implants, there is a growing interest in ceramic-based biomaterials, tantalum, and polyetheretherketone (PEEK) [16]. Various mechanical techniques, including shot peening and abrasion, are commonly employed during the initial stages of implant production to enhance the surface area and progressively refine the particle size of the outer layer. Methods such as machining, grinding, acid etching, refinishing, and sandblasting are utilized to modify the implant surface, thereby improving overall roughness and facilitating surface bioactivation. Furthermore, advanced techniques such as plasma spraying, anodization, laser treatments, electrochemical oxidation, and photofunctionalization serve to expand the bone-implant interface area and enable the application of various coatings that enhance bioactivity and tissue integration. Additionally, both physical and chemical treatments, including grit blasting with aluminum oxide or titanium oxide, hydrogen peroxide, as well as alkaline and acid reagents, have demonstrated efficacy in improving cellular adhesion, proliferation, and osteoblast differentiation [135]. For example, the DAE technique has been shown to enhance osteoblast responses, thereby improving initial cell attachment and proliferation, which are critical factors for successful bone integration [136]. The sandblasted, large-grit, acid-etched (SLA) implant surface, which synergistically combines the benefits of sandblasting and acid etching, exhibits superior bone-to-implant contact (BIC) compared to other surface modification techniques [137,138]. Moreover, implants can be treated with additive methods to develop coatings of bioactive molecules such as peptides, collagen, calcium phosphate, nitrides, naturally derived substances, and antimicrobial agents. These coatings increase the functional surface area, establish a bioreactive bond with bone tissue, and help prevent microbiological infections [135,139-141].

The surface roughness (Ra) and bioactive coating significantly influence biological responses such as cell adhesion, osseointegration, and susceptibility to bacterial colonization. Microtopography affects osseointegration at the cellular level, while nanotopography is believed to function at a protein level. These factors exert effects through physical, chemical, and biological mechanisms, enhancing the adhesion of osteogenic cells and promoting osseointegration. Surface roughness can be classified into

three categories: (i) macro-roughness (Ra ranging from millimeters to approximately 10 μm), which is directly associated with the overall design and structure of the implant, including macroscopic features like threads and porous structures that contribute to initial implant stability by enhancing primary fixation; (ii) micro-roughness (Ra around 1 μm), which is crucial for osseointegration, facilitating improved adhesion and growth of bone cells, thereby directly contributing to the long-term stability and success of dental implants; and (iii) nano-roughness (Ra < 200 nm), which enhances osseointegration by creating a favorable microenvironment for the proliferation of bone-forming osteoblasts and resisting bacterial adhesion and biofilm formation, thus reducing the risk of peri-implantitis [142]. Previous studies indicated that the optimal Ra should be approximately 1-2 μm within a hydrophilic composition to facilitate cellular locomotion, provide a supportive substrate for adhesion, and stimulate the proliferation of osteoblasts [143]. However, increased surface roughness may augment the surface area, potentially heightening the risk of microbial colonization and offering shelter to bacteria, which may hinder their removal by antibiotics. At the nanoscale, the optimal Ra for promoting osteogenic reaction has been reported to be within 70-100 nm, although the similarity to pores found in bone tissue is more closely aligned with micro-irregularities induced by SLA treatment (1-2 μm) [144]. Furthermore, studies have demonstrated that surface roughness below 0.2 μm is less likely to promote bacterial adhesion, as most bacteria exhibit sizes larger than this threshold [145].

Titanium is recognized as a bioinert material; however, its introduction into living tissues may provoke an immune response, necessitating a focus on promoting integration with hard tissues through an immune-modulated inflammatory process [146]. In the pursuit of enhancing dental implant integration, type I collagen has garnered considerable attention for surface modifications and coatings. This interest is due to its inherent properties that align closely with the requirements essential for promoting osseointegration, including osteoinduction and osteoconduction processes [147,148]. The unique biological characteristics of collagen present a substantial opportunity to improve the effectiveness of dental implants in facilitating bone integration while simultaneously addressing the ongoing challenge of bacterial colonization, which often results in peri-implantitis and complicates the success of the implant [149-151]. Moreover, within the realm of biomedical research, there is an increasing interest in equine collagen as a promising source of type I collagen, especially in the context of regenerative dentistry. This inclination is attributed to its high sequence homology with human collagen, low

immunogenicity, and significant resistance to degradation and mechanical stress [43,44,125,152]. Notably, equine tendons are particularly favored due to their high content of type I collagen, minimal protein contaminants, and advantageous physical-chemical properties in comparison to alternative sources [54,153]. However, it is important to note that the processing steps involved in collagen isolation and scaffold manufacturing can significantly influence the native collagen assembly, potentially affecting cellular responses and overall biological activity [53]. Previous research on collagens derived from bovine, fish, and equine sources has indicated a correlation between various production treatments and the structural characteristics of collagen at both submolecular and supramolecular scales. These variations are known to significantly impact the mechanical and biological properties of collagen-based medical devices [54,55].

The biocompatibility, availability, and advantageous mechanical properties of equine type I collagen have established it as a promising candidate for various biomedical applications. Recent studies have highlighted the potential of collagen derived from equine tendons as a biocompatible scaffold that facilitates the growth and differentiation of stem cells, thereby enhancing mineralization and bone formation [154-156]. Some researchers have also documented its role in soft tissue regeneration, hemostasis, and wound healing [157-161].

The quest for efficient biomaterials within the realm of regenerative medicine, particularly in dentistry, has garnered significant attention in recent years [5,56]. Stem cells derived from the apical papilla (SCAPs) have emerged as a promising resource for dental tissue regeneration due to their distinctive characteristics, including multipotency and the ability to differentiate into various cell lineages, such as osteoblasts [162,163]. The utilization of biomaterials that can enhance the osteogenic differentiation of SCAPs is essential for successful dental tissue regeneration and the repair of bone defects. However, there exists a notable dearth of research investigating the effects of equine collagen on SCAPs in the current literature.

Thus, the first study extensively evaluated the physical, chemical, and biological properties of type I collagen sourced from the tendon of *Equus caballus*. This research aimed to assess the impact of different concentrations of both native and hydrolyzed type I collagen coatings (0.1, 1, and 2 mg/cm³; Typeone Biomaterials S.r.l., Calimera, Italy) on SCAPs, with an emphasis on evaluating biocompatibility and osteogenic potential. The study also provided a comprehensive overview of the methodologies employed to characterize the various coatings prior to biological evaluations.

Subsequently, the incorporation of equine type I collagen as a surface coating presented a promising strategy for enhancing the biological interaction of titanium implants and increasing their resistance to microbial infection, which may contribute to the long-term viability of dental restorations. Given the limited literature on the application of equine collagen in implant dentistry, this research may represent a pioneering investigation in this field [43,44]. In summary, the primary focus of the second study was to optimize the surface characteristics of dental implant screws utilizing a type I collagen coating from equine origin. This involved applying varying concentrations of native equine tendon-derived type I collagen to create coatings on a spectrum of titanium surface textures, ranging from smooth to micro- and nano-roughened surfaces. The research comprehensively assessed the cytotoxicity, osteogenic potential, and antibiofilm properties of these surfaces through in vitro models involving SCAPs and common oral pathogens. Through this approach, the objective was to evaluate the ability of collagen to promote bone matrix deposition, enhance osteogenic differentiation, and mitigate biofilm formation when applied as a coating on dental implants. This research aimed to provide an in-depth understanding of the interactions among surface properties, collagen coatings, and biological systems to optimize implant characteristics for improved clinical outcomes.

The Osteoconductive Properties of Collagen Coating

4.2.2.1. MATERIALS AND METHODS

Collagen Characterization

The native type I collagen in gel form (COL N, 0.8% in 0.5 M acetic acid) and a prototypal partially hydrolyzed type I collagen in solution form (COL I, 0.8% in PBS 1×) were supplied by Typeone Biomaterials S.r.l. (Calimera, Italy) and produced according to a proprietary manufacturing process. Prior to the development of coatings, a preliminary assessment of the intrinsic properties of these collagens was conducted to ascertain their influence on the biological and structural performance of the scaffolds.

The molecular weights of COL N and COL I were determined using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Approximately 0.05 mL of 0.8% collagen suspensions underwent a reducing treatment utilizing 2 M Laemmli buffer and urea in a 1:1 ratio for a duration of 60' at a temperature of 50 °C, prior to being loaded into the wells of a polyacrylamide gel (5% stacking gel, 12% resolving gel) [43,164,165]. Protein standards with molecular weights ranging from 10 to 250 kDa were also incorporated. The electrophoresis was performed using a Mini-Protean Tetra Cell System (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 70 V for 30', followed by an increase to 120 V for approximately 2 h. The polyacrylamide gel was then rapidly stained in accordance with a previously optimized protocol [55,159]. The estimation of protein molecular weight was conducted with the GelAnalyzer 19.1 software (www.gelalyzer.com, accessed on 01 February 2023), developed by Istvan Lazar Jr., PhD, and Istvan Lazar Sr., PhD, CSc.

The secondary organization of COL N and COL I was examined using Fourier-transform infrared spectroscopy (FTIR). Diluted collagen suspensions (0.2%) were cast and air-dried on germanium lenses for 24 h at RT [166]. Absorption spectra were acquired utilizing an FTIR-6300 spectrometer from Jasco GmbH (Pfungstadt, Germany) across the frequency range of 4000 cm⁻¹ to 400 cm⁻¹, with a resolution of 4 cm⁻¹ and a total of 64 scans. The resulting spectra were subsequently analyzed using Origin software from Origin Lab Corporation (Northampton, MA, USA) [159,164,166].

Sample Preparation

Before cell seeding, collagen coatings of types COL N and COL I were prepared by casting collagen suspensions onto the bottoms of 24-well culture plates to achieve final

concentrations of 0.1, 1, and 2 mg/cm³ [55]. The plates were subsequently maintained under a sterile hood overnight to facilitate solvent evaporation. Following this, a series of four washes using PBS 1× were performed, each lasting 10', to eliminate solvent and any residual materials. The plates were then allowed to dry under a sterile hood overnight. Subsequently, they were either stored in the dark at a temperature of 2-8 °C or subjected to ultraviolet (UV) irradiation for a duration of 30', followed by an equilibration period in cell culture medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, DMEM/F-12; cat. 10565018, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA, supplemented with 10% FBS and 1% P/S) for an additional 30' prior to immediate usage. After completing these preparatory steps, the medium in each collagen-coated well, as well as in the empty well designated as the standard control growth surface (CTRL), was replaced with 1 mL of fresh medium containing 5×10^4 cells.

Wettability

The behavior of collagen coatings under physiological-like conditions was evaluated through contact angle analysis and assessment of the swelling degree (SD). The coatings were carefully detached from the bottoms of culture plates and subsequently stored in the dark at temperatures ranging from 2 to 4 °C until their use. The hydrophilic or hydrophobic characteristics of the coatings were determined utilizing the sessile drop method [167]. Approximately 0.01 mL of PBS 1× was dispensed onto the coatings, and the contact angle was measured using the FTA1000 analysis system (First Ten Angstroms, Newark, NJ, USA). The ability of the coatings to absorb and retain water was assessed by monitoring the weight of the samples over an extended period under physiological-like conditions (PBS 1×, 37 °C) [55]. Excess water was gently removed with filter paper prior to weighing. The SD (%) was calculated as the ratio of the coatings' wet weight at predetermined time points (Wt) to their initial dry weight (W0) using the following formula [159,165,166]:

$$SD\% = \frac{(W_t - W_0)}{W_0} \times 100$$

Each sample underwent triplicate testing to ensure the accuracy and reliability of the results.

Degradation Resistance

The resistance of coatings to degradation over time was assessed under

physiological-like conditions. Samples of COL N and COL I, which had been previously detached from culture plates, were incubated in 5 mL of 0.5 U/mL of collagenase derived from *Clostridium histolyticum* (cat. C0130, Sigma-Aldrich, Milan, Italy) in PBS 1× at 37°C [159]. At designated intervals, a small volume of the incubation medium was withdrawn and analyzed using the Bradford assay to quantify the dissolved protein content. Degradation resistance (D%) was calculated by comparing the remaining weight of the samples at each time point to their initial weight, employing the following formula [55,165]:

$$D\% = \frac{W_t}{W_0} \times 100$$

Each sample underwent triplicate testing to ensure the accuracy and reliability of the results.

Mechanical Properties

The mechanical properties of the coatings were assessed utilizing a ZwickLine universal testing machine (Zwick/Roell, Ulm, Germany), which was equipped with a 100 N load cell to conduct uniaxial tensile tests. Coating samples were carefully detached from the bottoms of culture plates, precisely cut to dimensions of 0.5 cm x 2.5 cm, and subjected to testing under displacement control at a rate of 0.1 mm/s until failure occurred. Prior to the commencement of the testing, a preload of 0.1 N was applied to all samples [55,164,166]. The determination of the elastic modulus (E), stress at break (σ_{max}), and strain at break (ϵ_r) was carried out using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA). Each sample underwent testing in triplicate to ensure the reliability of the results.

Cell Cultures

The pulp tissues were obtained from impacted third molars of three systemically healthy patients, aged between 18 and 25 years, who underwent extraction for orthodontic purposes at the Clinic for Oral Surgery, School of Dental Medicine, University of Belgrade.

SCAPs were isolated using previously established methodologies [168]. In brief, the extracted teeth were thoroughly rinsed with PBS 1× containing a 1% P/S. The apical papilla was carefully excised from the root apex, minced, and subsequently transferred into T-25 flasks. The tissues were cultured in DMEM/F-12 supplemented with 10% FBS and

1% P/S. The cell cultures were maintained under standard conditions of 37 °C, with an atmosphere of 95% air and 5% CO₂, all within a humidity level of 95%. The complete medium was replaced every two to three days. Cell cultures were passaged upon reaching 80% confluence, and all subsequent experiments utilized cells from the third to the sixth passage.

All participating patients provided informed consent, and the study was conducted in accordance with the Declaration of Helsinki, receiving approval from the Ethical Committee of the School of Dental Medicine, University of Belgrade (protocol number: 36/19).

To induce osteogenic differentiation, cells were seeded in 24-well plates at a density of 5×10^4 cells/well. The day following seeding, the cells were incubated for periods of 7, 14, and 21 days in an osteogenic differentiation medium (OM; cat. 130-091-678, StemMACS OsteoDiff Media human, Miltenyi Biotec Inc., Waltham, MA, US), with medium changes conducted every three days.

Flow Cytometry Analysis for Antigen Expression

The expression of specific mesenchymal markers in SCAPs was evaluated using flow cytometry analysis. This analysis employed FITC-labeled mouse monoclonal antibodies targeting CD34 (cat. 1F-297-T100, Exbio, Vestec, Czech Republic) and CD90 (cat. 1F-652-T100, Exbio, Vestec, Czech Republic), as well as PE-labeled mouse monoclonal antibodies for CD73 (cat. 1P-675-T100, Exbio, Vestec, Czech Republic), CD45 (cat. 1P-160-T100, Exbio, Vestec, Czech Republic), and CD105 (cat. 1P-298-T100, Exbio, Vestec, Czech Republic), in accordance with the manufacturer's recommendations.

Cells at passage five were harvested using TrypLE™ Express solution (cat. 12604013, Thermo Fisher Scientific, Waltham, MA, USA). Following a wash with PBS 1× supplemented with 10% FBS, the cells were counted. Subsequently, 1×10^6 cells were resuspended in 1 mL of 10% FBS solution in PBS 1× and incubated with the designated antibodies for 45' at 4 °C. The cells were then rinsed twice with PBS 1× and analyzed employing a tabletop flow cytometer (BD FACSMelody™; BD Biosciences, San Jose, CA, USA), which was equipped with BD FACSCorus software.

3-(4, 5-Dimethylthiazolyl-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

The MTT assay, conducted in accordance with ISO10993-5, was employed to assess cellular metabolic activity. Following incubation periods of 24 h, 48 h, and 72 h, as well as 7 days, 14 days, and 21 days of culture, the supernatant was carefully removed.

Subsequently, 300 μ L of MTT solution was added to each well and incubated for 3 h at 37 °C. The resulting formazan crystals were then dissolved in 300 μ L of DMSO by gentle shaking for 10' at 37 °C. Cell supernatants were obtained, and 80 μ L/well of the sample was dispensed into a flat-bottom 96-well plate in triplicate. The optical density (OD) was measured at 540 nm using a microplate reader (RT-2100c; Rayto, Shenzhen, China). Cell viability (%) was subsequently calculated using the following formula:

$$\frac{(OD_{sample} - OD_{blank})}{(OD_{control} - OD_{blank})} \times 100$$

Alizarin Red S Staining (ARS)

Alizarin Red S staining (ARS) serves as a method for detecting calcium deposits, which are indicative of mineralization produced by differentiated osteoblasts. These deposits are discerned as bright orange-red stained areas. In this procedure, cells were initially washed with PBS 1 $\times$ and subsequently fixed with 4% paraformaldehyde for a duration of 30'. Following fixation, the cells underwent a 15-minute staining process with 2% ARS (cat. A5533, Sigma-Aldrich, St. Louis, MO, USA) at RT. A quantification step was then implemented, which entailed the incubation of the cells with 200 μ L of 1% HCl in 70% ethanol for 30'. The supernatants from the cell cultures were collected, and 80 μ L of each sample was transferred into individual wells of a flat-bottom 96-well plate in triplicate. The absorbance of the samples was measured at 450 nm utilizing a microplate reader (RT-2100c). Cells that were cultured exclusively with OM served as control samples (CTRL).

Alkaline Phosphatase (ALP) Activity

Alkaline phosphatase (ALP) is an enzyme that catalyzes the hydrolysis of phosphate esters in an alkaline buffer, resulting in the production of an organic radical and inorganic phosphate. In the present study, a colorimetric assay to assess ALP activity was performed at 7 days, 14 days, and 21 days post-osteogenic differentiation. The ALP assay kit (cat. MAK447, Sigma-Aldrich, St. Louis, MO, USA) utilized p-nitrophenyl phosphate (pNPP) as a substrate, which exhibits a colorimetric change to yellow (λ_{max} = 405 nm) upon dephosphorylation by ALP.

To conduct the assay, cell supernatants were first removed, followed by washing the cell layers with PBS 1 $\times$. The cells were then permeabilized overnight at 4 °C using a 0.1% Triton X-100 solution. Next, 100 μ L of a 1 M diethanolamine buffer (pH 9.8, 0.5

mM MgCl₂; cat. D8885, Sigma-Aldrich, St. Louis, MO, USA) containing 1 mg/mL pNPP was added to each well and incubated for 30' at RT in the absence of light. After this incubation period, 20 µL of stop solution (3 M NaOH) was introduced into each well, followed by the addition of 80 µL of the sample in triplicate to a flat-bottom 96-multiwell plate. The absorbance was subsequently measured at 405 nm using a microplate reader (RT-2100c). Cells cultured in OM served as the CTRL group for the study. The percentage of ALP activity relative to the CTRL was calculated using the following formula:

$$\frac{(OD_{sample})}{(OD_{control})} \times 100$$

RNA Isolation, Reverse Transcription (RT), and real-time RT-Polymerase Chain Reaction (real-time RT-PCR)

The RNA isolation procedure was conducted at predetermined time points subsequent to osteoinduction. Total RNA was extracted from cells utilizing TRIzol (cat. 15596026, Thermo Fisher Scientific, Waltham, MA, USA). The concentration and quality of the RNA were subsequently assessed using the BioSpec-nano spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). The RNA was then reverse transcribed (RT) into complementary DNA (cDNA) for quantitative polymerase chain reaction (qPCR) analysis, employing the RevertAid First Strand cDNA Synthesis kit (cat. K1621, Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol and utilizing oligo (dT)₁₈ primers. Following this, real-time PCR analysis was performed using the LineGene K Plus Fluorescence Real-time PCR detection system (BIOER Technology, Shanghai, China) with the real-time PCR master mix (Maxima SYBR Green/ROX qPCR Master Mix; cat. K0223, Thermo Fisher Scientific, Waltham, MA, USA). The sequences of the human-specific primers are provided in **Table 5**. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized for normalization purposes, and the relative gene expression was calculated employing the comparative 2^{-ΔΔCT} method. This methodology assesses the ratio between the expression of the gene of interest and that of GAPDH. For each primer, a control consisting of no template cDNA and a control without reverse transcriptase were included.

Table 5. Primers with corresponding sequences used in the study.

Primer name		Sequences (5' → 3')
BMP4 ¹	Forward	CTG GAT GAG TAT GAT AAG GTG GTA
	Reverse	GGG TGA GTG GAT GGG AAC
RUNX2 ²	Forward	ACA AAC AAC CAC AGA ACC ACA AGT
	Reverse	GTC TCG GTG GCT GGT AGT GA
OCN ³	Forward	TTG GAC ACA AAG GCT GCA C
	Reverse	CTC ACA CTC CTC GCC CTA TT
BSP ⁴	Forward	AAA GTG AGA ACG GGG AAC CT
	Reverse	ACC ATC ATA GCC ATC GTA GCC
GAPDH ⁵	Forward	TCA TGA CCA CAG TCC ATG CCA TCA
	Reverse	CCC TGT TGC TGT AGC CAA ATT CGT

¹Bone morphogenetic protein 4; ²Runt-related transcription factor 2; ³Osteocalcin; ⁴Bone sialoprotein; ⁵Glyceraldehyde 3-phosphate dehydrogenase.

Statistical Analysis

The statistical analysis was conducted using the GraphPad Prism Software Analysis version 9. Following an assessment and validation of the normality of the distribution through the Shapiro-Wilk test, ordinary one-way ANOVA, two-way ANOVA, or unpaired t-tests were employed, followed by Tukey's post-hoc test. Statistical significance was determined at a p -value ≤ 0.05 . All experiments were performed in both technical and biological triplicate. The data were expressed as the mean $\pm$ standard error of the mean.

4.2.2.2. RESULTS

Characterization of the Two Types of Collagen

In this study, type I collagens isolated from equine tendons were processed according to the company's proprietary methodologies. SDS-PAGE was employed to ascertain the molecular weight of the collagens and establish a correlation with the biological response elicited by the coatings. The electrophoretic pattern (**Figure 24a**) demonstrated that COL N exhibits two distinct bands corresponding to the type I collagen $\alpha 1(I)$ and $\alpha 2(I)$ chains, with estimated molecular weights of approximately 140 kDa and 130 kDa, respectively [43].

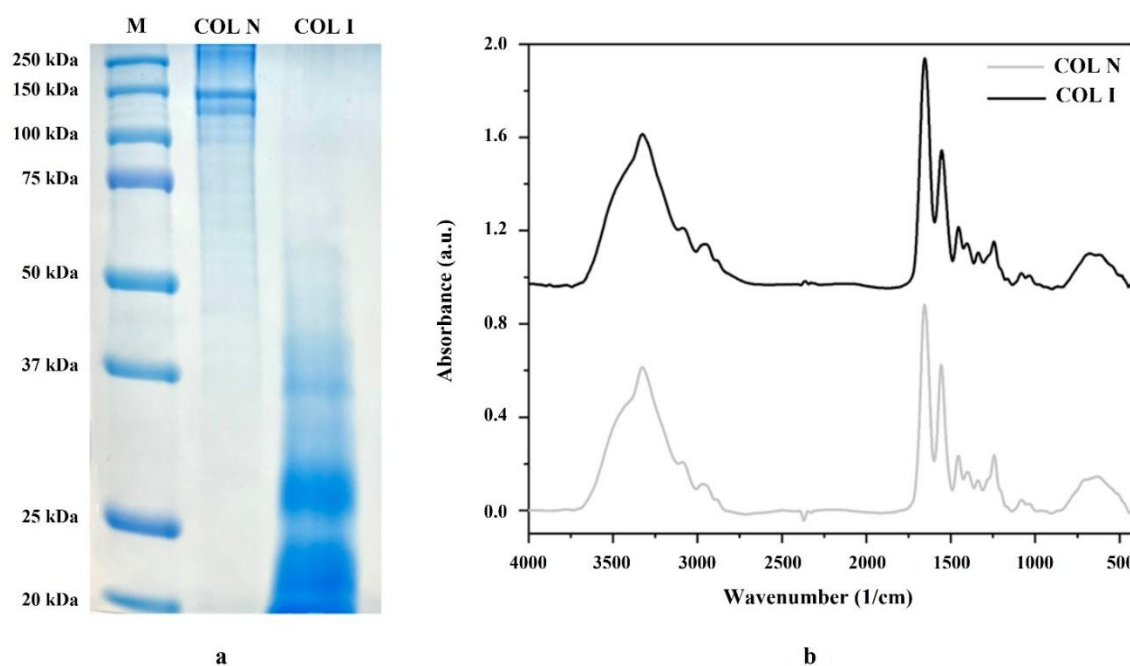


Figure 24. Comparative analysis of the properties of native type I collagen (COL N) and prototypical partially hydrolyzed type I collagen (COL I) derived from equine tendons, focusing on molecular weight and available chemical groups. **(a)** Electrophoretic profiles of COL N and COL I suspensions in relation to standard protein markers, which range from 10 to 250 kDa; **(b)** Fourier-transform infrared spectroscopy (FTIR) spectra for COL N and COL I suspensions. M: standard proteins.

Analysis of band intensities indicated that the intensity of the $\alpha 1(I)$ band was nearly double that of the $\alpha 2(I)$ band, reflecting the characteristic triple helical structure of collagen. Subsequently, COL I was generated from COL N to formulate a partially hydrolyzed variant designed to enhance the exposure of chemical groups. The electrophoretic pattern of COL I revealed protein fragments with molecular weights below 50 kDa, specifically ranging from approximately 40 kDa to 20 kDa.

Additionally, the material's characteristics were evaluated through FTIR spectroscopy, which identified distinct peaks associated with the chemical groups of type I collagen in both samples, particularly amide groups [43] (**Figure 24b**). In COL N, amide I was detected at 1656 cm^{-1} , amide II at 1555 cm^{-1} , and amide III at 1242 cm^{-1} ; whereas COL I exhibited amide I at 1656 cm^{-1} , amide II at 1550 cm^{-1} , and amide III at 1236 cm^{-1} . The FTIR spectra revealed the preservation of the strict hierarchical organization in COL N, while indicating a loss of quaternary structure in COL I. Notably, the triple helical content was assessed using the amide III to $-\text{CH}_2$ ratio, with values exceeding 1 confirming the high preservation of collagen structure, and values below 1 indicating a loss of tertiary structure [55,153]. The amide III/ $-\text{CH}_2$ ratio for COL N was approximately 2.2, affirming

the presence of collagen fibers as previously documented [43,55]. In contrast, the amide III/-CH₂ ratio for COL I was approximately 0.51, corroborating the degradation of triple helix integrity [153].

Physicochemical Characterization of the Coatings

The physical-chemical properties of COL N and COL I coatings were evaluated with respect to wettability, SD, degradation resistance, and mechanical properties. Initial evaluations involved determining the hydrophilic or hydrophobic nature of the samples under physiological-like conditions. As presented in **Table 6**, COL N exhibited a more hydrophobic character compared to COL I ($p < 0.0001$), although collagen concentration strongly influenced the behavior of the coatings.

Table 6. Properties of native type I collagen (COL N) and prototypical partially hydrolyzed type I collagen (COL I) derived from equine tendons, in terms of wettability and constitutive bond. n.d.: not detectable.

Sample type	Concentration (mg/cm ³)	Contact angle (°)	Elastic modulus (MPa)	Maximum stress (MPa)	Strain at break (%)
COL N	0.1	72 ± 4	n.d.	n.d.	n.d.
	1	88 ± 8	1.7 ± 0.2	4.2 ± 1.4	74 ± 18
	2	105 ± 9	2.1 ± 0.3	5.0 ± 1.1	85 ± 14
COL I	0.1	63 ± 5	n.d.	n.d.	n.d.
	1	69 ± 6	0.040 ± 0.010	0.006 ± 0.002	20 ± 9
	2	87 ± 6	0.035 ± 0.006	0.011 ± 0.003	74 ± 21

Specifically, contact angle values increased with higher collagen concentrations in both scenarios ($p < 0.008$ for COL I and $p < 0.01$ for COL N). Notably, COL I coatings consistently demonstrated hydrophilic properties ($\theta < 90^\circ$), with an inverse correlation observed with respect to collagen concentration. In contrast, COL N displayed hydrophilic characteristics at lower concentrations, transitioning to a hydrophobic character at concentrations of 1 mg/cm³ and 2 mg/cm³ ($\theta > 90^\circ$, $p = 0.017$), effectively indicating that its physicochemical properties are closely linked to concentration levels.

The capacity of the coatings to absorb and retain water over time was evaluated by calculating their SD. As illustrated in **Figure 25a**, all coatings successfully absorbed and retained water, achieving a range of 200-500% of their dry weight, depending on the type and concentration of collagen utilized.

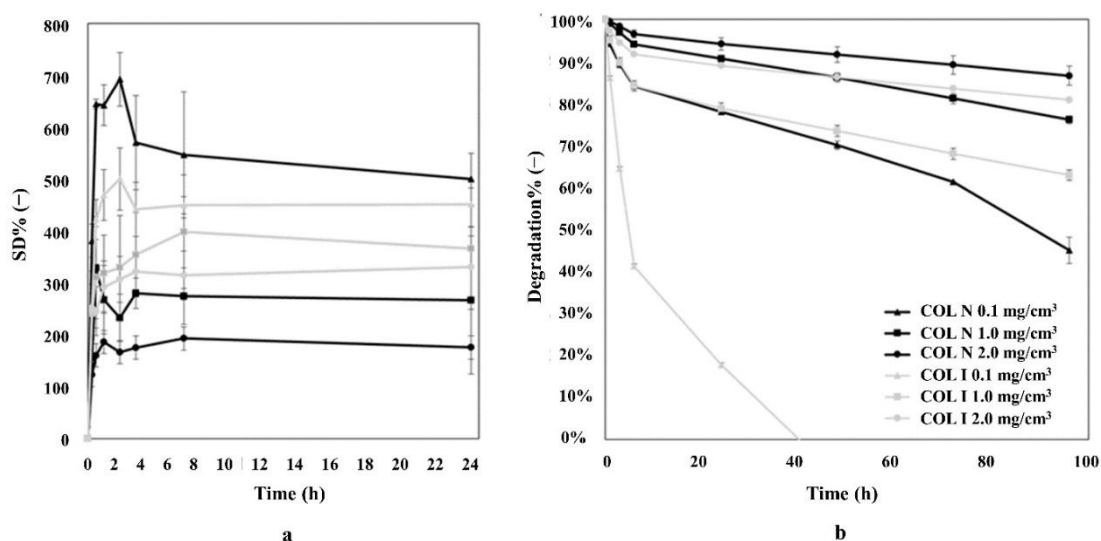


Figure 25. Physicochemical characterization of COL N and COL I. (a) Swelling degree (SD) in coatings; (b) Resistance to degradation over time.

The coatings were able to swell completely within 30' and reached a stabilization plateau within 1 h. In particular, COL N retained less water than COL I, likely due to the presence of collagen fibers and interchain interactions that contributed to a less hydrophilic matrix. Conversely, coatings derived from COL I, composed of shorter collagen polypeptide chains, exhibited less entangled matrices, which facilitated greater swelling compared to COL N using the same material quantity. Notably, coatings prepared with 0.1 mg/cm³ of COL N and COL I exhibited the highest SD% due to the low concentration of protein, resulting in less dense matrix structures that were more prone to water absorption.

The low concentration of materials, resulting in a diminished degree of interaction among proteins, rendered the two experimental classes more susceptible to enzymatic degradation. As illustrated in **Figure 25b**, the experimental classes with 0.1 mg/cm³ of COL I and COL N exhibited the lowest stability under physiological-like conditions. Specifically, COL I at a concentration of 0.1 mg/cm³ underwent complete degradation after 40 h, whereas COL N of the same concentration lost 50% of its weight at the 50-hour mark. It is important to note that collagen concentration was found to be inversely related to the extent of degradation observed in the samples. Coatings with higher collagen content demonstrated increased resistance to degradation, thereby exhibiting an extended half-life. Moreover, collagen concentration plays a crucial role in collagen organization. COL N coatings displayed greater resistance to degradation when compared to COL I coatings, a

difference attributed to the intrinsic properties of their raw materials. COL N, composed of type I collagen with a high degree of conservation of its native fibrillar structure, is less susceptible to enzymatic attack by collagenase in contrast to COL I, which comprises partially hydrolyzed collagen with more exposed functional groups.

The structural differences of the initial materials were reflected in their resistance to applied stresses, as indicated in **Table 6**. Due to the low protein concentrations in the 0.1 mg/cm³ coatings, tensile testing was not feasible. In contrast, samples with concentrations of 1 and 2 mg/cm³ for both types were analyzed, revealing considerable differences. COL N demonstrated greater resistance to applied stresses than COL I, with COL N exhibiting a maximum stress capacity of approximately 4-5 MPa—twice the values observed for COL I. Notably, while there was no significant effect of COL N concentration on maximum stress values, it effectively doubled the maximum stress threshold of COL I-derived coatings. Strain at break values were influenced by collagen concentration, with similar values recorded for both COL N and COL I at the 2 mg/cm³ concentration. However, COL N showed an increase of approximately 13% in the strain at break values corresponding to rising collagen concentration, while COL I experienced a considerably higher increment of about 73%. Lastly, COL I-derived samples were determined to be twice as elastic as those derived from COL N, with no statistically significant differences attributable to concentration.

Cell Culture Characterization

Flow cytometry analysis revealed that mesenchymal stem cell markers exhibited high expression levels in SCAPs, with CD73 at 97.38% ± 0.87, CD90 at 98.28% ± 0.92, and CD105 at 96.73% ± 0.78. In contrast, the presence of hematopoietic markers CD34 and CD45 was minimal, recorded at 0.04% ± 0.01 and 0.03% ± 0.01, respectively (**Figure 26**).

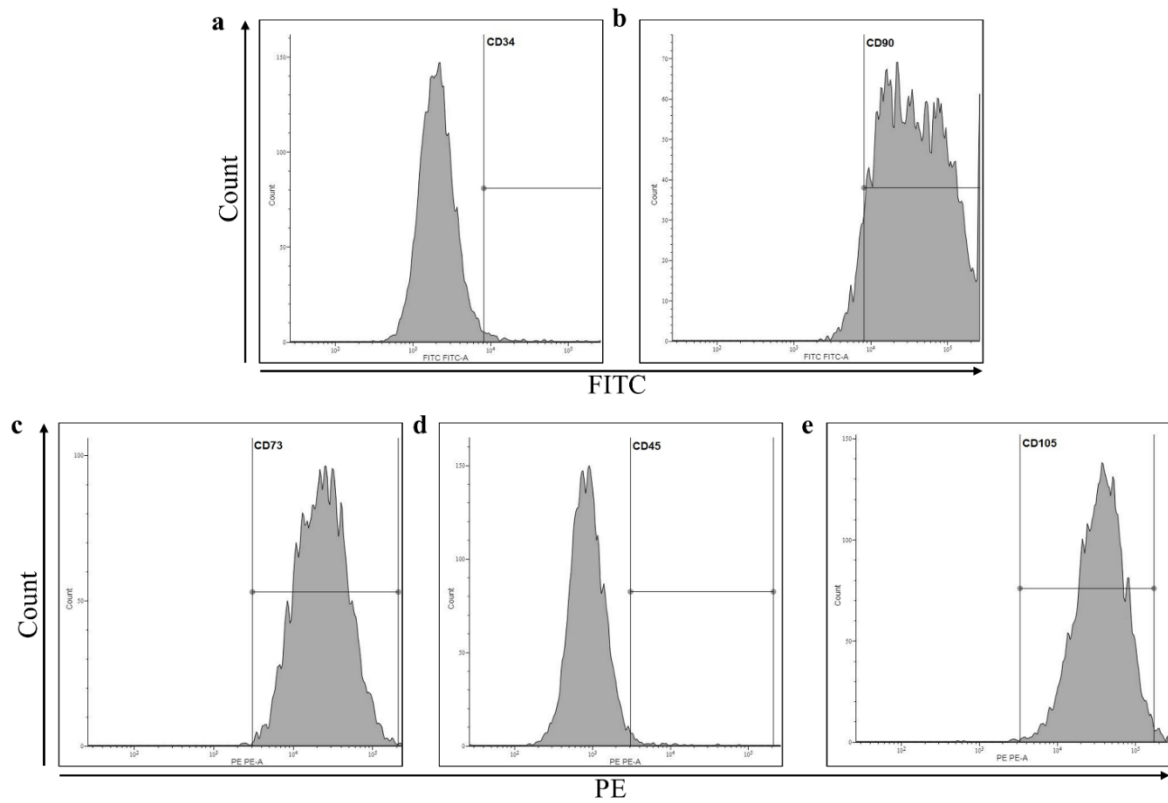


Figure 26. Immunophenotypic profile of mesenchymal stem cells from the apical papilla (SCAPs) evaluated using (a,b) FITC-labeled mouse monoclonal antibodies targeting CD34 and CD90, alongside (c-e) PE-labeled mouse monoclonal antibodies for CD73, CD45, and CD105. The SCAPs exhibited negative expression for hematopoietic markers CD34 and CD45, while demonstrating positive expression for the mesenchymal stem cell markers CD73, CD90, and CD105.

Biocompatibility of Collagen Coatings

The MTT assay was performed at specified intervals of 24 h, 48 h, and 72 h, as well as at 7 days, 14 days, and 21 days, utilizing concentrations of 0.1, 1, and 2 mg/cm³ of both COL N and COL I coatings. This evaluation aimed to assess their biocompatibility with SCAPs (Figure 27).

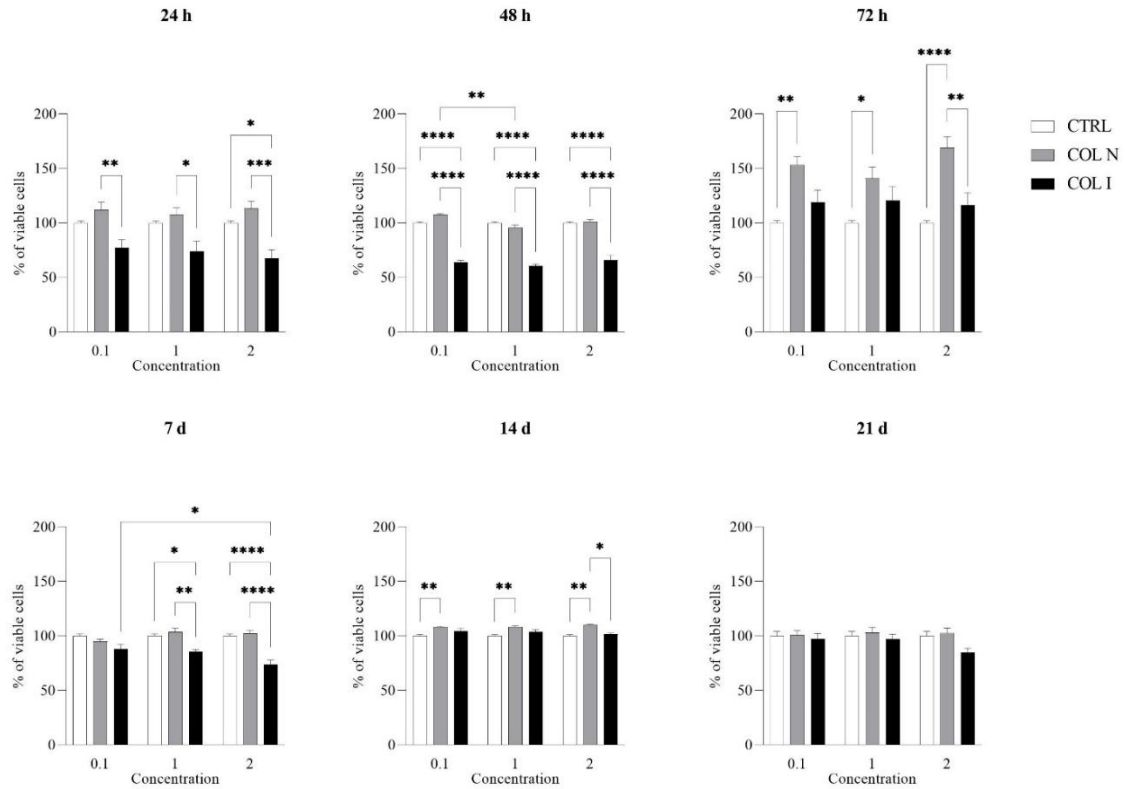


Figure 27. Viability of SCAPs cultured on COL N and COL I coatings in comparison to uncoated wells (CTRL) at intervals of 24 h, 48 h, and 72 h, as well as 7 days, 14 days, and 21 days. The data are expressed as the percentage of viable cells relative to the CTRL and specify the collagen concentration in mg/cm³. d: days. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

After a 24-hour incubation period, only cells cultured on COL I at a concentration of 2 mg/cm³ exhibited a statistically significant reduction in viability compared to the CTRL group (*p* < 0.05), while all other conditions did not demonstrate any significant differences. Notably, COL I at all concentrations resulted in significantly lower cell viability compared to the corresponding concentrations of COL N, with *p* < 0.01, < 0.05, and < 0.001 for 0.1, 1, and 2 mg/cm³, respectively. After 48 h, the viability of SCAPs cultured with COL N at any concentration displayed no statistically significant differences compared to the CTRL, whereas COL I consistently indicated a *p* < 0.0001 in relation to both the CTRL and COL N conditions. Interestingly, following a 72-hour incubation, COL I did not present significant differences when compared to any other experimental conditions, except for COL N at the highest concentration (*p* < 0.01). At this time point, cells cultured with COL N consistently demonstrated significantly higher viability compared to the CTRL, with *p* < 0.01, < 0.05, and < 0.0001 at 0.1, 1, and 2 mg/cm³ concentrations, respectively. After one week, only the lowest concentration of COL I did

not adversely affect cell viability, whereas higher concentrations resulted in a significant decrease in viability ($p < 0.05$ for 1 mg/cm³ and $p < 0.0001$ for 2 mg/cm³). Conversely, exposure to COL N resulted in significantly higher cell viability compared to COL I at concentrations of 1 and 2 mg/cm³ ($p < 0.01$ and $p < 0.0001$, respectively), without significant differences relative to the CTRL. Over extended periods, none of the experimental conditions indicated cytotoxicity. At the 14-day observation point, it should be emphasized that COL N demonstrated a significantly positive effect on cell viability across all concentrations ($p < 0.01$). Additionally, at its highest concentration, COL N exhibited a significantly greater effect than COL I ($p < 0.05$). After 21 days, none of the treatments demonstrated any significant differences when compared to the CTRL group.

In summary, with the exception of longer time periods, COL I generally showed lower biocompatibility compared to both the CTRL and COL N. Consequently, it was determined to proceed with further experiments utilizing only COL N across all concentrations.

The Effect of Collage Coatings on Bone Matrix Deposition

The osteogenic differentiation potential of COL N coatings was assessed at concentrations of 0.1, 1, and 2 mg/cm³ over periods of 7 days, 14 days, and 21 days using SCAPs. This evaluation was conducted in comparison with the CTRL group, in which the cells were subjected exclusively to OM treatment (**Figure 28**).

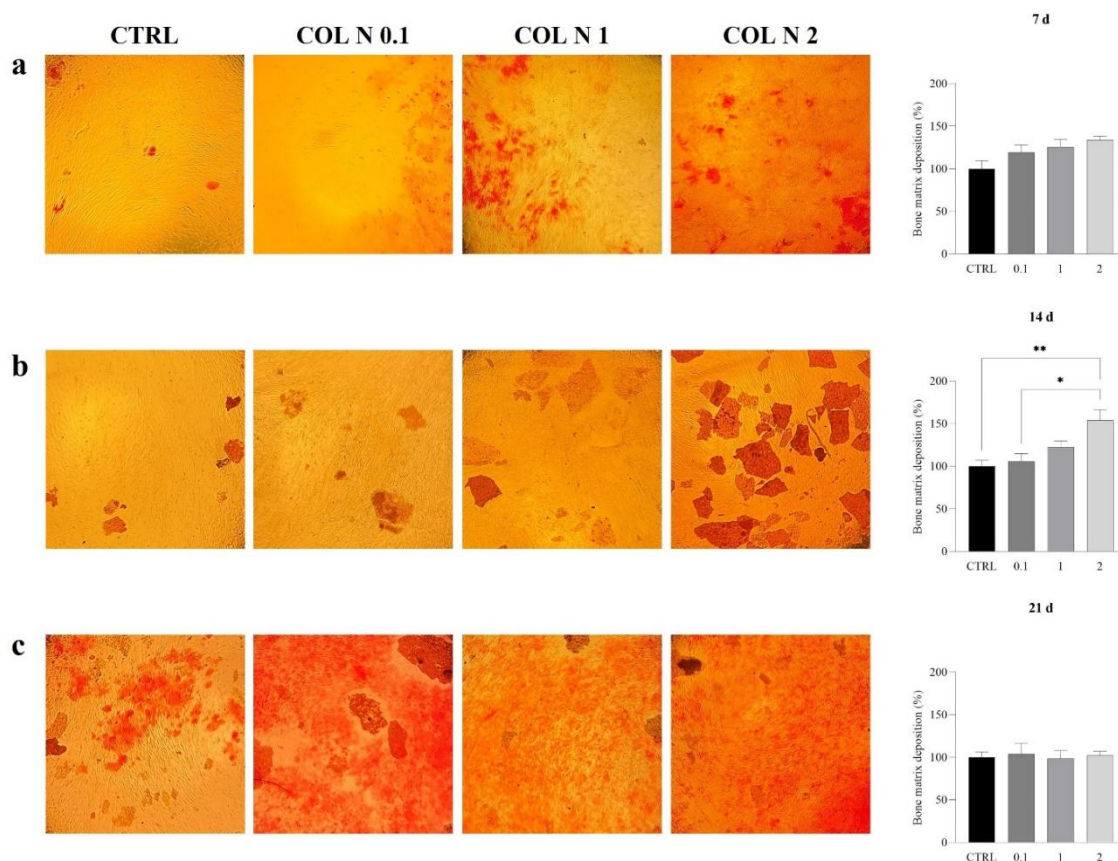


Figure 28. Inverted light microscopic representative images and relative quantitative analyses of Alizarin Red S staining (ARS) assays of SCAPs cultured on COL N coatings at concentrations of 0.1, 1, and 2 mg/cm³. **(a)** Mineral matrix deposition after 7 days, **(b)** 14 days, and **(c)** 21 days of culture. Original image magnification: 40×. The data are expressed as the percentage of bone matrix deposition in comparison to the CTRL group (cells treated solely with the osteogenic medium, OM) and indicate the concentration of collagen in mg/cm³. * $p < 0.05$, ** $p < 0.01$.

After 14 days of observation, only the COL N concentration of 2 mg/cm³ demonstrated statistically significant differences when compared to the CTRL condition ($p < 0.01$) and the lowest concentration ($p < 0.05$). It is important to note that no significant differences were detected at 7 or 21 days; however, a positive correlation between collagen concentration and bone matrix deposition was observed after 7 days. Additionally, an overall increase in the formation of mineralized nodules was evident across all conditions in comparison to the previous time periods, as illustrated in **Figure 28c**.

Collagen Coatings and Their Impact on ALP Activity Modulation

The osteoconductive potential of COL N coatings at various concentrations was further evaluated by analyzing the ALP activity of SCAPs over 7 days, 14 days, and 21 days. This assessment was conducted in comparison to the CTRL group, which consisted

of cells subjected exclusively to OM treatment, as illustrated in **Figure 29**.

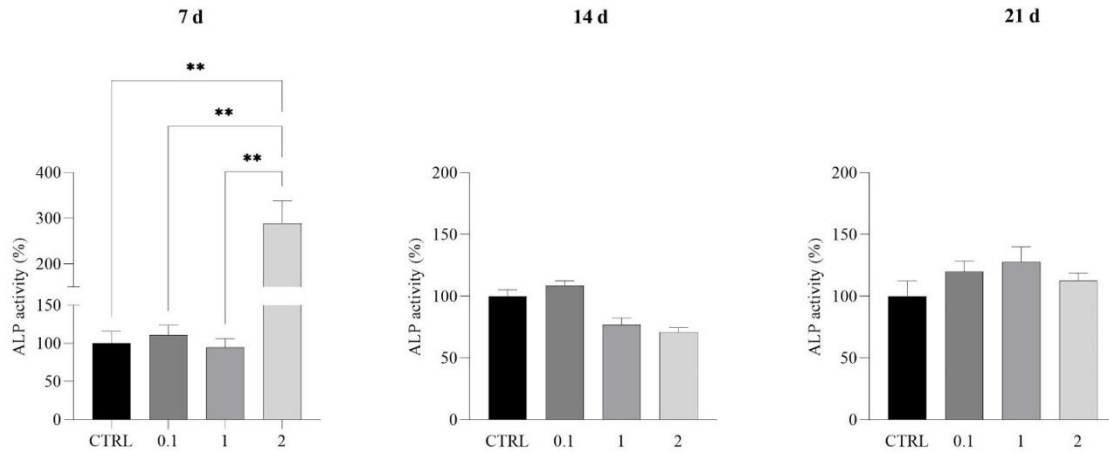


Figure 29. Alkaline phosphatase (ALP) activity in SCAPs cultured on COL N coatings at concentrations of 0.1, 1, and 2 mg/cm³ after 7 days, 14 days, and 21 days. The data are presented as a percentage of ALP activity relative to the CTRL group (cells treated solely with the OM). The graphs indicate the concentration of collagen in mg/cm³. ** $p < 0.01$.

Upon evaluation at both the 14-day and 21-day time points, no significant differences were observed among the experimental groups. However, at the 7-day time point, the application of COL N at a concentration of 2 mg/cm³ resulted in significantly elevated values in comparison to the CTRL group and other tested concentrations of COL N ($p < 0.01$).

Osteogenic Markers Regulation by the Selected Collagen Coating

The enhanced osteoconductive potential of COL N at a concentration of 2 mg/cm³, as demonstrated by ARS and ALP activity assays, warranted an investigation into the gene expression of four osteogenic markers: bone morphogenetic protein 4 (BMP4), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), and bone sialoprotein (BSP). This test focused exclusively on SCAPs cultured on COL N at a concentration of 2 mg/cm³ over periods of 7 days, 14 days, and 21 days.

Figure 30 illustrates a consistently elevated expression of these markers for SCAPs cultured on COL N2 coating compared to the CTRL group, irrespective of statistical significance, with the exception of the BSP marker at the 7-day time point.

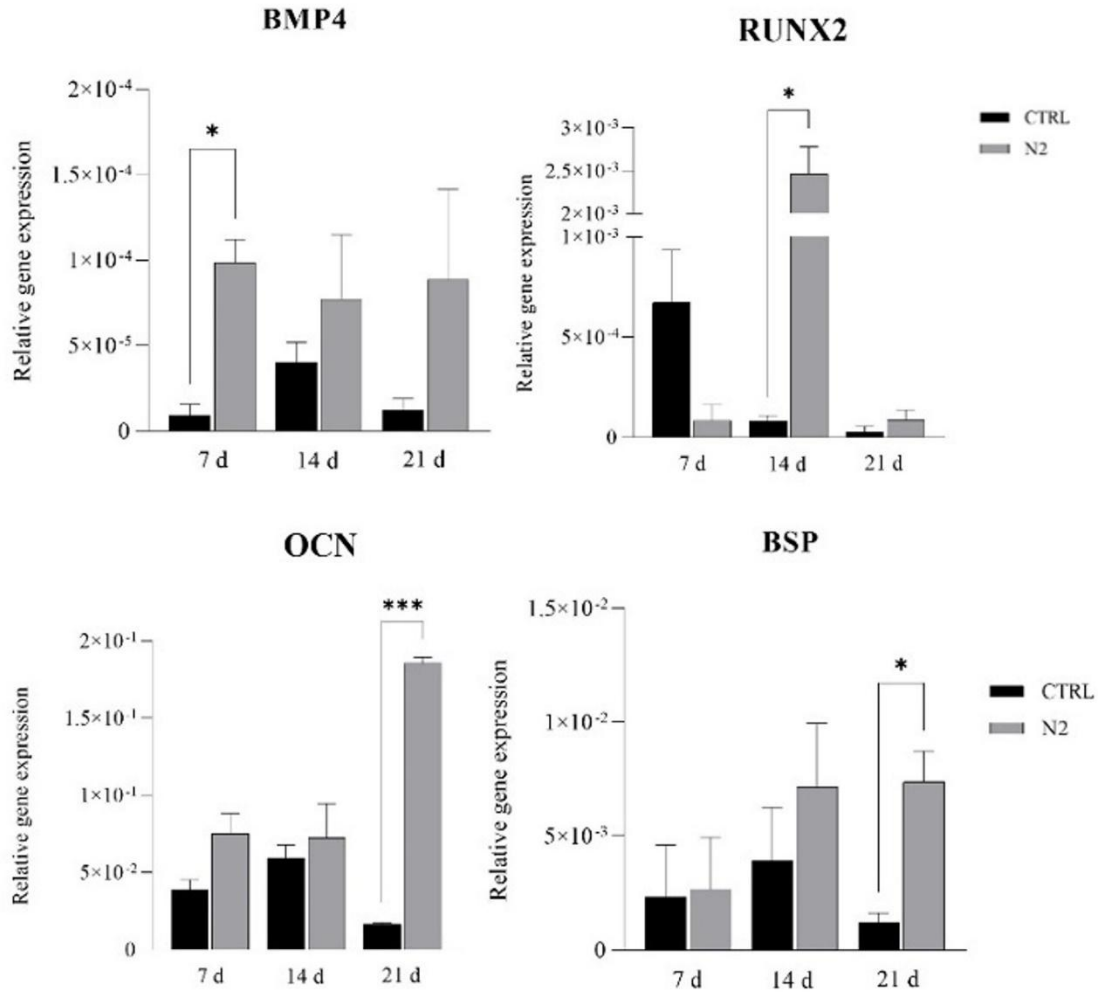


Figure 30. Effects of the COL N coating at a concentration of 2 mg/cm³ (N2) on the mRNA expression of osteogenic markers—bone morphogenetic protein 4: BMP4, runt-related transcription factor 2: RUNX2, osteocalcin: OCN, and bone sialoprotein: BSP—in SCAPs after 7 days, 14 days, and 21 days. The results are presented as relative gene expression ($2^{-\Delta\Delta CT}$). The CTRL group refers to cells that were treated solely with the OM. * $p < 0.05$, *** $p < 0.001$.

Notably, significantly higher levels were recorded after 7 days for BMP4 ($p < 0.05$), after 14 days for RUNX2 ($p < 0.05$), and after 21 days for OCN ($p < 0.001$) and BSP ($p < 0.05$).

4.2.2.3. DISCUSSION

The research conducted a comprehensive investigation into the physical, chemical, and biological properties of various formulations of type I collagen derived from horse tendons. The primary objective was to assess the influence of differing concentrations of COL N and COL I coatings (0.1, 1, and 2 mg/cm³) on SCAPs, while also

providing a chemical characterization of these coatings. The study concentrated on evaluating the biocompatibility and potential to enhance bone formation, with the goal of advancing the development of regenerative biomaterials within the field of dentistry.

The findings demonstrated that SCAPs cultured on COL N-coated surfaces exhibited enhanced viability and a significant increase in mineralization, particularly at elevated collagen concentrations. Importantly, the collected data revealed a positive correlation between collagen concentration and both mineral matrix deposition and ALP activity. This indicates that COL N, at a concentration of 2 mg/cm³, not only provided a conducive environment for SCAPs but also actively promoted their osteogenic differentiation. These results were further substantiated by gene expression analyses of key osteogenesis markers. Specifically, all four osteogenesis-related genes examined (BMP4, RUNX2, OCN, and BSP) demonstrated significant upregulation in SCAPs cultured on COL N at its highest concentration. The overexpression of these genes was observed at distinct time points: BMP4 at 7 days, RUNX2 at 14 days, and OCN and BSP at 21 days, consistent with previous literature on SCAPs [169]. Furthermore, the differences noted between COL N and COL I may be attributed to variations in extraction and purification methodologies or differing manipulation processes, which could result in alterations to the bioactive conformational structure of the collagen [53].

Due to its favorable properties, type I collagen derived from both marine and animal sources is widely utilized to develop various biomaterials for biomedical applications [20,21]. Despite the predominance of bovine and porcine collagens, equine collagen presents several advantages, including minimal zoonosis transmission risks, a high degree of sequence similarity to human collagen, superior resistance when compared to other collagen types, and the absence of reported immune reactions or religious restrictions [153]. Furthermore, existing literature highlighted that the distinct hierarchical organization of the equine tendon facilitates the preservation of collagen's native structure post-extraction and processing, in contrast to tissues from other mammalian sources. This underscores its significant potential in biomedical fields, where maintaining natural binding sites and intrinsic bioactivity is paramount [55]. A research study conducted by Salvatore et al. [43] provided a thorough structural analysis of an insoluble fibrillar type I collagen extracted from horse tendon, referred to as COL N. This analysis was conducted alongside an examination of a commercially available collagen isoform derived from the same species and tissue. The SDS-PAGE results indicated comparable integrity and purity in relation to the collagen under investigation, exhibiting the two characteristic bands of

type I collagen. Additionally, structural analysis via FTIR spectroscopy revealed identical spectral profiles for COL N, further affirming the reproducibility of the extraction and purification methodologies employed. Moreover, the resistance of collagen to enzymatic degradation was notably higher than the commercial counterpart, with 56% degradation observed at a time point when the commercial collagen was entirely degraded. This resulted in a half-life of 5.8 h for the test collagen, compared to 1.3 h for the commercial variant. The study also evaluated the biocompatibility and cellular interactions of these collagens when applied as films on mouse embryonic fibroblasts. The cytocompatibility of the test collagen remained unaffected; however, the proliferation rate of NHI/3T3 cells exhibited a progressive increase over time in comparison to the industrial collagen. This finding suggested that fibroblasts and SCAPs interact with native collagen through specific cell receptors, offering insights into the preservation of the extracted collagen. Similar trends in increasing cellular growth rates have been documented in other studies involving equine collagen as compared to bovine and porcine collagen [170]. In the current study, COL N demonstrated a gradual increase in cell viability over time, achieving statistical significance after 72 h ($p < 0.01$ for COL N0.1, $p < 0.05$ for COL N1, and $p < 0.0001$ for COL N2) and after 14 days ($p < 0.01$) in comparison to the CTRL group and even relative to COL I after only 24 h.

In the context of bone regeneration, collagen fibers play a critical role during the initial phases of bone tissue formation by providing a scaffold for the deposition of apatite crystals, thereby facilitating ongoing bone growth. Collagen scaffolds not only support cellular differentiation and proliferation but also replicate the architecture of natural bone, which is essential for achieving optimal outcomes in bone grafting procedures [37]. Numerous experimental studies indicated that modifications in the biochemical composition of the ECM result in significant alterations to its mechanical properties, such as stiffness, which subsequently influence cellular morphology, growth, differentiation, migration, and gene expression, with force transmission mediated through focal adhesions [56,61,171]. Specifically, the estimated stiffness of bone, contingent upon the composition and structure of the collagen fibers, is reported to range from approximately 100 kPa to 1 GPa, closely aligning with the measured Young's modulus of COL N [172]. Furthermore, research indicated that the osteogenic differentiation potential of mesenchymal stem cells is enhanced fourfold when cultured on rigid matrices [173]. To date, various formulations utilizing equine tendon collagen have been developed and are presently employed in commercial applications for the regeneration of hard tissues. These formulations include

osteoconductive sponges, blocks, hemispheres, microparticles, and membranes, often integrated with osteoinductive molecules. They have been shown to promote and enhance bone healing by facilitating neoangiogenesis, thereby contributing to improved support for the process of bone regeneration [174-179]. In the domain of dentistry, a substantial body of literature exists that documents the successful application of equine collagen for bone regeneration in augmentation and ridge preservation procedures. Evidence indicated the formation of new bone, preservation of ridge volumes, and enhanced vascularization at the site, with implant success rates comparable to those achieved with autogenous bone grafts after one year [152,154,156,180-182]. Moreover, recent preclinical investigations conducted by Barrino, Alqutub, and Tumedei [178,179,183] have further validated these findings, demonstrating the osteoconductive properties of equine collagen type I, revealing increased cell proliferation, calcium deposition, and gene expression of osteogenic markers in various oral mesenchymal stem cells. Although some studies have explored the utilization of equine collagen in gel formulations for applications such as drug delivery, wound healing, and nerve regeneration, there remains a notable gap in research concerning its application in bone repair [161, 184-186]. Accordingly, to the best of our knowledge, this study represents the first detailed exploration of the osteoconductive potential of type I collagen derived from equine tendons, specifically in the format of films.

In the field of dental tissue engineering, SCAPs are extensively employed owing to their remarkable proliferation capabilities, self-renewal properties, and potential to differentiate into various cell lineages. Consequently, they are considered a highly promising source of stem cells for regenerative oral treatments, as they differentiate into odontoblasts and pulp cells [187]. Although SCAPs exhibit superior proliferation and mineralization potential in comparison to other dental stem cells [169], there is a notable scarcity of investigations assessing the osteogenic efficacy of collagen on SCAPs, particularly equine collagen. Existing research has explored the osteogenic differentiation of SCAPs induced by different forms of pure collagen or in conjunction with other molecules. Such studies have indicated strong cytocompatibility and a significant positive effect on cell motility, along with an early osteogenic differentiation response in the absence of additional stimuli [188,189]. Moreover, some research has reported enhanced cell viability, adhesion, and spreading of SCAPs in the presence of type I collagen formulations aimed at dentin-pulp regeneration [190-193]. Conversely, a study conducted by Leite et al. [194] reported a lack of bioactivity when evaluating a rat tail type I collagen coating on SCAPs, attributing this absence of response to insufficient collagen

concentrations necessary for stimulating cell adhesion, proliferation, and spreading. Consequently, the current investigation represents the first assessment of the osteogenic properties of SCAPs in response to type I collagen derived from equine sources. While SCAP-mediated tissue regeneration demonstrated substantial promise, the understanding of the interaction between SCAPs and the microenvironment remains constrained. Variations in the ECM topographic structure, geometry, tissue mechanics (such as stiffness and elastic modulus), as well as other parameters (force, temperature, and light) significantly influence this interaction. Nevertheless, the effects of these factors on cell proliferation, migration, differentiation, and tissue remodeling, particularly concerning SCAPs, remain inadequately elucidated [195].

The present study acknowledges several limitations that merit consideration. Although not extensively recognized, equids exhibit susceptibility to alphaviral equine encephalomyelitis (AEE), a mosquito-borne zoonotic infection that rarely transmits to humans. The infrequency of encephalitis cases combined with the low mortality rate associated with AEE prompts the exploration of equine by-products as potential sources for medical-grade collagen extraction devoid of zoonotic risks. Additionally, the notable hierarchical organization of collagen fibers in tendons, along with their dense packing, indicates that devices manufactured from reconstituted native horse tendon collagen may possess enhanced resistance to degradation and mechanical stress [44]. However, a considerable challenge in the development of collagen-based scaffolds lies in accurately replicating the sub- and supramolecular structures inherent to natural type I collagen. Variations in extraction and purification methods, as well as differences in sterilization processes, can induce structural modifications that affect the bioactive conformational integrity of COL I and COL N [164]. From a biological standpoint, the efficacy of coatings to facilitate stem cell growth and their differentiation into bone phenotypes was evaluated using assays of ARS, ALP activity, and the gene expression of osteogenic markers. For this latter purpose, bone phenotype biomarkers BMP4, RUNX2, OCN, and BSP—recognized for their roles in bone formation—were selected [169]. Literature suggested that BMP4 and RUNX2 are early-stage markers of osteogenic differentiation, whereas OCN and BSP are considered late-stage markers [196]. This aligned with the current findings, which demonstrate significantly elevated levels of BMP4 and RUNX2 expression at 7 days ($p < 0.05$) and 14 days ($p < 0.05$), respectively, alongside a significant upregulation of OCN and BSP markers at 21 days ($p < 0.001$ and $p < 0.05$, respectively). Furthermore, the ALP activity assays consistently indicated comparable values for cells

treated with COL N relative to the CTRL group, with a significant difference observed after 7 days at a COL N concentration of 2 mg/cm³ ($p < 0.01$ versus the CTRL group and COL N0.1, N1, and N2). This corroborated previous studies that typically identify ALP as an early differentiation marker that downregulates during mineralization [197]. However, additional *in vitro* investigations employing a larger sample of primary cell cultures are recommended, followed by pre-clinical and clinical studies utilizing this collagen. It is essential to evaluate the expression of markers not only at the RNA level but also at the protein level to conclusively validate gene expression.

In conclusion, the analyses conducted have consistently illustrated that the proprietary manipulation process specifically optimized for COL N effectively preserves the native structure of collagen when compared to COL I. Notably, investigations into the structural and biological properties of COL N, isolated from equine tendon at a concentration of 2 mg/cm³, have provided compelling evidence of its significant osteoconductive properties. The findings demonstrated that equine COL N, particularly at this concentration, not only fosters a biocompatible environment for SCAPs but also actively promotes their differentiation into osteogenic cells. This reinforces the potential of this biomaterial as a viable alternative to conventional mammalian collagen for the advancement of implantable medical devices and highlights its applicability as a scaffold in dental tissue engineering. In this context, a logical subsequent step involved employing this collagen formulation as a coating for implant surfaces. Given the increasing demand for biocompatible, bioactive, and biomimetic surfaces to enhance the integration of dental implants, the exploration of an equine type I collagen coating presents a promising area for further investigation, particularly in light of the limited existing literature on functional equine collagen coatings [198,199].

The Osteoconductive Properties of Collagen-Coated Implant Surfaces

4.2.2.4. MATERIALS AND METHODS

Materials

Native type I collagen derived from equine tendons (N) was utilized to develop implant surface coatings in accordance with the previously optimized protocol (refer to *Sample Preparation* in section 3.2.2.1.). Three distinct concentrations of N—specifically, 0.1, 1, and 2 mg/cm³ (designated as N0.1, N1, and N2, respectively)—were investigated.

This study examined the influence of N coatings on various implant disc surfaces (Implacil De Bortoli, Cambuci, São Paulo, Brazil) made of titanium grade 4 (dimensions: 5 mm × 2 mm) in accordance with the standards set forth by the American Society for Testing and Materials (ASTM)-F67 [200]. As defined by Petrini et al. [105], the macro, micro, and nano surface characteristics were assessed using SEM and atomic force microscopy (AFM). The analysis of discs revealed four distinct surface treatments:

- Machined (M): Untreated smooth surfaces with a Ra of 27.29 ± 5.66 nm;
- Sandblasted and DAE (S): This procedure involved sandblasting with titanium oxide microparticles of 50-100 µm, followed by conditioning with maleic acid (dual-acid attack), resulting in a Ra of 92.03 ± 6.32 nm;
- S with a linear macro-roughness (L): The S surface treatment incorporated a linear macro-geometry akin to that of Due Cone implants (Implacil De Bortoli, Cambuci, São Paulo, Brazil) [201];
- S with a waved macro-roughness (W): The S surface treatment featuring the inclusion of healing chambers, representing the macro-geometry of Maestro implants (Implacil De Bortoli, Cambuci, São Paulo, Brazil) [201].

Sample Preparation

All titanium samples underwent a thorough washing process with distilled water, followed by air drying and subsequent sterilization using an autoclave. Prior to cell seeding, collagen-based coatings derived from N were cast onto the surface of discs placed in 96-well culture plates at varying concentrations (N0.1, N1, and N2), corresponding to 0.06%, 0.58%, and 0.80% of N, respectively, in accordance with previously established methodologies (refer to *Sample Preparation* in section 3.2.2.1.). Briefly, the plates were maintained in a sterile hood overnight to facilitate solvent evaporation, followed by four washes with PBS 1×. The discs were allowed to dry under sterile conditions in a flow hood

for an additional overnight period at RT. Subsequently, the prepared discs were either stored at 4 °C in the dark or subjected to 30' of UV irradiation, followed by rehydration in a cell culture medium (DMEM/F-12) for an additional 30' prior to immediate use. Following this preparation, the medium in each well containing collagen-coated discs, as well as in the wells with uncoated discs, which served as the standard control for the growth surface (CTRL), was replaced with 200 µL of fresh medium containing 1×10^4 cells.

Characterization of Coated Surfaces Using SEM

The visualization of the collagen-coated discs (N0.1, N1, and N2 on M, S, L, and W discs) and their respective CTRL samples was performed utilizing an EVO® 40 SEM (Carl Zeiss AG, Oberkochen, Germany) operating in variable pressure mode, with an accelerating voltage set to 20 kV. The dried discs were mounted onto SEM stubs using double-sided carbon tape and were subsequently examined at magnifications of 40× and 1000× utilizing the HD BSD detector.

Contact Angle

The wettability of collagen coatings was evaluated utilizing the sessile drop method. In this procedure, 5 µL of distilled water were deposited onto both uncoated and N-coated titanium discs, and images were immediately captured using a Dino-Lite digital microscope (AnMo Electronics Corporation, New Taipei City, Taiwan). Contact angle measurements were obtained using the drop snake method in ImageJ software. Each sample underwent three replicates to ensure the precision and reliability of the results.

Cultivation and Characterization of Cells

Pulp tissues were collected from three healthy patients, aged 18 to 20, during third molar extraction at the Clinic for Oral Surgery, School of Dental Medicine, University of Belgrade. All ethical considerations were meticulously adhered to, with each patient providing informed consent. The study was conducted in accordance with the Declaration of Helsinki and received approval from the Ethical Committee of the School of Dental Medicine, University of Belgrade (protocol number: 36/19). SCAPs were isolated following established protocols [168,202] and cultivated as detailed in *Cell Cultures*, section 3.2.2.1. Briefly, the extracted teeth were subjected to a washing procedure utilizing PBS 1× containing 1% P/S. Subsequently, the apical papilla was carefully excised from the root apex, minced, and placed into T-25 flasks for cell culture. The cells were cultured in a

complete growth medium (DMEM/F-12 supplemented with 10% FBS and 1% P/S, maintained under standard conditions of 37 °C and 5% CO₂ in a humid environment). The complete medium was replaced every two to three days, and the cells were passaged when reaching 80% confluence, with experimental assays conducted using cells from the third to the sixth passage. For osteogenic differentiation, the cells were seeded in 96-well plates at a density of 1×10^4 cells/well and subsequently incubated in OM after 24 h, with evaluations at 7 days, 14 days, and 21 days, including medium changes every three days.

Additionally, flow cytometry analysis was utilized to evaluate the expression of specific mesenchymal markers in SCAPs, in accordance with the previously outlined protocol, reagents, and instrumentation (refer to *Flow Cytometry Analysis for Antigen Expression* in section 3.2.2.1.). In summary, the analysis incorporated the use of FITC-labeled mouse monoclonal antibodies targeting CD90 and CD34, as well as PE-labeled mouse monoclonal antibodies for CD73, CD105, and CD45, as recommended by the manufacturer. Cells at the fifth passage were harvested employing TrypLE™ Express solution, subsequently washed with PBS 1× supplemented with 10% FBS and counted to achieve a concentration of 1×10^6 cells. These cells were then incubated with the appropriate antibodies for 45' at 4 °C. Following incubation, the cells were rinsed with PBS 1× and analyzed using a BD FACSMelody™ flow cytometer, which was equipped with specialized software (BD FACSCorus).

MTT Assay

Cytotoxicity assessments were performed on SCAPs cultured on collagen-coated surfaces (N0.1, N1, and N2 coatings applied to M, S, L, and W discs) and their CTRL groups after intervals of 24 h, 48 h, and 72 h, as well as 7 days, 14 days, and 21 days. At each specified time point, the culture medium was discarded, and the cells were subjected to a complete medium incorporating MTT (5 mg/mL) for a duration of 3 h at 37 °C in the absence of light. Subsequently, DMSO was utilized to dissolve the formed formazan crystals, and the plate was shaken for 15' at 37 °C while remaining in the dark. The cell supernatants were then transferred to a flat-bottom 96-well plate. The OD was measured at 540 nm using a microplate reader (RT-2100c). Ultimately, the percentage of viable cells (%) was calculated utilizing the formula detailed in MTT Assay, section 3.2.2.1.

ARS Assay

One day post-cell seeding, both CTRL and experimental samples underwent a culture period of 7, 14, and 21 days in OM. Following osteoinduction, the effects of

various collagen-coated surfaces on mineralized nodule formation were evaluated utilizing the ARS assay, as outlined in ARS, section 3.2.21. At the conclusion of the experiment, the supernatants from the samples were transferred to a new 96-well plate, and their absorbance was measured at a wavelength of 450 nm using a microplate reader (RT-2100c). Cells and discs treated solely with OM, without applying the N coating, served as controls (CTRL).

ALP Activity

The ALP activity assay was conducted utilizing the ALP kit at 7, 14, and 21 days following osteogenic differentiation in the presence of OM. The procedure adhered to the protocol outlined in *ALP Activity*, section 3.2.2.1. Subsequently, the supernatants were transferred to a new 96-well plate, and the absorbance was determined at 405 nm using a microplate reader (RT-2100c). The CTRL group for this assay consisted of cells and discs cultured solely with OM and without N coating. The percentage of ALP activity relative to the CTRL was calculated by determining the ratio of the OD of the sample to the OD of the CTRL, followed by multiplying the result by 100.

Real-time RT-PCR

The expression levels of markers associated with osteogenic differentiation, including BMP4, RUNX2, OCN, and BSP, were evaluated using *real-time* RT-PCR. RNA isolation was conducted at 7, 14, and 21 days following osteoinduction, adhering to the previously established protocol (refer to *real-time RT-PCR* in section 3.2.2.1.). The sequences of human-specific primers are detailed in **Table 5**. The housekeeping gene GAPDH served as the reference for normalization, and the comparative $2^{-\Delta\Delta CT}$ method was utilized to achieve relative quantification of gene expression.

Cell Adhesion

The visualization of the cells cultured on the N-coated discs and their corresponding CTRL samples was conducted utilizing a TESCAN FESEM, Mira 3 XMU (TESCAN a.s., Brno, Czech Republic), operating at an accelerating voltage of 10 kV. The samples were carefully removed from the culture medium and rinsed twice in PBS 1× to eliminate any unattached cells. For the analyses, the discs were fixed in a solution of 2.5 % glutaraldehyde for a duration of 48 h, followed by dehydration using 3% acetic acid (cat. 695092, Sigma-Aldrich, St. Louis, MO, USA) and subsequent exposure to ascending concentrations of ethanol (25%, 50%, and 70%) in 3% acetic acid [203]. After

dehydration, the samples were air-dried and sputter-coated with a thin layer of gold using Polaron SC503 SEM (Fisons Instruments, Ipswich, UK). The adherent cells on the surfaces were examined at magnifications of 1300× and 7900×, and the images were captured using a BSD detector.

Bacterial Strains and Growth Conditions

The reference strains utilized for this study included *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 10231), *Streptococcus mitis* (ATCC 13770), and *Porphyromonas gingivalis* (ATCC 33277), sourced from Microbiologics KWIK-STIK, Manassas, VA, USA. These strains were activated and cultivated in accordance with the procedures detailed in **Table 7**, with the objective of establishing monomicrobial biofilms on the specimens.

Table 7. Conditions for growing the reference strains.

Reference Strain	Activation (Medium)	Growth (Agar)	Activation Time	Conditions	Incubation Time for Biofilm Formation
<i>Staphylococcus aureus</i>	Sabouraud dextrose broth ¹	Columbia agar with 5% sheep blood ²	24 h	Aerobic, 37°C	48 h
<i>Candida albicans</i>	Sabouraud dextrose broth ¹	Sabouraud agar ³	24 h	Aerobic, 37°C	48 h
<i>Streptococcus mitis</i>	Brain heart infusion (BHI) broth ³	Columbia agar with 5% sheep blood ²	48 h	Anaerobic, 37°C	48 h
<i>Porphyromonas gingivalis</i>	Schaedler broth with hemin and vitamin K1 ⁴	Brucella agar with 5% sheep blood ¹	72 h	Anaerobic, 37°C	5 days

¹Sigma Aldrich, MO, USA; ²ProReady, Belgrade, Serbia; ³HiMedia, Mumbai, Maharashtra, India; ⁴Becton, Dickinson and Company, Franklin Lakes, NJ, USA.

Biofilm Formation

Upon activation, a small quantity from each colony was transferred to the corresponding liquid medium (broth) and incubated for a duration of 5 h. Following this incubation, the resulting monomicrobial suspensions were subjected to centrifugation at 3000 rpm for 5'. The supernatant was subsequently discarded, and the pellet was

resuspended in sterile PBS 1× to achieve a turbidity equivalent to the 0.5 McFarland standard (approximately 10⁸ cells/mL for bacteria and approximately 10⁶ cells/mL for fungi, measured using a DEN-1 densitometer, Biosan, Riga, Latvia). The final cell density was adjusted to 10⁵ cells/mL with the corresponding broth. This suspension was employed for the formation of monomicrobial biofilms.

Sterile discs were positioned in a 96-well plate and coated with N as per the established protocol. Subsequently, to facilitate the formation of a primary pellicle that would enable biofilm development and mimic *in vivo* conditions, the specimens were immersed in 150 µL of artificial saliva (Pharmacy Belgrade, Belgrade, Serbia) at a temperature of 37 °C for 30'. Following this period, the artificial saliva was removed, and 200 µL of the standardized suspensions were introduced and incubated for either 48 h or 5 days, depending on the species under examination (**Table 7**).

2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) Assay

The metabolic activity of microorganisms was evaluated utilizing the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Following the designated period for biofilm formation (**Table 7**), the supernatant was carefully removed, and the samples were rinsed with PBS 1× to eliminate non-adherent cells. Subsequently, an XTT solution (Cell Proliferation Kit II, XTT; Roche Diagnostics GmbH, Mannheim, Germany) was prepared in accordance with the manufacturer's guidelines and introduced into each well. The plate was then incubated in the dark at 37 °C for a duration of 3 h. After a 15' agitation at 37 °C in the absence of light, the cell supernatants were transferred to a new 96-well plate. The ODs were measured at a wavelength of 492 nm using a microplate reader (RT-2100c). The OD of the medium containing N-coated discs devoid of bacteria was utilized as a reference to reduce interference from particles and aggregates present in the medium, which was then subtracted from the other measurements.

Colony Forming Units (CFU) Counting

The biofilms developed on each sample were quantified by counting the number of colonies present per mL (CFU/mL). To remove unattached microbial cells, the specimens were gently washed with PBS 1× and subsequently transferred into sterile microcentrifuge tubes containing 1 mL of sterile PBS 1×. The tubes were then shaken at 9000 rpm at 37°C using a Varioshake VS 15B (Lauda, Lauda-Königshofen, Germany) for a duration of 5'. Following this, serial ten-fold dilutions from the tubes were inoculated

onto suitable agar plates (**Table 7**). After incubating the agar plates at 37 °C under the specified conditions (**Table 7**), the CFU counting was performed.

SEM Analysis for Biofilm Adhesion

An additional qualitative analysis utilizing SEM on the N-coated discs and their CTRL samples was conducted using a TESCAN FESEM, Mira 3 XMU operating at 10 kV to visualize the formation of monomicrobial biofilms by *S. aureus*, *C. albicans*, *S. mitis*, and *P. gingivalis* on the surfaces. The methodology outlined in *Cell Adhesion*, section 3.2.2.4., was adhered to in the final analysis of the samples using Polaron SC503 SEM. The microorganisms adhering to the surfaces were examined at a magnification of 7900×, and images were acquired employing a BSD detector.

Statistical Analysis

The statistical analysis was performed utilizing GraphPad Prism Software Analysis version 9. Following the assessment of distribution normality through the Shapiro-Wilk test, a two-way ANOVA followed by Tukey's post-hoc test was employed for multiple comparisons. For the comparison of two groups, either unpaired t-tests with Welch's correction or unpaired Mann-Whitney tests were applied. Statistical significance was established at a p -value ≤ 0.05 . All experiments were conducted in both technical and biological triplicates, and the data were presented as the mean $\pm$ standard error of the mean.

4.2.2.5. RESULTS

SEM Analysis of the Coating

The surface morphology of both uncoated and N-coated titanium discs was analyzed using SEM. **Figure 31** presents a detailed examination of the samples, highlighting distinct macro-morphological differences among the bare titanium discs (CTRL).

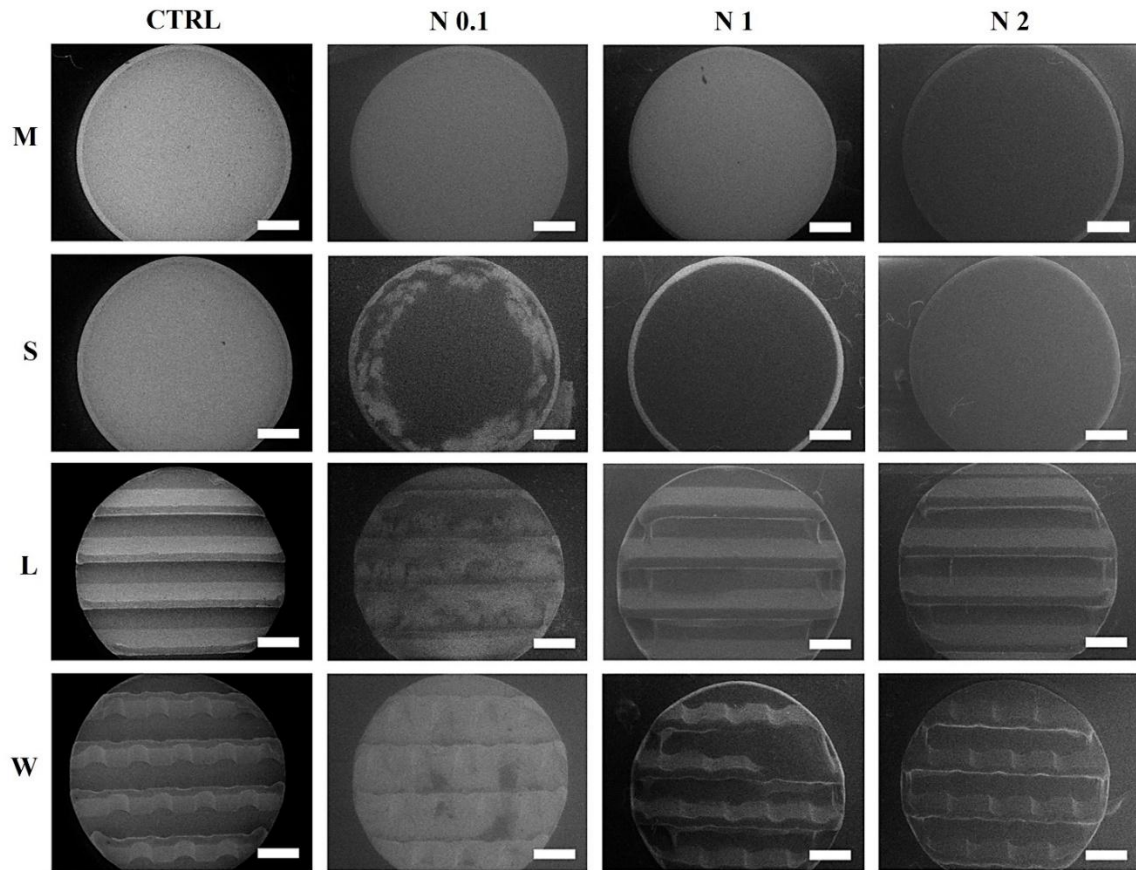


Figure 31. Representative SEM images of titanium discs illustrating various surface treatments: machined (M), sandblasted and DAE (S), S with linear roughness (L), and S with waved roughness (W). These discs were coated with differing concentrations of native type I collagen, specifically 0.1 mg/cm^3 (N0.1), 1 mg/cm^3 (N1), and 2 mg/cm^3 (N2), and compared to their respective uncoated controls (CTRL). Magnification: $40\times$. Scale bar: 1 mm.

It was observed that the application of collagen coating led to a slight reduction of surface characteristics, with this effect becoming increasingly pronounced at higher collagen concentrations. However, these elevated concentrations also resulted in a more uniform distribution of coating across the disc surfaces.

A comprehensive analysis of the micro-morphology of the coated discs is illustrated in **Figure 32**.

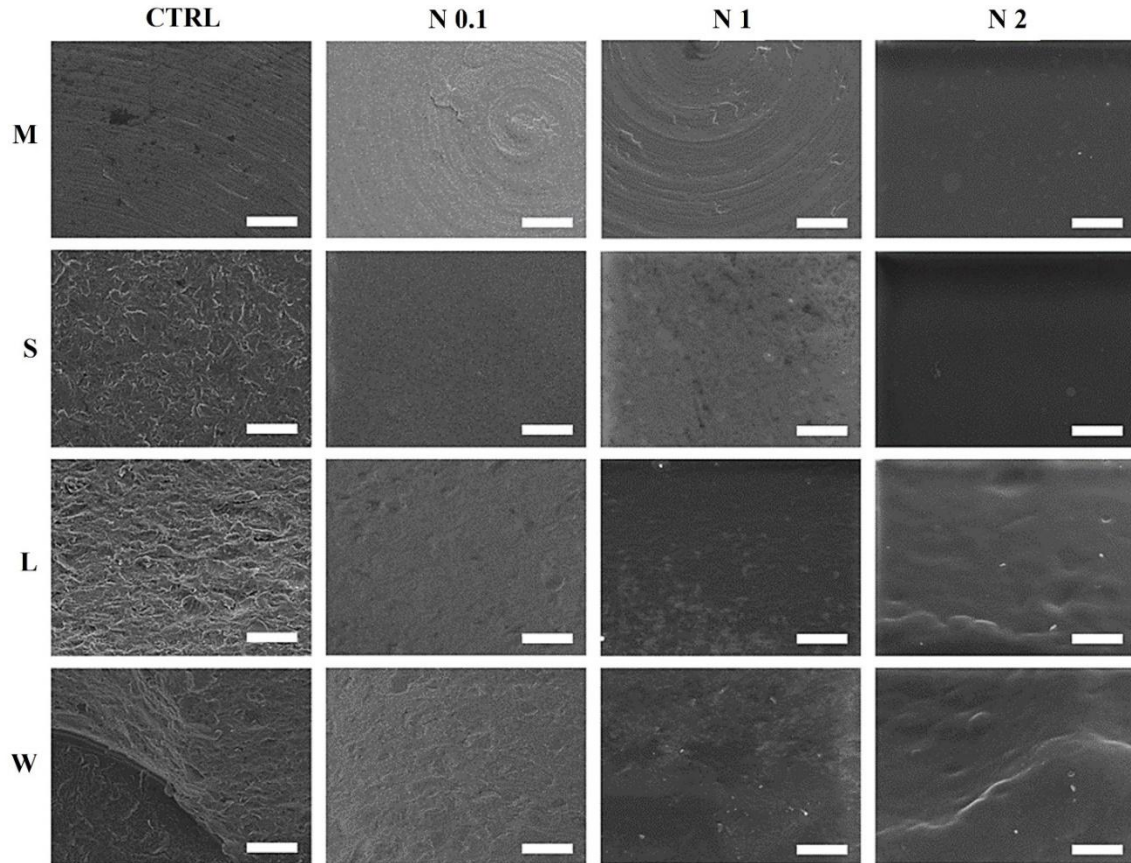


Figure 32. Representative SEM images of N-coated discs (N0.1, N1, and N2 on M, S, L, and W discs) and their corresponding CTRL samples. Magnification: 1000 $\times$. Scale bar: 50 μ m.

The M discs exhibited a relatively smooth surface, whereas the S discs displayed a rough, sanded texture. Additionally, the L and W discs incorporated the S treatment along with linear and wave-like grooves [105]. The deposition of collagen on the titanium discs was shown to alter their surface characteristics. Although the change was less pronounced at the lowest N concentration (N0.1), it was markedly evident at the highest N concentration tested (N2). Indeed, collagen casting completely transformed the surface micro- and nano-roughness of all discs. Notably, while the characteristics of collagen predominated in the N-coated M and S discs, the presence of grooves in the N-coated L and W discs allowed for the retention of the macro-geometry of the discs.

Wettability of the Coated Surfaces

The wettability of both CTRL and N-coated discs was evaluated using the sessile drop method. Contact angle (θ) measurements for all CTRL surfaces were recorded to be below 90 $^\circ$, indicating their hydrophilic nature [105]. It was observed that the surface

treatment of the discs greatly influenced their wettability properties. The characteristics of the M discs were found to be comparable to those of the S discs, with contact angle values of approximately 70°. In contrast, the L- and W-grooved discs exhibited enhanced hydrophilic properties, displaying contact angle values around 40° ($p > 0.05$), which were significantly lower than those noted for the M and S discs ($p < 0.01$ for M versus L; $p < 0.001$ for M versus W; $p < 0.0001$ for S versus L and W).

The deposition of a collagen layer influenced the surface properties of the discs in a concentration-dependent manner. For the M and W discs, the application of N0.1 did not result in a statistically significant alteration in contact angle values. Conversely, the same concentration applied to the S and L discs produced opposing effects. Specifically, the N0.1 coating led to a reduction in contact angle for the S discs ($p < 0.0001$) while yielding an increase for the L discs ($p < 0.0001$). Nonetheless, the hydrophilic characteristics of the discs were preserved across all samples with the lowest collagen concentration, except for the L disc, which demonstrated significantly higher contact angle values in comparison to the other N0.1-coated discs ($p < 0.0001$ versus M, S, and W discs). Furthermore, the N0.1-coated W disc exhibited significantly enhanced hydrophilic properties relative to the N0.1-coated M ($p < 0.0001$) and S discs ($p < 0.01$). However, significant differences were also observed between the N0.1-coated S and M discs ($p < 0.0001$). Additionally, statistically significant differences were found between the N0.1-coated discs and the N0.1 films ($p < 0.01$ versus M; $p < 0.0001$ versus S, L, and W).

As the concentration of N increased from 0.1 mg/cm³ to 1 mg/cm³, the N1 coating enabled the M surface to achieve a partially hydrophobic character ($\theta \geq 90^\circ$, $p < 0.0001$). This resulted in a statistically significant increase in contact angle values for all sample types ($p < 0.0001$), with the exception of the L discs, where values remained unchanged. Notably, only the N1-coated S and W surfaces retained their hydrophilic nature ($\theta < 90^\circ$). In this context, the topographical differences among the discs also influenced the contact angles, resulting in an increase of approximately 30-40° for the N1-coated M, S, and W discs. There were no statistically significant differences in wettability between the N1 films and the N1-coated M and S discs, indicating that the effects of the cast protein superseded those of the underlying material for flat surfaces, regardless of micro-roughness. Conversely, the macro- and micro-patterns on the W-grooved discs resulted in a reduction in contact angle compared to the N1 film ($p < 0.01$), similar to the observations for N0.1 deposition. In contrast, the N1-coated L surfaces presented significantly higher contact angle values compared to the N1 film ($p < 0.01$). Moreover, statistically significant

differences were noted for N1-coated S and W surfaces when compared to M ($p < 0.01$ versus S and $p < 0.0001$ versus W), as well as for N1-coated S and W discs when compared to L ($p < 0.001$ versus S and $p < 0.0001$ versus W).

For N films alone, contact angle values exceeded 90° when a concentration of 2 mg/cm^3 was tested in comparison with N1 and N0.1 ($p < 0.05$ and $p < 0.0001$, respectively). Under these conditions, the values obtained for the M and S discs were comparable to those of the N2 film, while those recorded for the L and W discs were significantly higher ($p < 0.01$). In comparing the N1-coated discs, no statistically significant differences were observed for the M discs; however, significant increases were observed for the S, L, and W discs ($p < 0.05$, $p < 0.05$, $p < 0.0001$, respectively). Although the increase in contact angle from N1 to N2 on the M discs was not statistically significant, the overall increment was approximately 30° from N0.1 ($p < 0.01$). Conversely, the contact angles of all other N2-covered discs were significantly higher than those of the corresponding N0.1-coated discs ($p < 0.0001$ for S; $p < 0.05$ for L; $p < 0.0001$ for W). The observed increases in contact angle values for L and W discs ($+20^\circ$ and $+50^\circ$, respectively) may be attributed to their grooved macro-morphology, with no statistically significant differences recorded among them with the N2 coating. Furthermore, no significant differences were identified between the N2-coated M and S discs; however, their values were significantly lower compared to those of the N2-coated L and W discs ($p < 0.01$ for M versus L and W; $p < 0.01$ for S versus L and W) (**Figure 33**).

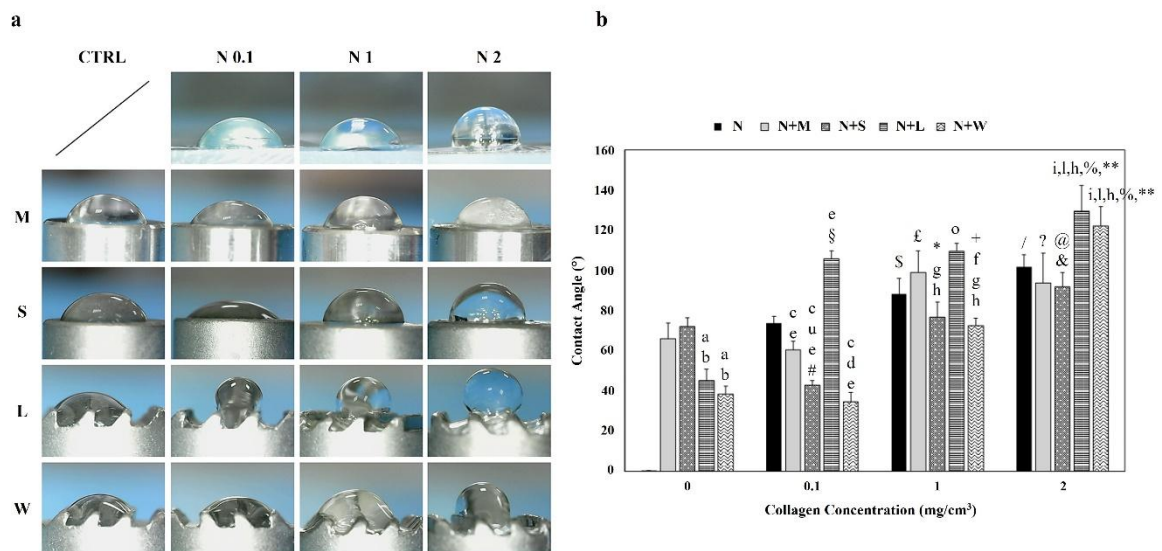


Figure 33. Wettability of the surfaces. (a) Representative snapshots and (b) relative histograms depicting the contact angle of water drops on N-coated discs (N0.1, N1, and N2 on M, S, L, and W discs) and their corresponding N films (CTRL). a: $p < 0.01$ for L versus M and $p < 0.001$ for W versus M; b: $p < 0.0001$ for L and W versus S; #: $p < 0.0001$ for

N0.1+S versus S; §: $p < 0.0001$ for N0.1+L versus L; c: $p < 0.0001$ for N0.1+M, N0.1+S, and N0.1+W versus N0.1+L; d: $p < 0.0001$ for N0.1+W versus N0.1+M and N0.1+S; u: $p < 0.0001$ for N0.1+S versus N0.1+M; e: $p < 0.01$ for N0.1+M versus N0.1; $p < 0.0001$ for N0.1+S, N0.1+L, N0.1+W versus N0.1; \$: $p < 0.0001$ versus N0.1; £: $p < 0.0001$ for N1+M versus N0.1+M; *: $p < 0.0001$ for N1+S versus N0.1+S; +: $p < 0.0001$ for N1+W versus N0.1+W; f: $p < 0.01$ for N1+W versus N1; o: $p < 0.01$ for N1+L versus N1; g: $p < 0.01$ for N1+S versus N1+M; $p < 0.0001$ for N1+W versus N1+M; h: $p < 0.001$ for N1+S versus N1+L; $p < 0.0001$ for N1+W versus N1+L; /: $p < 0.05$ for N2 versus N1; $p < 0.0001$ versus N0.1; i: $p < 0.01$ for N2+L and N2+W versus N2; @: $p < 0.05$ N2+S versus N1+S; %: $p < 0.05$ for N2+L versus N1+L; $p < 0.0001$ for N2+W versus N1+W; ?: $p < 0.01$ for N2+M versus N0.1+M; &: $p < 0.0001$ for N2+S versus N0.1+S; **: $p < 0.05$ for N2+L versus N0.1+L; $p < 0.0001$ for N2+W versus N0.1+W; l: $p < 0.01$ for N2+L and N2+W versus N2+M; h: $p < 0.01$ for N2+L and N2+W versus N2+S.

Flow Cytometry Analysis

In **Figure 34**, the flow cytometry analysis revealed elevated expression levels of mesenchymal stem cell markers: CD73 (PE-labeled; $98.14\% \pm 0.66$), CD90 (FITC-labeled; $97.16\% \pm 0.79$), and CD105 (PE-labeled; $96.48\% \pm 0.63$) in SCAPs. Furthermore, the analysis revealed minimal expression of hematopoietic markers, specifically CD34 (FITC-labeled; $0.1\% \pm 0.003$) and CD45 (PE-labeled; $0.07\% \pm 0.004$).

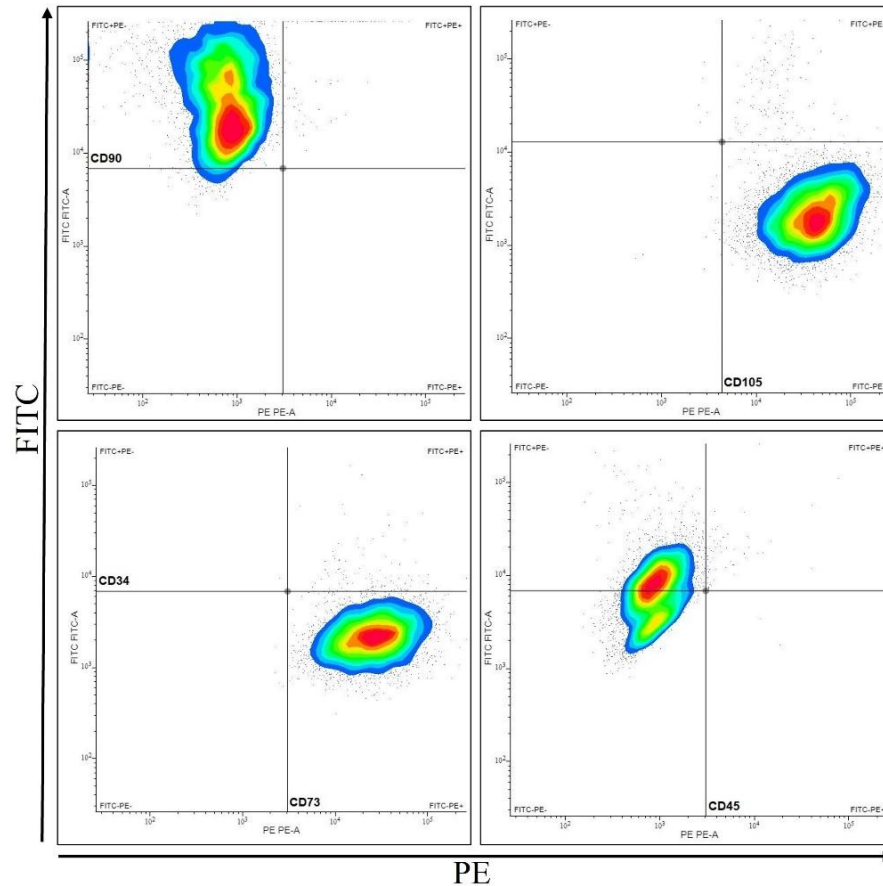


Figure 34. Cytometric analyses using FITC-labeled mouse monoclonal antibodies

targeting CD90 and CD34, which are markers for mesenchymal stem cells, as well as PE-labeled mouse monoclonal antibodies against CD73, CD105, and CD45, which are associated with hematopoietic lineages.

Stem Cells from the Apical Papilla (SCAPs) Viability on Collagen-Coated Discs

The MTT assay was conducted at 24 h, 48 h, 72 h, and at 7 days, 14 days, and 21 days on implant surfaces (M, S, L, and W), which were coated with N0.1, N1, and N2. This assessment aimed to evaluate their biocompatibility with SCAPs in comparison to the corresponding CTRL discs, as illustrated in **Figure 35**.

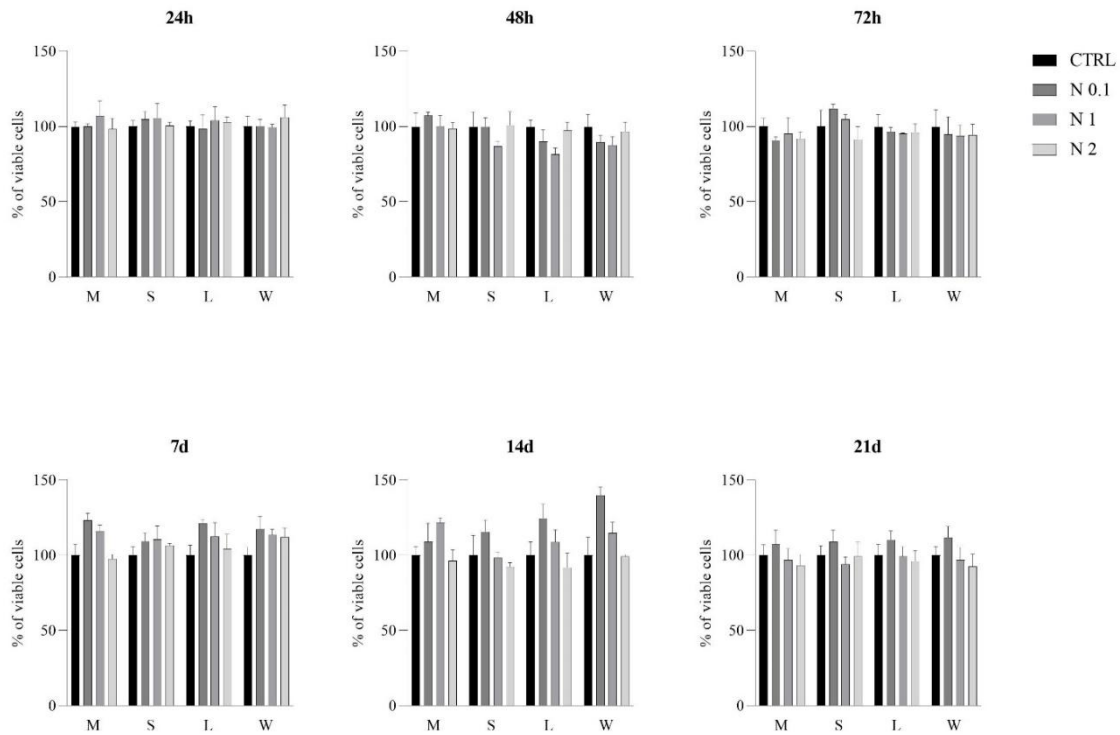


Figure 35. Cell viability of SCAPs cultured on N-coated discs (N0.1, N1, and N2 on M, S, L, and W discs) compared to uncoated discs (CTRL) over time periods of 24 h, 48 h, and 72 h, as well as 7 days, 14 days, and 21 days. The results are presented as the percentage of viable cells relative to the corresponding CTRL.

The analysis of treated samples did not indicate significant cytotoxicity in SCAPs, even at the highest concentrations of N and extended incubation times. This conclusion was supported by the absence of any significant reduction in cell viability below 70% compared to the corresponding CTRL across all sample groups. Notably, the experimental M groups exhibited slightly lower viability compared to their CTRL at 72 h across all N concentrations, at 14 days with N2, and at 21 days with both N1 and N2. Other coating concentrations and time points generally produced similar or higher results than CTRL. The evaluated S groups demonstrated reduced viability relative to their CTRL with N1 at

48 h and 21 days, as well as with N2 at 72 h and 14 days, whereas the highest viability values were recorded with N0.1 at both 14 days and 72 h. The coated L surfaces consistently exhibited comparable results to their CTRL, displaying lower viability only with N0.1 and N1 at 48 h and N2 at 14 days. Increased cell viability was noted with all N concentrations following 7 days, with N0.1 and N1 after 14 days, and with N0.1 after 21 days. The coated W discs demonstrated the highest viability compared to other discs when coated with N0.1 at 14 days. However, they exhibited slightly lower results than their CTRL with N0.1 and N1 at 48 h, across all N concentrations at 72 h, and with N1 and N2 at 21 days. In all other combinations and time points, they displayed comparable or higher viability than the CTRL.

Overall, **Figure 35** indicated that the N coating resulted in an ascending trend in cell viability for all groups until 14 days, followed by a slight decline after 21 days.

Bone Matrix Deposition on Collagen-Coated Discs

The osteoconductive potential of coatings N0.1, N1, and N2 was evaluated using both qualitative and quantitative methods over periods of 7, 14, and 21 days on SCAPs. This evaluation included a comparison with the corresponding CTRL groups, where the cells and uncoated discs were subjected exclusively to OM treatment, as illustrated in **Figure 36**.

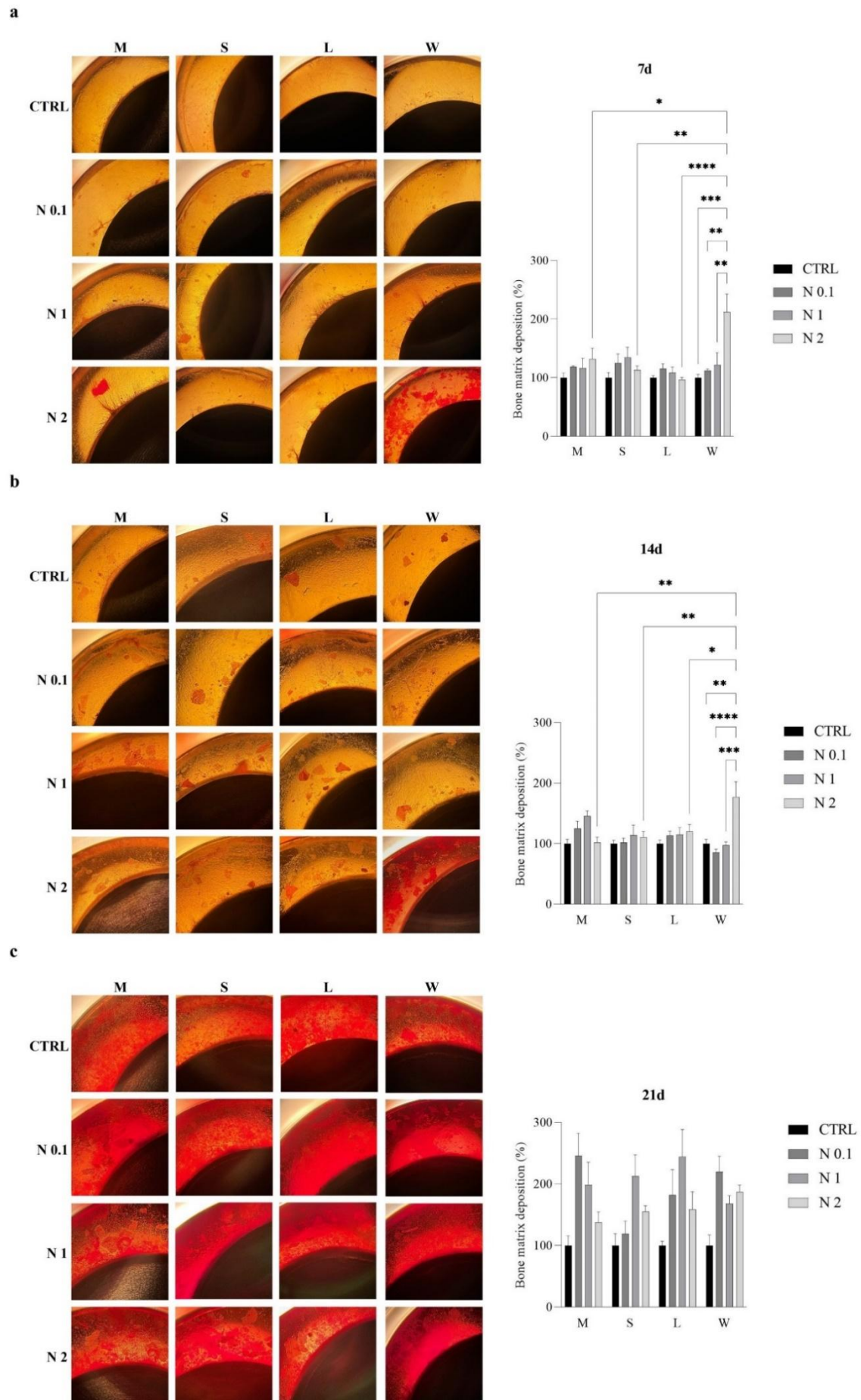


Figure 36. Representative inverted light microscopic images illustrating bone matrix deposition in the area immediately surrounding the N-coated discs, together with quantitative analyses following ARS assay on SCAPs. The percentage of bone matrix deposition is reported at (a) 7 days, (b) 14 days, and (c) 21 days. Various concentrations of N coating (N0.1, N1, and N2) and implant surfaces (M, S, L, and W) were evaluated. Original image magnification: 40×. The results are presented as the percentage of bone matrix deposition (%) relative to their controls (CTRL; cells and uncoated discs treated solely with the OM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The formation of mineralized nodules exhibited a direct correlation with time across all experimental conditions. Statistical significance was observed at 7 and 14 days; however, data indicated the maximum mineralization potential occurred after 21 days of treatment for all N-coated discs at any concentration. It is important to note that all tested conditions demonstrated higher values compared to their respective CTRL groups at each time point, with the exception of the W disc coated with N0.1 at the 14-day mark.

After 7 days (**Figure 36a**), the N2-coated W disc displayed a significantly higher percentage of bone matrix deposition when compared to its CTRL ($p < 0.001$) and also in comparison to the corresponding discs with N0.1 ($p < 0.01$) and N1 ($p < 0.01$) coatings. Significant differences were also evident between this and other discs with the same N2 coating, with $p < 0.05$ observed versus the M surface, $p < 0.01$ versus the S disc, and $p < 0.0001$ versus the L disc. A similar pattern was observed after 14 days, where the N2-coated W disc exhibited the highest mineralization potential, demonstrating statistically significant differences compared to the N0.1-coated W ($p < 0.0001$) and N1-coated W ($p < 0.001$) discs, as well as its CTRL ($p < 0.01$). Furthermore, as illustrated in **Figure 36b**, this condition yielded significantly higher results even when compared to other discs featuring the same collagen coating (N2) ($p < 0.01$ versus M and S, and $p < 0.05$ versus L). It is noteworthy that at 21 days, no significant differences were detected among the groups, although all tested conditions resulted in increased bone matrix deposition compared to their CTRL counterparts. Notably, at this time point, the discs coated with N0.1 and N1 exhibited the most favorable outcomes (**Figure 36c**).

Effect of Collagen-Coated Discs on ALP Activity

The ability of N-coated discs to enhance osteogenic differentiation at various concentrations was further assessed by analyzing the ALP activity of SCAPs over durations of 7, 14, and 21 days. This evaluation included a comparative analysis with their respective CTRL groups, wherein the cells and uncoated discs were solely subjected to OM treatment, as illustrated in **Figure 37**.

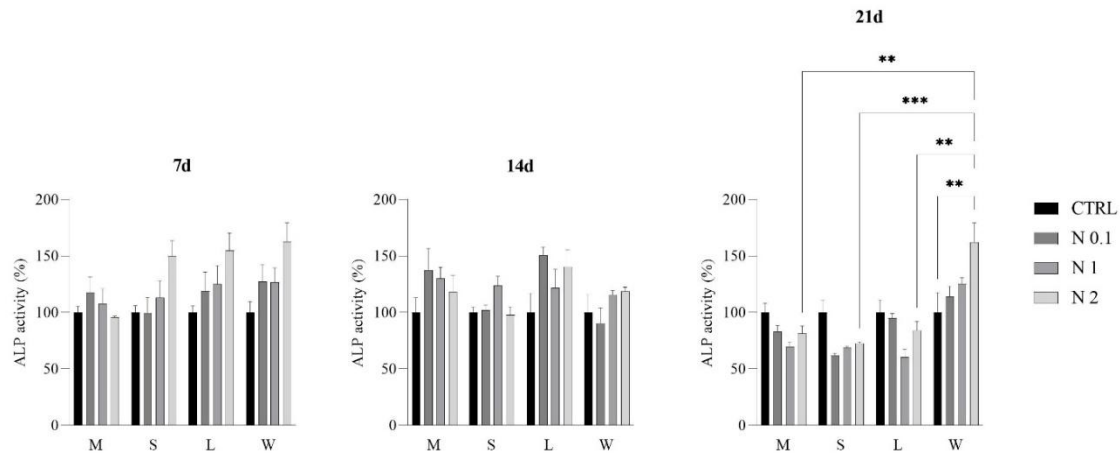


Figure 37. ALP activity of SCAPs evaluated on N-coated implant discs over periods of 7, 14, and 21 days. Various concentrations of N coating (N0.1, N1, and N2) and different implant surfaces (M, S, L, and W) were investigated. The results are presented as the percentage of ALP activity relative to the controls (CTRL; cells and uncoated discs treated solely with the OM). ** $p < 0.01$, *** $p < 0.001$.

In the analyses conducted over both the 7-day and 14-day periods, no statistically significant differences were observed among the groups assessed. Notably, the majority of the N-coated discs, particularly N2 at 7 days and N0.1, N1, and N2 at 14 days, demonstrated increased ALP activity in comparison to their respective CTRL groups. Specifically, only the N2-coated M disc at 7 days and the N0.1-coated W disc at 14 days exhibited marginally lower ALP activity relative to their CTRL counterparts. Importantly, the ALP activity of SCAPs cultured on N2-coated W disc after 21 days revealed significantly higher results when compared to the CTRL group ($p < 0.01$), although it did not present a statistically significant difference when compared to other N coatings. Moreover, this combination yielded significantly superior results in comparison to other discs coated with N2 ($p < 0.01$ versus M, $p < 0.001$ versus S, and $p < 0.01$ versus L).

Given the remarkable outcomes observed in the osteogenic differentiation tests, the combination of N2 and W disc was selected for further evaluation.

Modulation of Osteogenic Markers by the Selected Combination of Collagen-Coated Discs

The results presented in **Figure 38** demonstrated the gene expression of early-stage osteogenic markers, specifically BMP4 and RUNX2, in conjunction with late-stage markers, namely OCN and BSP. These markers were assessed in SCAPs that were cultured exclusively on N2-coated W discs for 7, 14, and 21 days. This evaluation aimed to substantiate the osteogenic potential of the selected combination of collagen concentration

and disc.

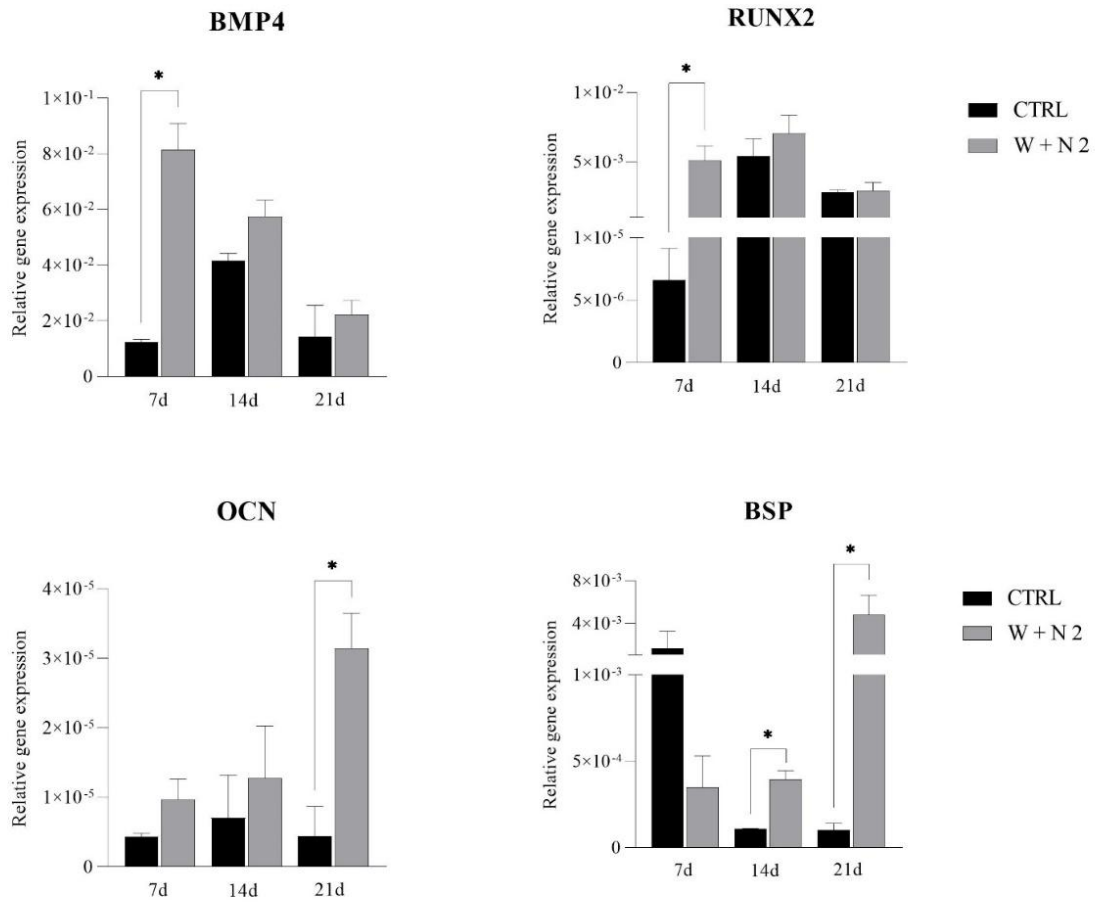


Figure 38. Influence of N2 coating on W discs (W+N2) regarding the mRNA expression of osteogenic markers (BMP4, RUNX2, OCN, and BSP) examined in SCAPs at 7, 14, and 21 days. The results are presented as relative gene expression ($2^{-\Delta\Delta CT}$). The control group denotes cells and uncoated discs treated exclusively with the OM (CTRL). * $p < 0.05$.

In relation to the early-stage biomarkers, significantly higher levels were observed after a period of 7 days ($p < 0.05$). In contrast, for the late-stage biomarkers, statistical significance was identified at 21 days for OCN and at both 14 and 21 days for BSP ($p < 0.05$).

Overall, consistently elevated results were noted for SCAPs cultured on N2-coated W discs in comparison to the CTRL groups, including both the cells and uncoated discs cultivated exclusively with the OM, at every time point assessed, with the exception of the BSP marker at the 7-day mark.

SCAPs Adhesion on the Selected Modified Surface

Figure 39 presents a qualitative analysis of SCAP adhesion on the N2-coated W

surface at various magnifications (1300× and 7900×) following a 24-hour incubation period. Notably, on the CTRL surface, the cells demonstrated limited attachment to the titanium substrate, as indicated by white arrows, in contrast to the N2-coated surface, where cells exhibited a dense coverage, demonstrating a tissue-like behavior. Additionally, the cells on the N2-coated W disc displayed a more intertwined and elongated morphology, along with an increased presence of lamellipodia and filopodia structures. The black arrows highlight the distinctive fibrillar pattern of the collagen, which is notably different from that associated with the identifiable W surface treatment.

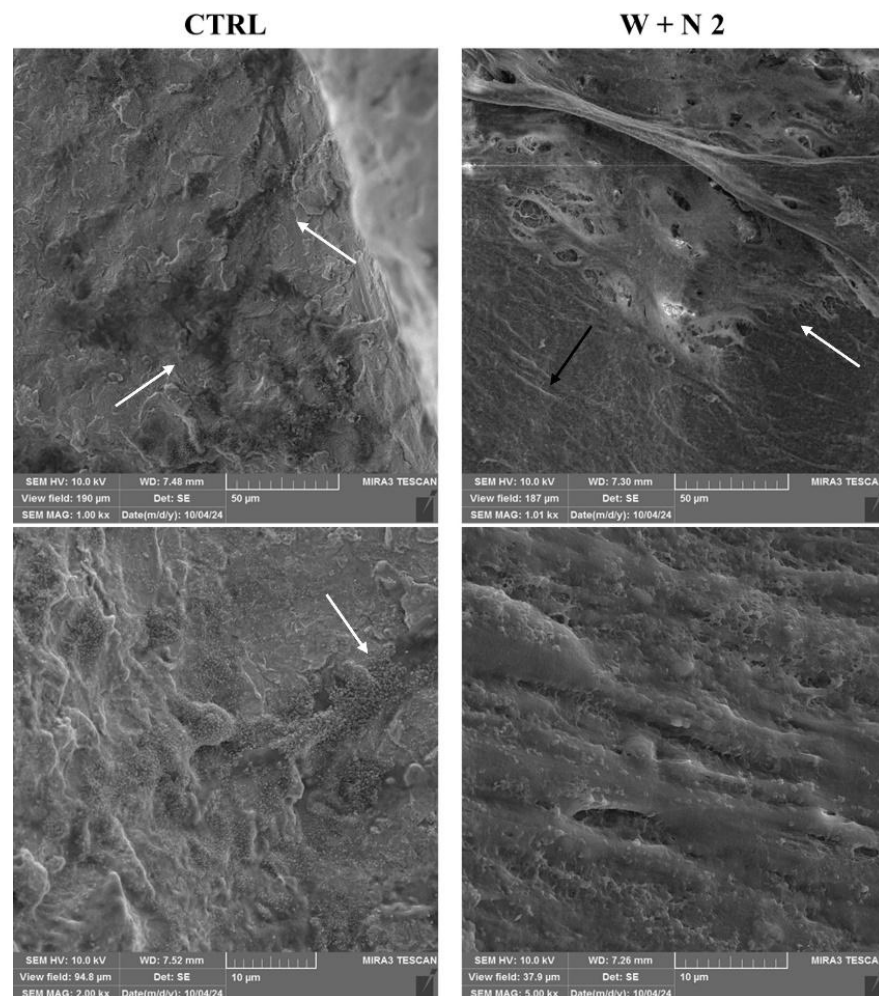


Figure 39. Representative SEM images illustrating the adhesion of SCAPs on both uncoated discs (CTRL) and W+N2 surfaces. Magnifications: 1300× (upper line) and 7900× (bottom line). Scale bars: 50 μm (upper line) and 10 μm (bottom line). White arrows: SCAPs, black arrows: N2 coating.

Microbial Metabolic Activity in Biofilms

The initial step in evaluating the biofilm susceptibility of the selected N2-coated W discs involved the quantification of metabolic activity in monomicrobial biofilms of *S.*

aureus, *C. albicans*, *S. mitis*, and *P. gingivalis* cultured on these discs. The assessment relied on the measurement of XTT formazan production, which indicated that the N2-coated W discs demonstrated lower levels of metabolically active bacteria and fungi compared to the uncoated discs (CTRL) after the respective incubation periods (**Table 7**). However, it is important to note that no statistically significant differences were observed between the two groups for each strain. The reduction in metabolic activity was most pronounced in *P. gingivalis*, followed by *S. aureus*, *S. mitis*, and *C. albicans* (**Figure 40**).

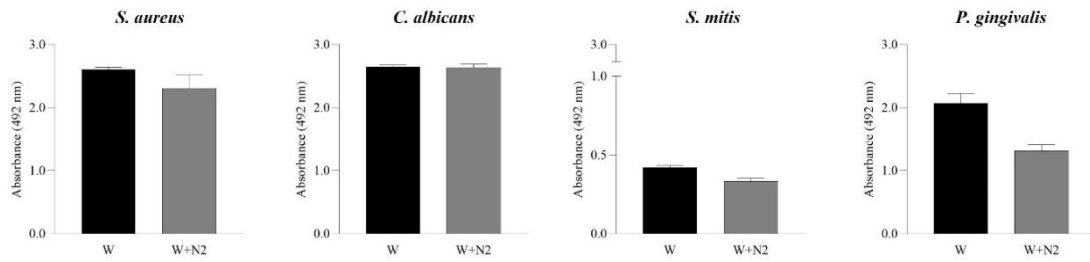


Figure 40. Absorbance measured at 492 nm for sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) analyzed in relation to monomicrobial biofilms consisting of *S. aureus*, *C. albicans*, *S. mitis*, and *P. gingivalis*. These biofilms were cultivated on W+N2 discs and were compared against those cultured on uncoated W discs (CTRL). The OD of the medium containing W+N2 discs devoid of bacteria was used as a baseline to reduce potential interference within the medium. The baseline was then subtracted from the other absorbance readings to ensure the accuracy of the results.

Biofilm Quantification by CFU Counting

The quantification of bacterial and fungal cells within monomicrobial biofilms established on N2-coated W titanium discs was executed by measuring the CFU/mL following the formation of the biofilms. The results are illustrated in **Figure 41**.

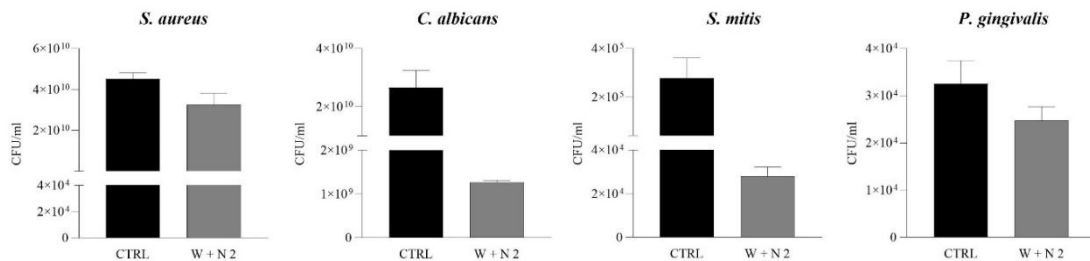


Figure 41. Results of the colony-forming units (CFU) analysis indicating the presence of adhered monomicrobial biofilms of *S. aureus*, *C. albicans*, *S. mitis*, and *P. gingivalis* on W+N2, in comparison to the uncoated discs (CTRL).

The reduction in monomicrobial biofilm CFU observed on N2-coated W discs, in

comparison to the uncoated discs (CTRL), closely mirrored the outcomes of the XTT assays, although the difference did not achieve statistical significance. Notably, the antibiofilm effects illustrated by the graphical data were more pronounced than those represented in the XTT analyses, particularly against *C. albicans* and *S. mitis*. In the context of CFU counting, the most relevant impact was evident in *C. albicans*, followed by *S. mitis*, *S. aureus*, and *P. gingivalis*.

Bacterial and Fungal Adhesion

Representative SEM micrographs of the specimens covered by monomicrobial biofilms are presented in **Figure 42**.

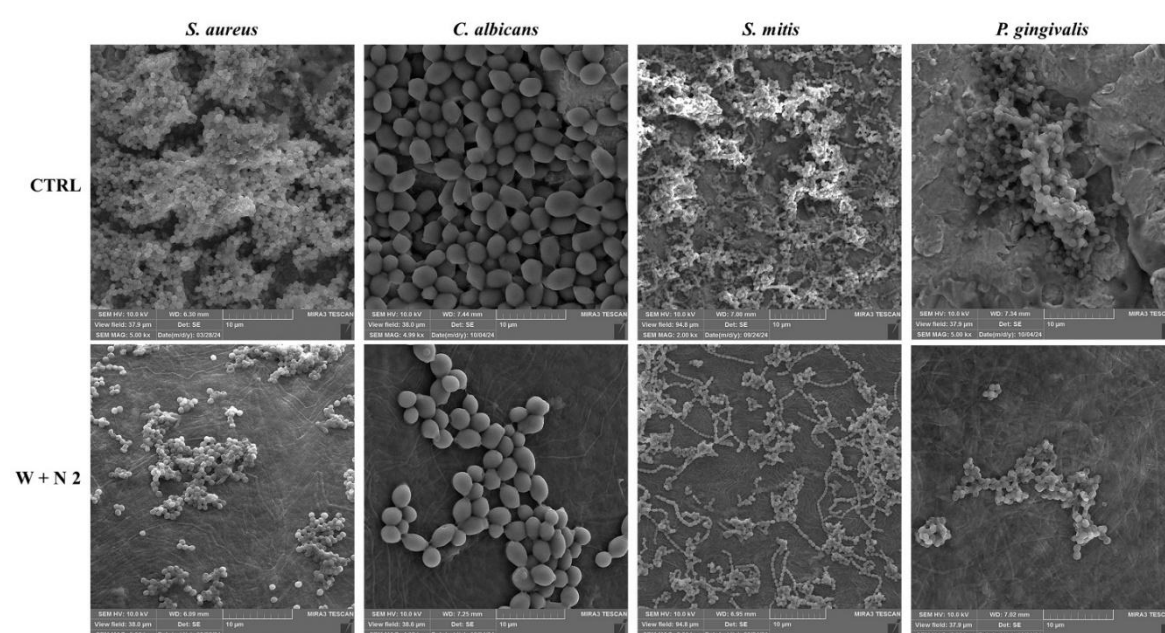


Figure 42. Representative SEM images depicting monomicrobial biofilms consisting of *S. aureus*, *C. albicans*, *S. mitis*, and *P. gingivalis* on the surfaces of both uncoated discs (CTRL, upper line) and W+N2 discs (bottom line). Magnification: 7900×. Scale bar: 10 µm.

Following the designated incubation period (**Table 7**), a multilayer biofilm developed, accompanied by the production of ECM on the surfaces of uncoated W discs (CTRL), which were not entirely covered by microorganisms. In contrast, SEM images of N2-coated W samples revealed a consistent N2 coating featuring discernable fibrillar structures across all samples. Additionally, isolated areas of organized microorganisms were identified on the coated surfaces, with minimal or nonexistent ECM.

Microscopic SEM analysis of the *S. aureus* CTRL group demonstrated the formation of monomicrobial biofilms characterized by cluster-shaped microorganisms

embedded within the ECM. Conversely, the N2-coated surface exhibited fewer small, round-shaped microorganisms. Micrographs of *C. albicans* indicated nearly complete coverage of the biofilm mass on the surface of the CTRL specimen. In contrast, images of the N2-coated W specimen displayed only a minimal presence of chain-grouped fungi, characterized by the pseudohyphal growth of *C. albicans*. On the N2-covered surface, the presence of chain-organized *S. mitis* cells was markedly reduced in comparison to the CTRL, and there was minimal or no observable ECM. Similar patterns were noted in the images of the *P. gingivalis* biofilm, where rod-shaped bacteria were distributed across the CTRL surface and embedded in the ECM. The visual representation of the covered discs exhibited a comparable arrangement, demonstrating a reduced presence of ECM and providing a clearer view of the linearly organized microorganisms.

4.2.2.6. DISCUSSION

This research investigated the effects of utilizing N collagen as a coating material on various titanium implant surfaces. The study aimed to thoroughly evaluate the cytotoxicity, osteogenic potential, and anti-biofilm properties of N in vitro, with the objective of enhancing clinical outcomes in dental care through the design and implementation of bioactive dental implants.

The viability assay demonstrated the biocompatibility of all N concentrations across the different titanium surfaces, revealing no statistically significant differences. This finding confirmed the high biocompatibility of both titanium and equine type I collagen. Although titanium exhibits favorable properties such as low density, high strength, durability, and corrosion resistance, its osteoinductive and osteoconductive abilities are relatively modest. This limitation impacts cell migration and can result in poor integration with the host tissue [204]. Numerous *in vitro* and *in vivo* studies have established that the application of biomolecules, including type I collagen and other adhesive proteins or peptides that mimic the ECM, onto the surfaces of titanium-based implants is among the most effective strategies for enhancing the bioactivity of this material and promoting early osteogenesis [147,205,206]. Furthermore, it has been documented that collagen type I increases the hydrophilicity of titanium, thereby improving its osteoconductive and osteogenic properties, which are critical for optimal osseointegration [207]. The interaction between cells and materials is significantly influenced by surface properties. It is widely accepted that moderate wettability is ideal for facilitating cell adhesion and proliferation.

Nonetheless, it has been demonstrated that microroughness initially induces hydrophobicity but ultimately leads to increased hydrophilicity compared to titanium surfaces that lack microstructuring. This initial hydrophobic state may arise from air being trapped within the smallest micropores, resulting in a heterogeneous surface that cannot be spontaneously wetted; however, this can transition to complete wettability over time [208]. In the present study, contact angle measurements indicated significantly higher values than 90° when N2 was applied, exhibiting a concentration-dependent effect, particularly on L- and W-grooved surfaces compared to other surfaces and the corresponding N0.1- and N1-coated discs. This observed increase in the hydrophobic behavior of the coated discs may be attributed to the greater number of crosslinks present in the N2 coating. Such bonding formation reduces the availability of chemical groups that can interact with water molecules, leading to enhanced hydrophobicity and improved resistance to enzymatic degradation [159,207]. Additionally, it is important to note that the sessile drop method utilized a dry coating without the rehydration process prior to cell seeding (refer to *Sample Preparation* in section 3.2.2.4.), which may have further contributed to the decrease in hydrophilicity across all surfaces.

The microstructure and the nanostructure of titanium surfaces considerably influence the behavior of mesenchymal precursors of osteoblasts, thereby affecting cellular proliferation and differentiation towards the osteoblastic lineage. Research has also indicated that surfaces subjected to acid-alkali treatment exhibit a greater capacity for collagen adsorption [206,209]. For instance, studies have demonstrated that modifying the surface of titanium implants utilizing SLA treatment, which increases surface roughness, can positively impact cell growth, adhesion, and differentiation compared to smoother surfaces [137,210,211]. However, it is crucial for cells to organize their cytoskeleton, adhere to an implant surface, and manage microbial adhesion, with a threshold of 0.2- μ m Ra surface roughness being necessary; excessively rough surfaces can diminish overall adhesive strength and promote biofilm formation [212]. The S surface treatment previously revealed a higher average nano-roughness compared to the M surface (92.03 ± 6.32 nm versus 27.29 ± 5.66 nm of Ra), while the L and W surface treatments exhibited enhanced hydrophilicity, cell adhesion, and proliferation [105,213]. Although SEM analysis demonstrated that collagen coating slightly reduced surface characteristics, particularly on M and S discs—with this effect becoming more pronounced at higher collagen concentrations—higher concentrations facilitated a more uniform coverage of the disc surfaces, while maintaining the macro-geometry of the L and W discs.

In the present study, the deposition of bone matrix, ALP activity, and gene expression of osteogenic markers have been evaluated to ascertain the osteogenic differentiation of SCAPs and the osteoconductive potential of collagen-coated surfaces. Notably, ARS staining enabled the visualization of a greater number of calcium deposits surrounding the coated discs and the respective CTRL groups over time, thereby allowing for the quantification of bone matrix deposition across each experimental group. Although all conditions tested demonstrated increased bone matrix deposition when compared to their corresponding CTRLs at each time point, statistically significant differences were observed at 7 days ($p < 0.001$) and 14 days ($p < 0.01$) for the N2-coated W disc relative to its CTRL. Furthermore, significant differences were noted when comparing N2-coated W discs against W discs coated with different N concentrations ($p < 0.01$ versus N0.1-coated W disc and N1-coated W disc at 7 days; $p < 0.0001$ and $p < 0.001$ versus N0.1-coated W disc and N1-coated W disc, respectively, at 14 days), as well as with other N2-coated discs ($p < 0.05$ versus N2-coated M disc, $p < 0.01$ versus N2-coated S disc, and $p < 0.0001$ versus N2-coated L disc at 7 days; $p < 0.01$ versus N2-coated M disc and N2-coated S disc, and $p < 0.05$ versus N2-coated L disc at 14 days). The ALP activity assay further confirmed the high osteoconductive potential of the N-coated discs. At 7 days, all experimental conditions exhibited enhanced results compared to their corresponding CTRLs, with the N2-coated S, L, and W discs yielding the highest values, although no significant differences were noted when compared to the CTRLs. It was only after 21 days that the N2-coated W disc demonstrated statistically significant higher values when compared to its CTRL ($p < 0.01$) as well as other N2-coated discs ($p < 0.01$ versus N2-coated M disc and N2-coated L disc, and $p < 0.001$ versus N2-coated S disc). These promising findings allowed for the affirmation of osteogenic marker expression, particularly on the N2-coated W surface, where significant up-regulation ($p < 0.05$) of key early-stage (BMP4 and RUNX2) and late-stage (OCN and BSP) markers was noted in comparison to the CTRL. The data presented herein reflected and affirmed the osteogenic potential of N2 as assessed in our prior *in vitro* study, aligning with existing literature regarding the osteogenic properties of type I collagen as a coating for dental implants [147,214-217], as well as the relatively limited studies on equine collagen [178,179]. For instance, research conducted by Bellone et al. [199] and Cecconi et al. [218] substantiated the efficacy of equine collagen, although used in conjunction with other biomaterials such as hydroxyapatite, in significantly enhancing bone regeneration and implant osseointegration *in vitro* and in animal models involving New Zealand white rabbits. These

studies reported significantly enhanced viability and migration of bone marrow stromal cells, increased ALP activity in osteoblasts, as well as significant improvements in BIC% and trabecular thickness with coated implants compared to uncoated ones after 45 and 90 days. To the best of our knowledge, these represent the only studies available in the literature regarding the application of equine collagen type I as a coating for dental implants within a bone-derived formulation. However, a broader range of *in vivo* studies addressing the osteogenic potential of equine collagen scaffolds exists [154,155,219]. Notably, significant reductions in bone resorption and enhanced new bone formation using equine collagen type I have been demonstrated over both short-term and long-term periods in comparison to negative controls. Additionally, recent investigations by Mozzati et al. [220] have indicated that the combined use of a bone substitute composed of equine collagen type I and magnesium hydroxyapatite can effectively induce new bone formation as early as two months post-application, thereby accelerating socket closure and tissue maturation following tooth extraction in a human cohort.

Biofilm can be defined as a structured microbial community composed of cells that are adhered to a substratum and embedded in an ECM produced by the organisms themselves. Foreign implants introduced into a patient's body present a colonization surface for bacteria, facilitating the adherence of both bacteria and plasma proteins, which, in turn, leads to the formation of bacterial biofilms. These biofilms confer increased resistance to antibiotics among the embedded bacteria and diminish their susceptibility to host immune defenses. Consequently, they are a source of persistent bacteremia and localized tissue damage. Clinical practice and experimental studies have consistently demonstrated that antibiotic therapy alone is frequently inadequate to eliminate biofilm infections. Therefore, the ideal implant surface should minimize bacterial adhesion, confer effective sterilization, and not interfere with bone healing and osteointegration processes, effectively preventing biofilm formation. To address these challenges, significant efforts have been directed toward reducing bacterial adhesion to implants through surface modifications that lower adhesive properties or through the design of antibacterial coatings [221]. Specifically, passive coatings achieve reductions in bacterial adhesion by modifying the implant surface chemistry or structure without the local release of fungicides, employing mechanisms such as steric or electrostatic repulsions and low surface energy by altering surface topography. In contrast, active coatings release pharmacologically active pre-incorporated bactericidal agents, thus reducing the risk of infection [19]. In the present study, the adhesion and metabolic activity of monomicrobial biofilms comprising early and

late oral colonizers have been assessed on a selected collagen-coated surface (N2-coated W surface) to mitigate frequent complications such as peri-mucositis and peri-implantitis. The findings indicated a reduction in biofilm formation on the treated surface compared to the CTRL group, suggesting a potential role for N2 in passively repelling microorganisms, even though collagen type I does not possess intrinsic antimicrobial properties [222]. Although some studies asserted collagen's intrinsic ability to combat infections [223-225], it has been primarily utilized as a carrier vehicle for various antimicrobial and bioactive molecules due to its biostability and superior biological activities, which significantly enhance its resistance to microbial infections [149]. Comparative analyses revealed that conventional titanium implant surfaces demonstrated greater susceptibility to bacterial colonization when compared to modified collagen-coated surfaces [226-228].

It is important to acknowledge the limitations present in this study. Recent literature has identified functionalization methods that may enhance the resistance and stability of collagen coatings over time compared to traditional adsorption techniques [229-231]. Among them, the immobilization of type I collagen on implant surfaces has been proposed as a strategy to improve cellular responses and expedite the healing processes of both soft and hard tissues; however, functionalized implant surfaces have yet to be introduced into the market [232,233]. Furthermore, to fully exploit the diverse applications of collagen, there is a potential to develop collagen-modified titanium implant surfaces that incorporate antimicrobial and other bioactive molecules [149]. Such innovations could support the maintenance of peri-implant soft tissue health by ensuring a bacterial seal and preventing bacterial colonization, thus addressing the risk of peri-implantitis. The integration of topographical and biochemical surface modifications presents a significant advancement in surface engineering, particularly through the combination of type I collagen with other osteoinductive biomaterials, which could further enhance osseointegration and bone formation [230,234].

In conclusion, the application of N2 coating to W titanium implant surfaces has resulted in a reduction of surface wettability, while concurrently increasing protein adsorption and cellular attachment. This interaction supported the osteoconduction of titanium implant surfaces and has been shown to slightly reduce the formation of monomicrobial biofilms. A comparison of these findings with relevant published literature suggested that the native conformation of N2 may be a crucial factor influencing the efficacy of the biological processes studied. The potential impact of collagen-coated dental implants in clinical settings also warrants exploration to validate and further analyze the

outcomes observed in animal experiments. In summary, collagen-coated dental implants may serve as a competitive alternative to traditional titanium surfaces by offering enhanced osteogenic properties and reducing the risk of infection. The observed benefits in improved osseointegration, along with reduced biofilm adhesion, underscore the potential for these coatings in future dental implant applications. Continued research is essential to establish comprehensive effectiveness metrics and promote broader clinical adoption.

4.2.3. *IN VITRO* BIOMECHANICAL ASSESSMENTS

The loss of teeth, especially in the posterior maxillary region, leads to the atrophy of the alveolar ridges. As a result, it is imperative to ensure an adequate quantity and quality of alveolar bone to facilitate precise 3D positioning of dental implants and to achieve optimal aesthetic and functional outcomes. The long-term stability of dental implants relies on the establishment of a close and dynamic relationship between the implant surface and the surrounding bone [235]. This relationship is directly influenced by several factors, including the initial stability of the implant, the quality and quantity of the bone tissue, the macro- and micro-design features of the implant, as well as the surface characteristics and thread profile. Collectively, these factors are essential in promoting successful osseointegration of the implant [236]. Moreover, achieving a high level of primary stability allows for the immediate loading of implants, thereby minimizing the need for multiple surgical procedures and reducing patient discomfort [237].

Surface modifications of implants, including the application of roughness, biomaterial coatings, or the incorporation of growth factors, can significantly enhance bone regeneration. These modifications lead to a reduction in the time required for osseointegration, thereby improving the initial stability of the implant [238]. Research focused on the mechanical properties of collagen-coated implants has demonstrated that such coatings can enhance the mechanical performance of titanium implants. Specifically, the principal biomechanical advantage of collagen coatings on titanium implants is the promotion of osseointegration, which denotes the direct structural and functional connection between the implant and the surrounding bone [215]. Additionally, collagen coatings may provide enhancements in tensile strength, primary stability, and the overall durability of implant surfaces [239-241]. Numerous animal studies have already confirmed that collagen-coated dental implants are associated with significantly increased bone growth and a higher BIC [242,243].

Misch et al. [244,245] classified bone density into four distinct categories based on density, as well as the cortical and trabecular microstructure: (i) D1 bone: dense cortical bone featuring poor or absent trabecular bone, typically observed in the mandibular symphysis; (ii) D2 bone: characterized by thick cortical and dense trabecular bone tissue, found in the anterior mandible and anterior maxilla; (iii) D3 bone: identifies as having thin cortical and less dense trabecular bone, commonly encountered in the posterior mandible and maxilla; (iv) D4 bone: noted for poor or absent cortical bone alongside thin trabecular

tissue, which occurs in the posterior maxilla; and (v) D5 bone: indicative of immature bone tissue. Various mathematical and biomechanical models have been developed to simulate human bone for *in vitro* testing of implant-supported prostheses under load conditions [246-249]. In the present context, polyurethane is defined as an isotropic and homogeneous material recognized as a standard prototype by the ASTM for the assessment of force distribution around dental implants across various anatomical sites [250]. The synthetic foam blocks, which replicate the characteristics of oral bone, are categorized into four groups according to the Misch classification [244,245]: (i) 40 pounds per cubic foot (PCF), corresponding to D1 bone; (ii) 30 PCF, representing D2 bone; (iii) 20 PCF, simulating D3 bone; and (iv) 10 PCF, emulating D4 bone.

Numerous *in vivo* and *in vitro* investigations have evaluated the primary stability of implants by quantifying the maximum insertion torque (IT) during the implant placement procedure [251-253]. In the context of dental implantology, the term IT refers to the force necessary for maximum clockwise movement when displacing material. Elevated IT values have been demonstrated to enhance primary stability by reducing implant micromotion. Generally, insertion forces of 30 Ncm or greater are routinely utilized for implant placement in both healed ridges and fresh extraction sockets, particularly when immediate loading of the implant is intended in the treatment protocol. IT values between 30 Ncm to 50 Ncm are generally regarded as the optimal range for immediate implant placement, even though research has shown that an $IT \geq 50$ Ncm contributes to the reduction of micromotion without inflicting damage to the surrounding bone. However, high IT values may compromise the structural integrity of implants, irrespective of the type of implant connection used, and cause osseous pressure necrosis, which can subsequently lead to clinical complications [254-257]. In contrast, removal torque (RT) is clinically defined as the force required to extract the implant from the bone, serving as a reliable method for evaluating primary stability. Importantly, RT indirectly offers insights into the extent of BIC [258]. Furthermore, resonance frequency analysis (RFA) is essential for determining the implant stability quotient (ISQ) following implant placement. The ISQ values range from 0 to 100, with a designated cutoff value of 47 ISQ indicating good implant stability. However, the classification of implant stability is as follows: values ≤ 60 ISQ suggest low stability, values ranging between 60 and 70 ISQ indicate medium stability, and values > 70 ISQ are associated with high stability [259]. This non-invasive technique is critical for assessing the risk of implant failure and provides valuable information regarding the predictability of dental implant outcomes [260].

Based on these considerations and following the encouraging *in vitro* results, the N2 coating has been selected for further investigation on the 3D implant corresponding to the specified W surface (refer to *Materials* in section 3.2.2.4.). The objective was to preliminarily evaluate the impact of collagen coating on the *in vitro* biomechanical behavior of this implant utilizing rigid polyurethane foam blocks. These artificial bone substrates simulated three distinct artificial bone densities—30, 20, and 10 PCF—representing D2, D3, and D4 bone types, respectively, and were employed to analyze IT, RT, and RFA.

4.2.3.1. MATERIALS AND METHODS

Dental Implants

The 3D implant models (CM AR Maestro, Implacil De Bortoli, Sao Paolo, Brazil) exhibited a conical design featuring progressive trapezoidal threads and an internal Morse cone fitting, along with an identical surface treatment and macro-geometry to the W discs. Notably, the macro-geometry displayed a straight head without micro-threads, while the body incorporated grooves and healing chambers (resembling waves on W discs). These design elements were intended to enhance the stability of blood clots and facilitate early bone formation without causing bone compression or necrosis in the peri-implant area during insertion, while simultaneously preserving high levels of stability. Clinically, these chambers serve as osteopromotive regions that accelerate osseointegration by creating spaces for the accumulation of bone chips and facilitating blood clot formation, which ultimately contributes to improved secondary implant stability [261,262]. In the present study, titanium Maestro implants coated with N2 collagen were designated as test (COATED), while uncoated implants were designated as control (CTRL). All implants utilized in the investigation possessed a length of 9 mm and a diameter of 4 mm.

In **Figure 43**, the two implants being examined are illustrated.

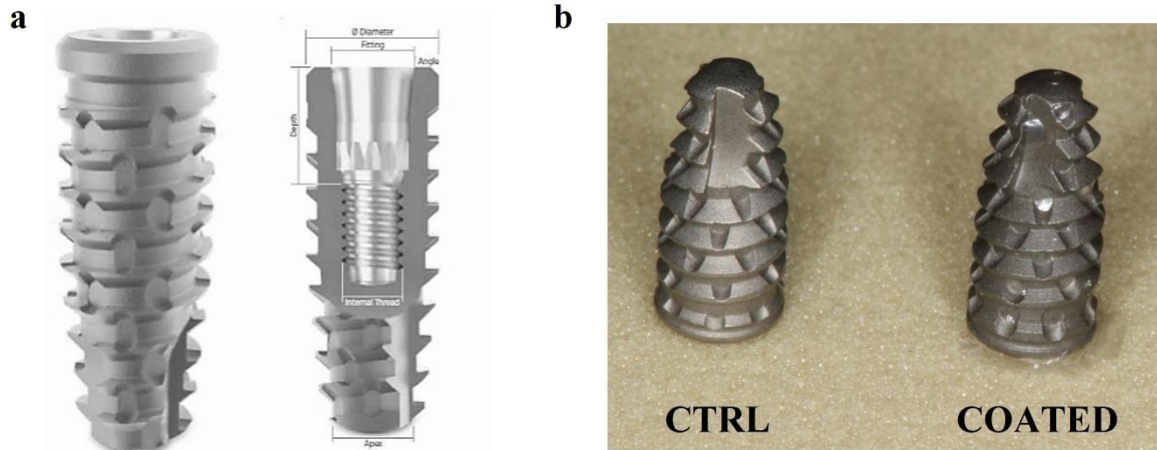


Figure 43. Design of the implants under investigation (CM AR Maestro, Implacil De Bortoli, Sao Paulo, Brazil). **(a)** A three-dimensional (3D) reconstruction of the Maestro implant design; **(b)** Representative images of the uncoated (CTRL) and N₂-coated (COATED) Maestro implants tested in this study.

The preparation of the N₂-coating required for the production of COATED implants was conducted in accordance with a previously established protocol (refer to *Sample Preparation* in section 3.2.2.4.). The integrity and uniformity of the coating were assessed using toluidine blue staining [263]. To summarize, the CTRL implant did not absorb the dye, whereas the COATED implant did, as the uncoated areas exhibited no staining, while the collagen-coated regions readily absorbed the cationic toluidine blue dye. This demonstrated the effectiveness of the coating process (**Figure 44**).

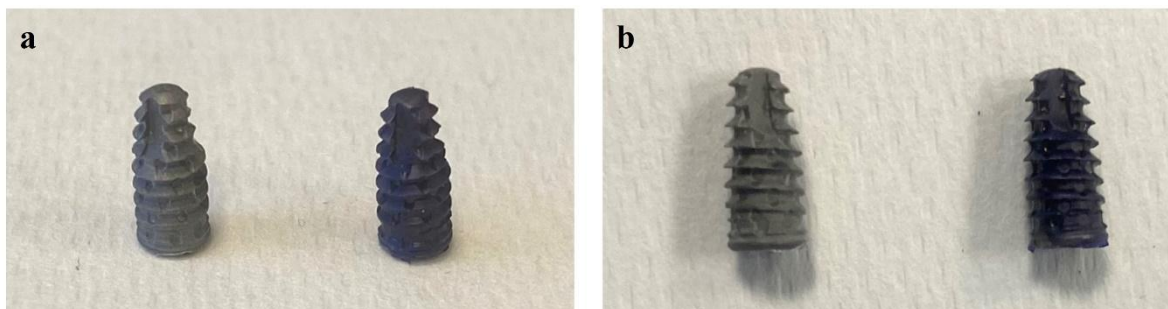


Figure 44. Images depicting the fixtures used for testing, showcasing unstained CTRL implants (on the left) and toluidine blue stained COATED implants, which illustrate the uniformity of the coating. **(a)** Apical view; **(b)** Lateral view.

Bone Models

Recent findings have indicated that polyurethane possesses biomechanical properties analogous to those of human bone tissue, including linear elasticity and isotropic constitutive symmetry [264]. These characteristics render polyurethane an appropriate material for conducting biomechanical assessments on dental implants, specifically in

evaluating IT, RT, and RFA. To accomplish this, various types of solid rigid polyurethane foam blocks (Sawbones Europe AB, Malmö, Sweden), each with uniform dimensions (120 mm × 170 mm × 40 mm), were selected to mimic different bone densities as classified by Misch [244,245]. The following types were utilized: (i) 10 PCF polyurethane block (cod. 1522-01) with a density of 0.16 g/cm³, simulating extremely low-density bone characterized by poor mechanical properties, typically observed in the posterior maxilla or in patients with advanced osteoporosis (D4 bone type). This bone type has sparse or absent cortical bone and thin trabecular tissue; (ii) 20 PCF polyurethane block (cod. 1522-03) with a density of 0.32 g/cm³, representing low-density bone commonly found in the posterior mandible and maxilla or in individuals with moderate osteoporosis. This variety presents a thin cortical bone and trabecular bone of slightly improved quality relative to D4 bone (D3 bone type); and (iii) 30 PCF polyurethane block (cod. 1522-04) with a density of 0.48 g/cm³, which features thicker cortical bone and denser trabecular bone compared to D3 and D4 bone types, thereby providing enhanced conditions for primary implant stability. Such bone is typically found in the anterior maxilla and mandible, representing mildly osteoporotic bone (D2 bone type), indicative of bone quality that is moderately compromised yet better than optimal. This type of bone is often observed in younger elderly patients or those presenting with mild osteoporosis.

The artificial bone blocks utilized in this study are illustrated in **Figure 45**.



Figure 45. Solid rigid polyurethane foam blocks used in the present study. (a) A block with a density of 10 pounds per cubic foot (PCF); (b) A block designated as 20 PCF density; and (c) A block designated as 30 PCF density.

Implant Drill

The drilling procedure was calibrated by a single operator. Notably, the manufacturer's drilling sequence was followed, starting with an initial lance drill and then using Ø 2 mm and Ø 3.5 mm conical drills for bone bed under-sizing, particularly due to low bone densities. Drilling was executed at a calibrated speed of 800 rpm, with an operating of 30 Ncm (**Figure 46**).

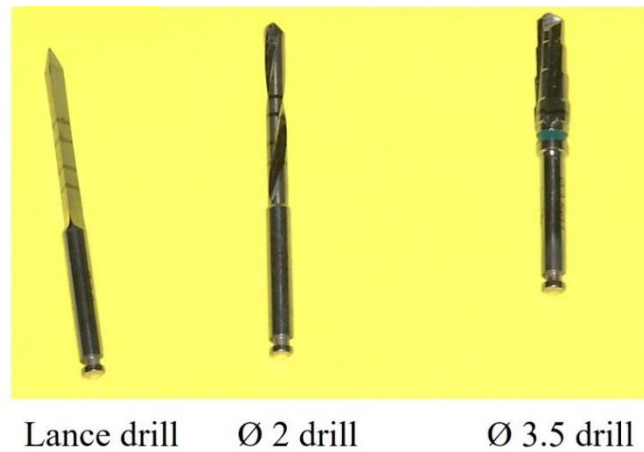


Figure 46. Details of the drills used for the implants in this study.

A Bien Air Chiropro (Bien Air SA, Bienne, Switzerland) surgical implant motor was used for this procedure. The final implant insertion was performed at a speed of 20 rpm with a maximum calibrated torque value of 75 Ncm. Then, the IT and the RT were measured during the last 1 mm of implant placement, which was considered at 2 mm below the surface of the polyurethane block. A Smart Peg n° 16 (Osstell AB, Gothenburg, Sweden) was used to assess the RFA values in both the buccolingual (BL RFA) and mesiodistal (MD RFA) directions.

Experimental Design and Statistical Analysis

In the present investigation, a total of 120 implants (20 per group) were included for testing on three polyurethane blocks with varying densities (10, 20, and 30 PCF). This configuration will provide 40 drilling sites for each block (**Figure 47**).

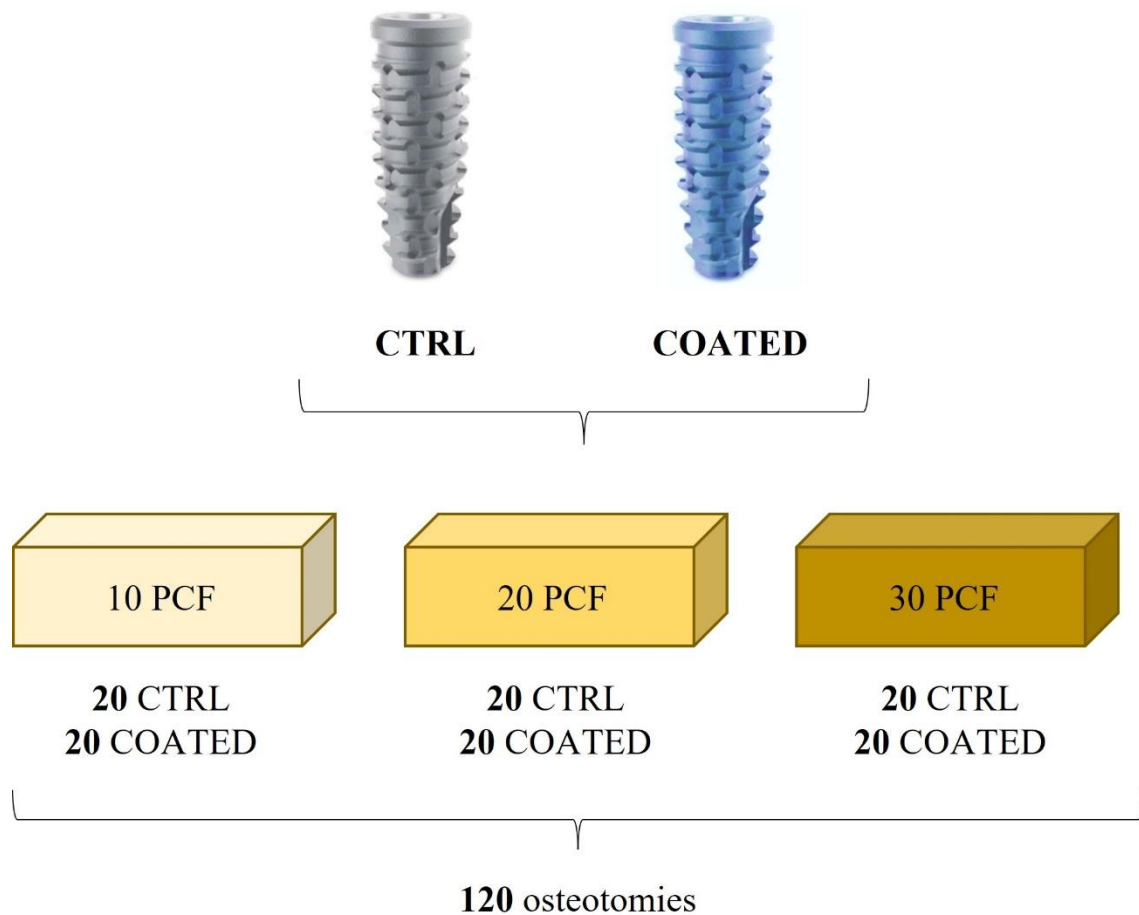


Figure 47. Scheme of the study design.

The sample size calculation and power analysis were performed utilizing the G*Power 3.1.9.4 program (Heinrich Heine Universität Düsseldorf, Düsseldorf, Germany). The analysis involved the F tests family and a “fixed effects, special, main effects, and interactions analysis of variance (ANOVA)” with an effect size of 0.3, α err of 0.05, power (1- β) of 0.8, numerator df of 2, and 6 groups. The dependent variables for the sample size calculation included IT, RT, BL RFA, and MD RFA measurements. In contrast, the independent variables were the implant type (CTRL and COATED) and the polyurethane density (10, 20, and 30 PCF). The statistical significance was set at p-values ≤ 0.05 , with a minimum sample size requirement of 111 implants, encompassing 19 samples per group.

Additionally, the study utilized a 3×2 factorial design and applied a two-way ANOVA model followed by Tukey’s post-hoc test. Descriptive statistics were proposed as means $\pm$ standard deviations. GraphPad Prism Software Analysis version 9 was used for statistical analyses.

4.2.3.2. RESULTS

Insertion Torque (IT) Measurements

The values of IT exhibited a direct proportionality to the density of the polyurethane, with the lowest values recorded in the lowest-density block (10 PCF). Specifically, the IT values measured were 15.50 ± 0.45 Ncm for the CTRL implants and 15.31 ± 0.55 Ncm for the COATED implants, exhibiting no statistically significant differences. Conversely, the highest IT values were observed in the block characterized by 30 PCF density, with mean values of 73.50 ± 4.12 Ncm for CTRL implants and 62.21 ± 1.79 Ncm for COATED implants. Notably, the test implants presented significantly lower values ($p < 0.0001$) in comparison to the CTRL group. The 20 PCF density block yielded intermediate IT values for all implant types, specifically 32.46 ± 2.63 Ncm for CTRL implants and 31.14 ± 1.25 Ncm for COATED implants, without significant differences between the two groups. Statistically significant higher values ($p < 0.0001$) were documented for each implant type inserted in the highest-density block when compared to their corresponding implants positioned in the 10 and 20 PCF density blocks. Furthermore, each implant type placed in the 20 PCF density block demonstrated significantly elevated IT ($p < 0.0001$) in comparison to its counterpart implanted in the 10 PCF density block.

In summary, the COATED implants displayed comparable IT values under all experimental conditions, with the exception of the 30 PCF density block, which yielded significantly lower IT values. Nonetheless, these implants exhibited acceptable IT values (15–62 Ncm), indicative of mechanical implant stability, particularly in cases involving extremely low-density bones (**Figure 48**).

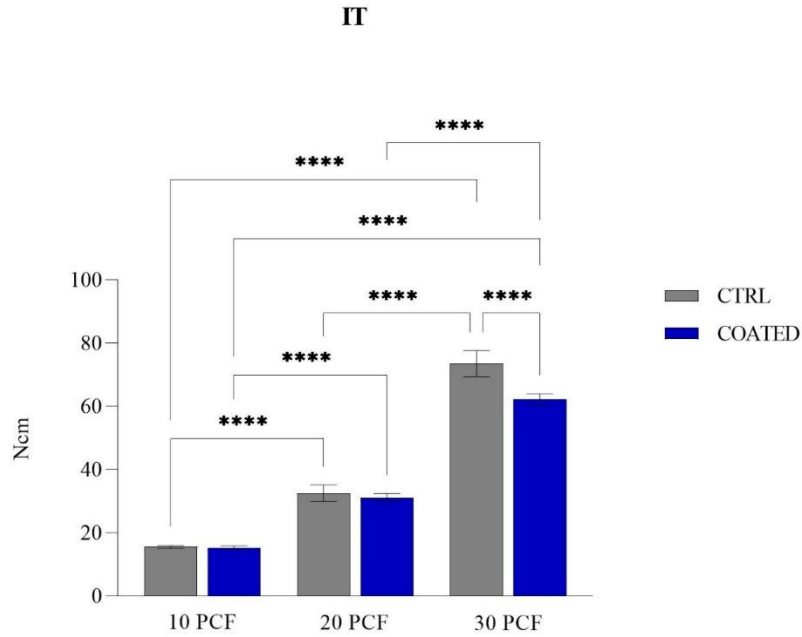


Figure 48. Insertion torque (IT) values recorded for CTRL and COATED implants across various artificial bone densities. The data are shown as the mean $\pm$ standard deviations. **** $p < 0.0001$.

Removal Torque (RT) Measurements

The RT values exhibited a proportional relationship with the density of the polyurethane blocks, demonstrating the highest values in the 30 PCF density block. Specifically, the mean RT was recorded at 58.61 ± 5.46 Ncm for the CTRL implants, while the COATED implants yielded a mean of 47.61 ± 4.56 Ncm. Conversely, the lowest RT values were observed in the 10 PCF density block, where the mean RT for CTRL implants was 17.39 ± 0.84 Ncm, and for COATED implants, it was 17.02 ± 1.11 Ncm. Notably, no significant differences were identified between the implant groups within the lowest-density block; however, in the highest-density block, the CTRL implants demonstrated a significantly greater RT compared to the COATED implants ($p < 0.001$). The 20 PCF density block yielded intermediate RT values across all implants, with measurements of 25.06 ± 2.03 Ncm for CTRL implants and 25.26 ± 2.23 Ncm for COATED implants, which did not exhibit any significant differences. In terms of IT, statistically significant higher values ($p < 0.0001$) were observed for each implant type placed in the highest-density block when compared to their respective implant types inserted in the 10 and 20 PCF density blocks. Furthermore, each implant type in the 20 PCF density block demonstrated significantly higher RT values ($p < 0.05$ for CTRL implants and $p < 0.01$ for COATED implants) relative to their counterparts in the 10 PCF density block (**Figure 49**).

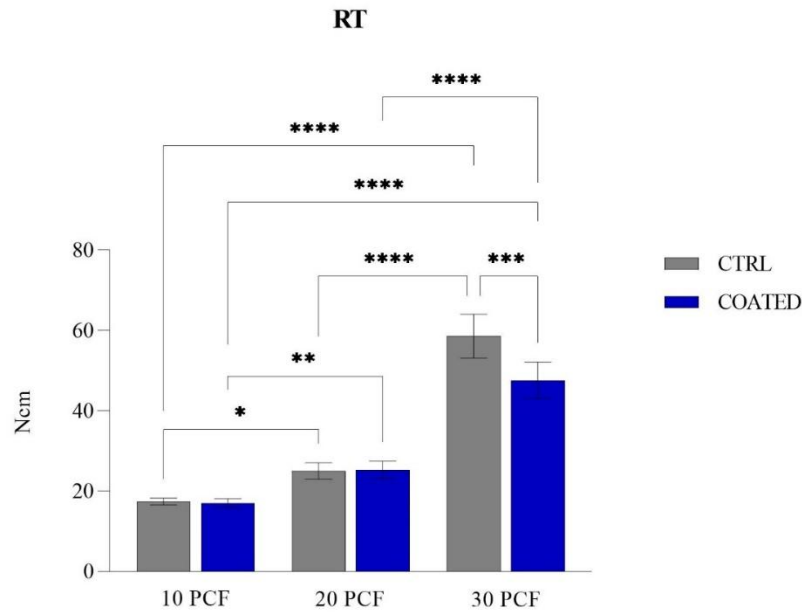


Figure 49. Removal torque (RT) values recorded for CTRL and COATED implants across various artificial bone densities. The data are shown as the mean $\pm$ standard deviations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Overall, the test implants displayed slightly lower RT values across all experimental conditions, with the exception of the 30 PCF density block, which exhibited significantly lower RT measurements. It is noteworthy that the RT registered lower results for both implant types than the corresponding IT values exclusively in the 20 and 30 PCF density blocks. In the 10 PCF density block, the RT values were marginally higher than the corresponding IT values for both implant categories. Additionally, the difference between IT and RT values was slightly more pronounced for CTRL implants (approximately 15 Ncm in the 30 PCF density block) in comparison to the test implants, which ranged between 14–15 Ncm.

Resonance Frequency Analysis (RFA) Measurements

In the assessment of stability values, it was noted that discrepancies related to bone density exhibited a more uniform distribution compared to the trends observed in IT and RT. Importantly, no statistically significant differences were found between the groups in any of the artificial bone blocks. However, as per the other evaluated biomechanical parameters, the ISQ values were directly proportional to the density of the blocks. Indeed, the highest ISQ results were recorded in the bloc with a density of 30 PCF, followed by intermediate results in the block with a density of 20 PCF, and the lowest values in the block with a density of 10 PCF. Specifically, statistically significant higher ISQ values (p

< 0.0001) were determined for each implant type placed in the highest-density block compared to their counterparts implanted in the 10 and 20 PCF density blocks. Similarly, for each implant type in the 20 PCF density block, significant differences were noted when compared to the corresponding implants positioned in the 10 PCF density block.

The CTRL implants demonstrated slightly lower results than the test implants across all experimental conditions, with the exception of the MD RFA at the 20 PCF density block. Specifically, at the 10 PCF density block, the CTRL implants yielded results of 40.80 ± 0.45 ISQ for BL RFA and 43.21 ± 1.79 ISQ for MD RFA, compared to the COATED implants that exhibited 43.21 ± 2.86 ISQ for BL RFA and 43.01 ± 2.12 ISQ for MD RFA. Conversely, at the highest-density block, the BL RFA and MD RFA values for the CTRL implants were both 69.60 ± 0.55 ISQ, whereas the COATED implants reported 70.21 ± 0.45 ISQ and 69.81 ± 0.45 ISQ, respectively. Intermediate values at the 20 PCF density block for the CTRL implants were recorded as 61.81 ± 1.64 ISQ and 62.80 ± 1.92 ISQ, while the COATED implants yielded values of 62.40 ± 0.55 ISQ and 62.21 ± 0.45 ISQ in both the BL and MD orientations (**Figure 50**).

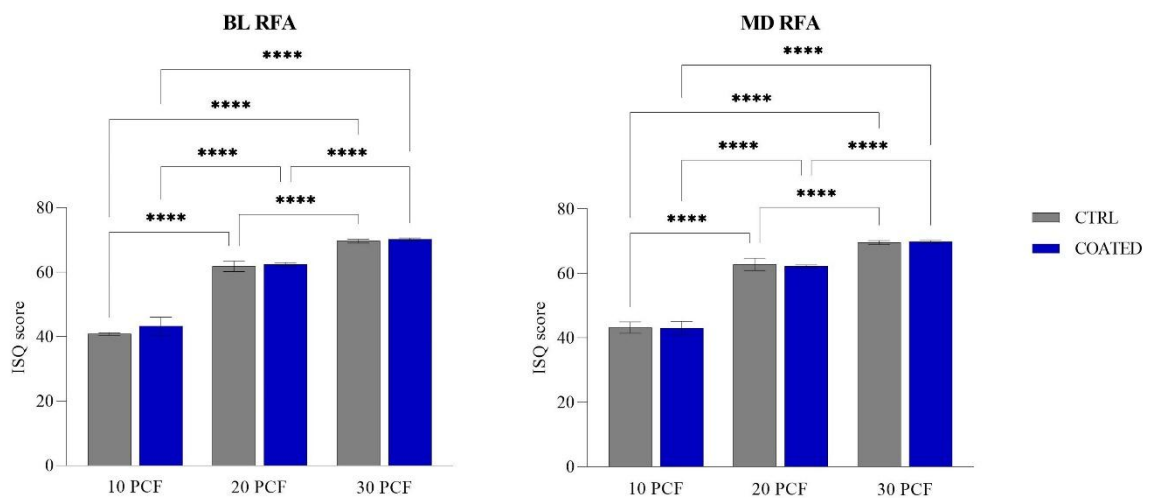


Figure 50. Resonance frequency analysis (RFA) measuring the implant stability quotient (ISQ) values in the buccolingual (BL) and mesiodistal (MD) orientations for CTRL and COATED implants across various artificial bone densities. The data are shown as the mean $\pm$ standard deviations. **** $p < 0.0001$.

The implants consistently exhibited high primary stability in the block with 30 PCF density, moderate stability in the block with 20 PCF density, and low stability in the block with 10 PCF density. The latter stability is deemed adequate, particularly in light of the extremely low density of the artificial block.

4.2.3.3. DISCUSSION

The primary objective of this study was to evaluate the effects of the N2 collagen coating on the biomechanical behavior of the W surface-corresponding implant, specifically in terms of IT, RT, and ISQ. An *in vitro* biomechanical assessment was conducted using various densities of artificial bone blocks composed of polyurethane, which simulated bone types D2, D3, and D4.

The findings of this study indicated that the application of N2 coating significantly decreased IT ($p < 0.0001$) and RT levels ($p < 0.001$) when compared to the CTRL group, particularly at higher polyurethane densities (30 PCF). This reduction was achieved without compromising the initial ISQ and even exceeding 60 ISQ at the highest density, thereby demonstrating high stability. Notably, the IT of the COATED implant remained below the established safe threshold of approximately 30-50 Ncm required for immediate loading [257], even in the context of the highest bone density (62.21 ± 1.79 Ncm). This effectively mitigates the risk of bone necrosis and fracture during implant placement, in contrast to the higher IT levels recorded in CTRL implants (73.50 ± 4.12 Ncm). A general consensus previously suggested that elevated IT values may compromise the structural integrity of both the implants and the surrounding bone, potentially leading to compression bone necrosis, a recognized risk factor for bone fracture and peri-implant disease. Compression of bone beyond physiological limits can induce ischemia, subsequently resulting in bone necrosis. Additionally, excessive torque may exert high strain levels in the adjacent bone [265]. Certain researchers contended that when the strain imposed on the bone exceeds a tolerable threshold, it may inflict irreversible damage characterized by plastic deformation and microcracking, ultimately causing early bone loss and implant failure. Moreover, bone compression is particularly likely to occur in denser bone types, such as type D1 or type D2 bone. Another factor contributing to early implant failure is the overheating of bone tissue during insertion, which can trigger necrosis surrounding the implant. It is well-established that bone necrosis may occur when temperatures reach 47°C for a duration of one minute or longer [256,257]. In contrast, other studies posited that IT values equal to or exceeding 50 Ncm could contribute to a reduction in micromotion without damaging the adjacent bone [254,255]. It is also crucial to highlight that the presence of healing chambers within the tested implants may significantly facilitate bone decompression during the placement of Maestro implants, thereby alleviating the risk of excessive bone stress and necrosis, as previously

documented in various biomechanical and histomorphometric studies [261,262]. In this context, research by Gehrke et al. [266,267] has indicated that implants equipped with healing chambers, despite exhibiting lower IT values, demonstrated less vertical bone loss in comparison to those with higher IT values, along with enhanced ISQ, BIC, and other histological parameters. Clinically, these healing chambers act as osteopromotive regions, expediting osseointegration by providing space for the accumulation of bone chips and facilitating the formation of blood clots, which ultimately enhances secondary implant stability [261,262].

Moreover, due to the absence of comparative data from existing biomechanical evaluations of collagen-coated implants available in the literature, this study proposes a potential explanation for the significantly reduced IT. The collagen coating is known to create a smoother or more lubricated interface when interacting with bone, thereby diminishing friction during the insertion process. In contrast, uncoated titanium presents a rougher surface, which can lead to increased resistance during insertion. As a result, the collagen coating may facilitate a more even distribution of stresses, thereby reducing the risk of micro-damage to the bone and minimizing resistance associated with localized deformation [268]. Additionally, the collagen coating can "mask" the roughness of the implant, temporarily smoothing the interface for easier placement while retaining the long-term advantages of rough surface characteristics for osseointegration. Although no studies specifically addressing IT or ISQ values for collagen-coated implants have been identified, some research indicated that pull-out tests or extraction torque assessments conducted in animal models suggest these implants may enhance bone repair and BIC more effectively than their uncoated counterparts, showing superior values after healing periods exceeding six weeks [238,269,270].

It is important to underline that, as detailed in the literature [271], a high IT does not invariably correspond with a high ISQ. Instead, elevated values of RFA may prove more advantageous for immediate loading protocols, thereby enhancing BIC. Notably, regardless of bone density, COATED implants consistently demonstrated slightly higher ISQ values (ranging from 43 to 70 ISQ) in comparison to CTRL implants (ranging from 40 to 69 ISQ). Although no significant differences were observed between the two, significant variations were evident based on bone density ($p < 0.0001$). This led to the inference that collagen-coated implants provided comparable primary stability to uncoated implants, despite presenting lower IT values. These findings indicated that a high level of primary stability can be achieved, particularly in areas of high bone density, with a lower IT value

being adequate for immediate implant placements. Furthermore, this phenomenon may be attributed to the incorporation of healing chambers on the Maestro surface, which are strategically designed to enhance the stability of blood clots and promote early bone formation while concurrently preserving high stability levels, thus expediting the osseointegration process for improved secondary implant stability. These results have been further corroborated by other *in vitro* biomechanical studies comparing Maestro implants to alternative implant macro-geometries [272,273]. Specifically, it has been established that the integration of healing chambers within the macro-structure of dental implants can significantly reduce IT and RT values without adversely affecting ISQ values. This outcome suggests the potential to mitigate compression and damage to peri-implant bone while sustaining consistent levels of primary stability.

The outcomes of this study are subject to specific limitations due to the utilization of an artificial bone model. Consequently, it is essential to acknowledge that the results may not be directly applicable to clinical settings. Although polyurethane foam blocks are recognized as a reliable *in vitro* model for implant research, serving as alternatives to cadaver or animal bones owing to their consistent and reproducible testing attributes, they fail to fully replicate the complex characteristics of natural bone [264]. Several factors warrant consideration for thorough data analysis, including the lack of individual human variability, the natural response of bone, and the intricate microenvironment associated with both healthy and pathological bone states. Additionally, the surgical technique should be considered to enable a more accurate interpretation of these results. It is, therefore, imperative to validate these findings through animal and clinical studies to facilitate their future application and implementation. To further optimize the use of polyurethane blocks as a model for human bone in studying implant behavior, conducting biomechanical assessments through finite element analysis (FEA) studies is advisable [274,275]. In conclusion, despite the limitations inherent in an *in vitro* study using non-human bone tissue, it can be inferred that these preliminary results may offer valuable insights into the biomechanical behavior of these implants, ultimately aiming to assist clinicians in refining their surgical rehabilitation strategies.

The findings of this research indicated that the integration of titanium can be enhanced through the proposed combination of surface coating and the concept of healing chambers, potentially facilitating stable implant fixation and allowing for early or immediate loading of the device, while also promoting the osseointegration process. To the best of our knowledge, this study represents the first investigation into the combination of

a collagen coating with an implant macro-geometry featuring healing chambers. The application of type I collagen as a coating for titanium implants may not only improve biocompatibility but also enhance mechanical interlocking between the implant and bone, thereby facilitating a more rapid and effective healing process. As ongoing research aims at optimizing collagen coatings, it is anticipated that such advancements may enhance implant performance and longevity. Ultimately, the objective is to improve patient outcomes through the implementation of advanced surface modification techniques that bolster the safety and efficacy of dental implant treatments. Furthermore, comprehensive studies examining the mechanics of collagen constructs—considering the mechanisms of stress transfer across different hierarchical levels—would significantly improve our understanding of the *in vivo* behavior of these materials.

4.2.4. SIMULATION OF *IN VIVO* BIOMECHANICAL BEHAVIOR

The alveolar bone possesses the ability to adapt to mechanical stresses through a process known as remodeling. It is essential to subject the bone to mechanical stimuli in order to maintain its structural integrity and density. The loss of a tooth, however, results in a lack of stimulation to the residual bone, which subsequently leads to a decrease in density and structural integrity. This reduction may manifest as a decrease in width, height, and thickness [276]. Over time, the bone loses its original morphologic characteristics and begins to acquire different properties, as indicated by the classifications of Misch or Lekholm and Zarb [277,278]. Additionally, factors such as the individual's age, overall health, gender, and the duration since tooth extraction play a significant role in determining the quality and quantity of bone and influence how the bone adapts to and responds to masticatory forces. For instance, when the bone is subjected to repeated loading or stress, it may adapt by altering its shape or density to enhance its strength. Conversely, a loss of bone density, as observed in conditions such as bone atrophy or osteoporosis, results in a decrease in bone strength, rendering it more vulnerable to fractures and other injuries [278].

Therefore, to ensure a high success rate and stability of dental implants, it is imperative to establish a favorable biomechanical environment that facilitates the effective distribution of masticatory loads to the surrounding tissues [279]. The success of dental implants is critically dependent on the initial stability and long-term osseointegration of the implant with the surrounding bone. Previous research has demonstrated that surface modifications using biomaterials, such as various coating techniques, can enhance bone strength and improve the initial rate of osseointegration [280]. In this context, as discussed in section 3.2.3., research assessing the mechanical properties of collagen-coated implants has demonstrated that such coatings can considerably enhance the mechanical performance of titanium implants. The primary biomechanical advantage of collagen coatings on titanium implants is improved osseointegration, which refers to the direct structural and functional connection established between the implant and the surrounding bone [215]. Furthermore, collagen coatings facilitate enhancements in tensile strength, stability, and the overall durability of implant surfaces [239-241]. These enhancements render the implants more robust and biocompatible throughout the healing process, potentially facilitating improved stress distribution across the implant surface [281]. It is essential to

recognize that the use of removable implants in biomedical applications may pose challenges due to the pronounced difference in Young's modulus between metallic implants and bone tissues. This discrepancy can induce a stress-shielding effect, which may lead to suboptimal osseointegration. Therefore, it is advisable for materials employed in such applications to possess a lower Young's modulus in order to mitigate the stress-shielding effect, which could otherwise result in excessive bone resorption due to diminished mechanical stimulation in the bone regions replaced by metallic medical devices. Ultimately, this approach is likely to enhance the performance of the prosthesis and ensure its permanent attachment to the surrounding bone [282].

Many interrelated factors influence load transfer at the bone-implant interface, including the type of loading, material properties of the implant and prosthesis, implant geometry, surface structure, design aspects such as diameter and length, as well as the quality and quantity of the surrounding bone, and the nature of the bone-implant interface [283]. Consequently, a deeper understanding of how stress is distributed from dental implants to the surrounding bone is crucial for the advancement of future dental prosthesis applications.

In this regard, FEA serves as a valuable tool for simulating the behavior of implanted devices within bone structures, thereby allowing researchers to model dental implants and their interactions with bone with enhanced accuracy and efficiency. This capability helps to address the challenges associated with *in vivo* testing [284]. Specifically, the assessment of von Mises stress distribution is particularly significant, as it serves as a critical indicator of dental implant stability and the integrity of the surrounding bone—factors that directly affect the longevity and success of implant procedures. Indeed, the biomechanics associated with the chewing process generates axial and bending forces that propagate a series of moments to the dental implant and subsequently to the adjacent bone. Excessive stress can lead to bone resorption and potential implant failure [285]. According to Frost's theory [276], bone initiates growth and remodeling processes in response to biomechanical demands based on the intensity and direction of applied stress. Moderate mechanical stress may stimulate bone remodeling, thereby enhancing bone density and strength while promoting Semaphorin-3A secretion and osteocyte proliferation. In contrast, excessive stress may result in pathological damage, as described in a study conducted by Uto et al. [286]. Typically, within a stress range of 11–30 MPa, the bone remains in a state of equilibrium, known as the lazy zone, maintaining stable conditions referred to as the homeostatic state. Exceeding this equilibrium may result in

bone deposition, whereas stress levels beyond a certain threshold may induce bone microfractures and subsequent resorption due to overload. Conversely, stress levels lower than the equilibrium value can lead to decreased mineral density and bone atrophy. Numerous studies have been conducted to analytically characterize bone remodeling as a function of load, emphasizing the critical importance of efficient load transfer from the implant to limit bone resorption [287]. It is also essential to consider that bone type significantly affects implant stability and stress distribution. Azcarate-Velázquez et al. [288] conducted an FEA to investigate the influence of bone quality on two different types of dental implants, revealing that a decline in bone quality adversely affects stress distribution and results in the overloading of the cortical bone at the implant collar. Consequently, treatment plans should incorporate strategies to mitigate stress, thereby reducing the likelihood of initial bone loss, such as surface treatments and the method of implant placement [289].

Currently, there is a lack of FEA studies examining the biomechanical behavior of collagen-coated implants. Few studies have reported on the effects of other bioactive coatings on dental implants using the finite element method (FEM), particularly concerning stress distribution within both the bone and the implant under masticatory loads. These studies have indicated that the application of coatings such as hydroxyapatite, monticellite, and TiN significantly reduces induced stress concentrations and total implant displacements, in addition to demonstrating superior bonding strength [290-292]. Thus, the present study aimed to be the first of its kind to simulate the mechanical behavior of a collagen-coated implant (N2 collagen-coated Maestro implant) within the surrounding tissues under specific loading conditions via FEA. The objective was to analyze the stress and strain distribution on the implant and adjacent bone by applying a perpendicular load of 150 N, akin to a static masticatory force, along with a horizontal load of 100 N to assess the resultant micro-movements through numerical methods, facilitating a correlation with the ISQ measurements obtained from *in vitro* biomechanical tests performed on artificial bone.

4.2.4.1. MATERIALS AND METHODS

Modeling

A computer-aided design (CAD) software (Autodesk Inventor 2023, San Francisco, CA, USA) was used to develop a 3D prototype of a segment of the mandibular

bone derived from a cone-beam computed tomography (CBCT) scan (NewTom Giano HR, Cefla, Imola, Italy) [248]. The dimensions of the modeled block were defined as 10 mm × 14 mm × 24.22 mm, corresponding to the anatomical region of the first right molar. Additionally, Maestro dental implants (9 × 4 mm) were modeled, including both uncoated implants (CTRL) and N2-coated implants (COATED) with varying coating thicknesses between 0.082 and 0.482 mm, with these specifications determined based on SEM measurements of the implant surface (**Figure 51**).

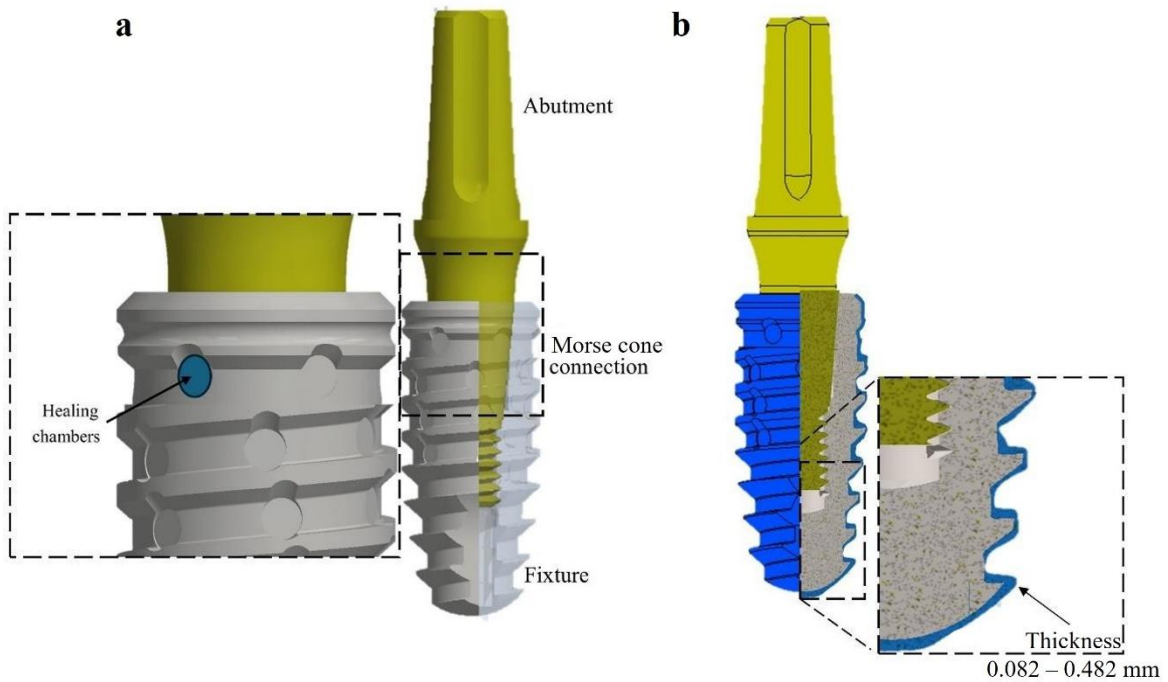


Figure 51. A 3D model of Maestro implants utilized in this study, featuring a straight abutment, developed using computer-aided design (CAD) software (Autodesk Inventor 2023, San Francisco, CA, USA). (a) The uncoated implant (CTRL) showing the details of the healing chambers present on the implant body; (b) The N2-coated implant (COATED) illustrating the variation in the thickness of the coating.

Subsequently, following the biomechanical *in vitro* study previously conducted on polyurethane blocks, three distinct bone block models were developed, classified as D2, D3, and D4 in accordance with Misch's classification [245]. These models corresponded to the polyurethane blocks identified as 30 PCF, 20 PCF, and 10 PCF, utilized during the *in vitro* test. Both the bone and polyurethane models were employed to subtract the geometric thread profile after the insertion of the implant in a crestal position, thereby ensuring an accurate fit between the implant and the bone, as illustrated in **Figure 52**.

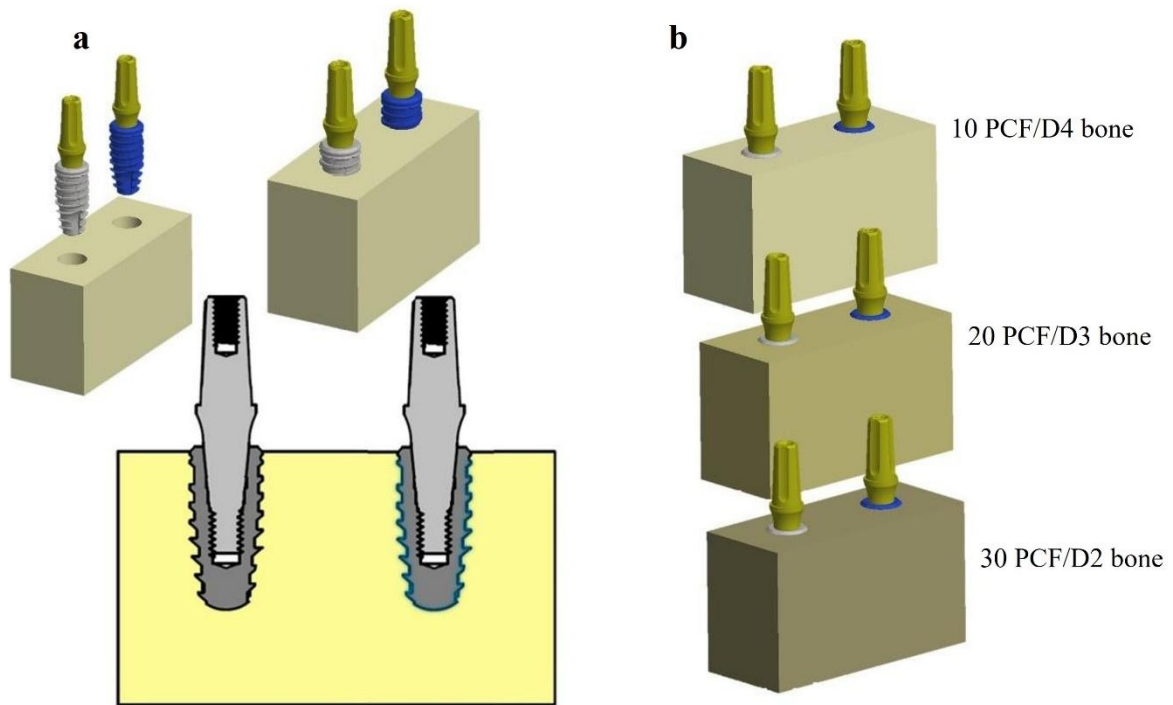


Figure 52. A detailed description of the 3D models analyzed in this study. **(a)** A 3D model of the bone incorporating both the CTRL and COATED implants (upper line) and a section illustrating the crestally inserted implants within the bone model (bottom line); **(b)** 3D models of both implant types positioned within polyurethane block models of varying densities.

Materials

To accurately simulate the mechanical behavior of materials, various Young's moduli (E), which serve as indicators of material stiffness, and Poisson's Ratios (ν), which measure material deformability, were assigned. The characteristics of the bone in the mandibular region and polyurethane blocks represented the three distinct bone classes examined in this study. The material selected for the implants was the titanium alloy Ti6Al4V. For the collagen coating, type I collagen (N2) was utilized, with the corresponding properties supplied by the manufacturer. This native collagen, derived from horse tendons, is characterized by well-organized collagen fibers, which afford the collagen substantial mechanical stability and resistance [43]. For the purposes of this investigation, all examined materials were assumed to demonstrate linear elastic and isotropic behavior, thereby simplifying the computational models [293,294]. Detailed mechanical properties of the materials employed in this study are presented in **Table 8**.

Table 8. Mechanical properties of the materials used [250,295,296].

Material	Young's Modulus (MPa)		Poisson's Ratio (ν)
D2 Bone	5500.00		0.30
D3 Bone	1600.00		0.30
D4 Bone	690.00		0.30
Ti6Al4V	110000.00		0.30
N2	2.10		0.16
Polyurethane (PCF)	10 (0.16 g/cm ³)	5800.0	0.30
	20 (0.32 g/cm ³)	2100.0	0.30
	30 (0.48 g/cm ³)	4450.00	0.30

Pounds per cubic foot (PCF).

Finite Element Method (FEM)

The models constructed in CAD were imported into Ansys Workbench 2023 R1 software (Canonsburg, PA, USA) for FEA, with file formats converted for numerical evaluation (**Figure 53**). The mechanical properties of the structural materials (**Table 8**) were implemented into the software based on data obtained.

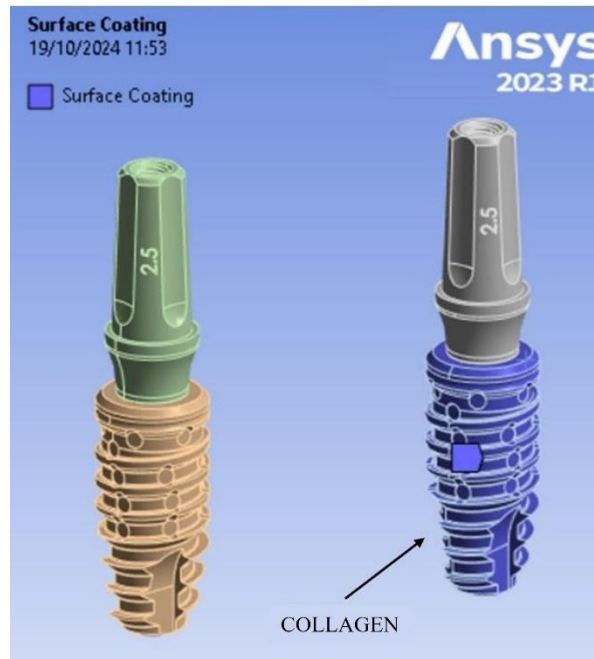


Figure 53. Application of the N2-coating on the surface of Maestro implants into Ansys Workbench 2023 R1 software (Canonsburg, PA, USA) for finite element analysis (FEA).

The FEA involves discretizing the continuous body into smaller elements known as meshes, followed by the application of elasticity equations to each element to assess the body's behavior. The mesh size is critical for achieving accurate results [297]. In this study, a linear tetrahedral mesh with an element size of 0.5 mm was generated for all components. The mesh size was further refined for the collagen coating to facilitate more

precise convergence on von Mises stress, reducing the element size to 0.3 mm due to the thinness of the coating (**Figure 54**).

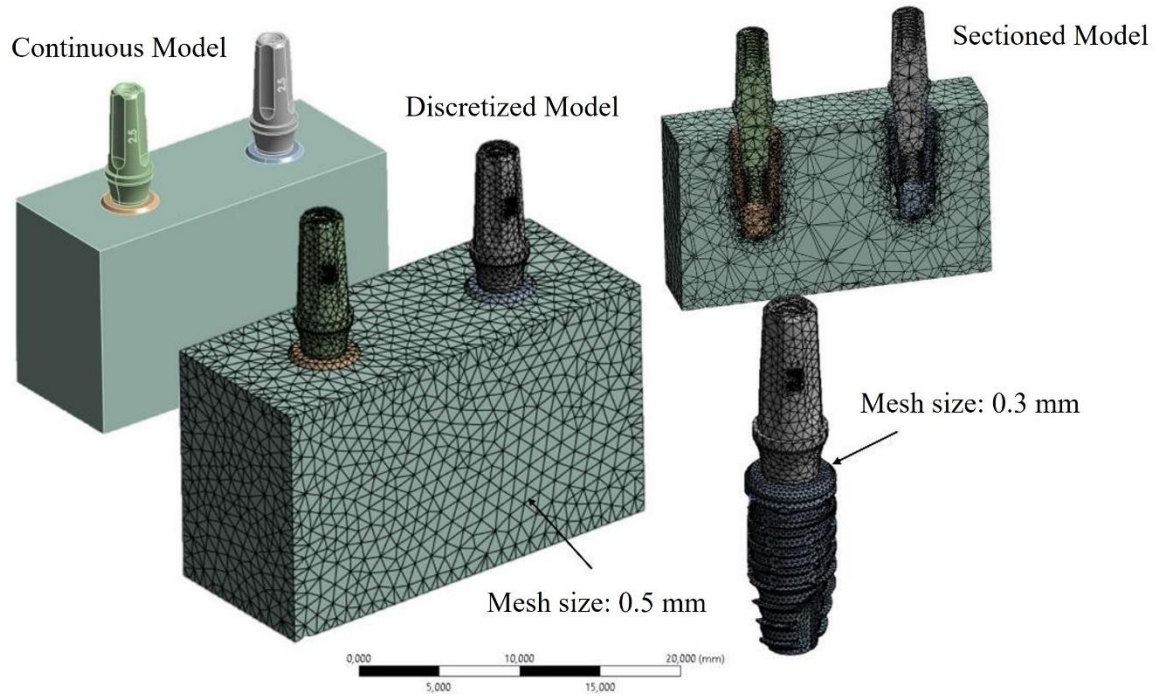


Figure 54. Discretization of the continuous model with mesh sizes of 0.5 mm and 0.3 mm.

Loads and Constraints

All faces of the block, except for the upper face, were subjected to constraints in all directional movements. Physical interactions were evaluated in the contact zones between the implant and the bone structure, the abutment and the implant, as well as the coating and the external surface of the implant, utilizing fixed contact types. Specifically, it was assumed that upon the insertion of implants into the bone models, all surrounding bone surfaces adhered to the implant's outer surface. This assumption supports the principle of ideal osseointegration, thereby streamlining the modeling process [293]. Full osseointegration indicates that the implant is stabilized through bony attachment, which facilitates optimal load transfer during functional loading. In clinical practice, achieving successful osseointegration represents a fundamental objective in implant therapy, thus proving a coherent framework for FEA simulations that model an optimal scenario in which the implant operates as a unified entity with the bone. The use of simplified models is particularly beneficial in exploratory studies, as it enables an initial understanding of stress behavior without imposing excessive demands on computational resources.

The loading conditions for the implants encompassed a vertical load of 150 N applied to the upper surface of the abutment to simulate masticatory forces [298],

alongside a horizontal load of 100 N to assess the micro-mobility of dental implants, as shown in **Figure 55**. This evaluation is useful for determining primary stability and calculating the ISQ, in accordance with the study conducted by Pagliani et al. [299]. It is essential to note that the application of a horizontal load may not directly correlate with ISQ values, as the ISQ is derived from dynamic measurements rather than static loading conditions.

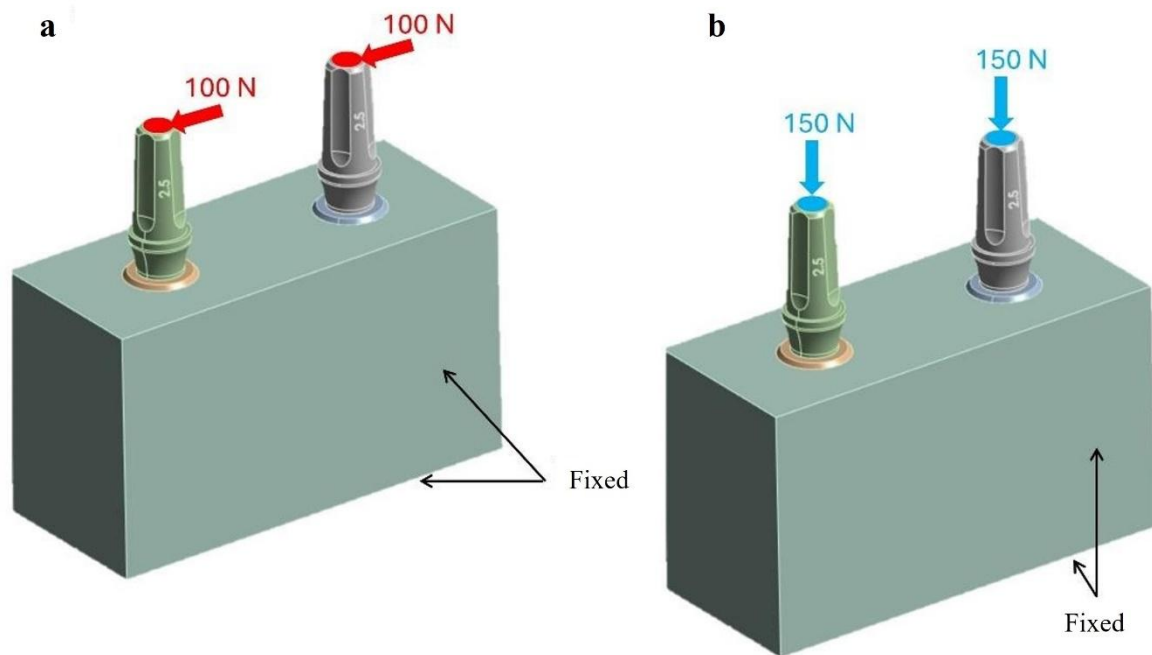


Figure 55. The 3D model showing (a) the vertical load and (b) the axial load applied to the abutments.

This study analyzed the stress induced by applied masticatory forces on both implant surfaces and surrounding bone, in conjunction with the assessment of implant micro-movements under axial loads. The von Mises criterion, which is based on equivalent stress, was employed to evaluate the system's stress, demonstrating particular effectiveness in scenarios involving multi-directional and time-varying loads. This approach also accounted for shear and normal stresses, thereby providing a more accurate criterion than alternative models in the presence of complex loading conditions [300]. Data derived from mathematical solutions have been converted into visual representations employing color gradients that transition from red to blue, with blue indicating minimum stress and red signifying maximum stress. All intermediate shades present in this gradient represent varying levels of stress. Subsequently, stress values were measured at various points collected from the models under investigation and then compared. The levels of von Mises equivalent stress were employed to identify regions exhibiting the highest stress

concentrations within the analyzed models [301]. This investigative methodology facilitated a comprehensive understanding of the biomechanical behavior of the bone–implant system in both COATED and CTRL implant configurations.

Assessment of Implant Stability Quotient (ISQ) Using FEM

In 2013, a study conducted by Pagliani et al. [299] demonstrated a nonlinear correlation between implant micro-mobility and ISQ. The study revealed that micro-mobility decreases by approximately 50% when the ISQ increases from 60 to 70. Through interpolation using nonlinear regression analysis, the authors established a mathematical relationship between ISQ and micro-mobility, expressed by the following formula:

$$ISQ = 74.94 - 5.21 * \ln (Micro\ mobility - 0.24)$$

This formula delineates the ISQ as a function of micro-mobility, defined as the ratio of the implant’s micro-displacement to an applied force (mm/N), which quantifies the extent of implant movement under a transverse load. Consequently, employing the FEM allows for the calculation of micromovements for the implants analyzed. This established formula was then used to assess the ISQ, facilitating a comparison of these results with those derived from previous *in vitro* testing. **Figure 56** illustrates a summary of the methodological steps involved in the numerical evaluation of the ISQ parameter through this method.

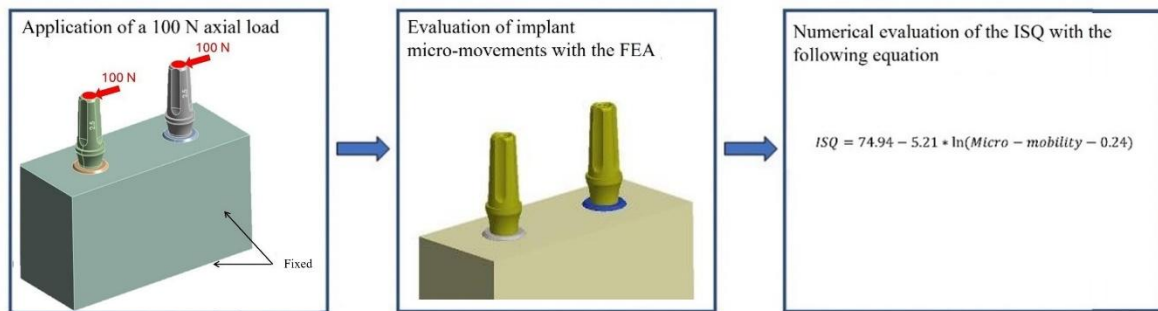


Figure 56. Steps for the numerical assessment of ISQ: (i) Application of a 100 N horizontal load to the implants inserted in polyurethane blocks; (ii) Assessment of micro-movements of the implants using the FEA, focusing on the highest micro-movements at the neck of the implants ('Probe' command); (iii) Input of the micro-movement results into the formula that correlates the ISQ with the micro-movements.

4.2.4.2. RESULTS

Evaluation of Stress on Bone Blocks

Using the FEM, it was possible to evaluate the stress distribution on the bone blocks following the application of a masticatory load. This analysis allowed for an understanding of how the use of the N2 coating influenced the stress on the bone compared to an uncoated implant. The visual results are presented in **Figure 57**.

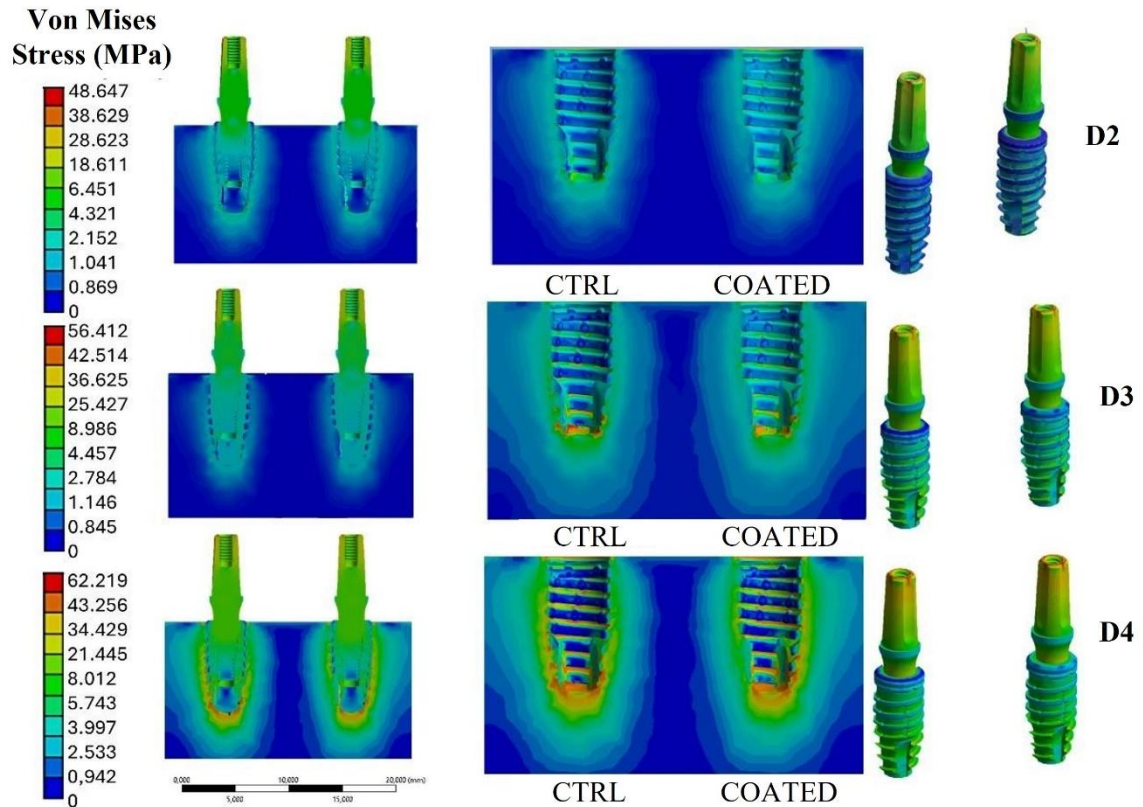


Figure 57. Distribution of von Mises stress on implants and various bone types.

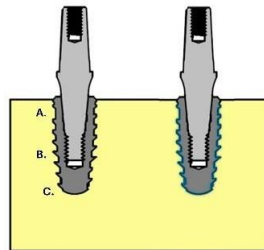
In reference to **Figure 57**, it is evident that maximum stress levels increased concomitantly with a decrease in bone quality. Specifically, stress rose from 48.647 MPa for a D2 bone type to 62.219 MPa for a D4 bone type. The stress distribution was observed to be similar across all bone types for both implants. However, it was noted that the application of collagen as a coating resulted in enhanced force distribution on the bone in contact across all configurations. This phenomenon was particularly prominent in the D4 bone type, where stress values ranged from 33.723 MPa in the crestal region to 51.449 MPa in the apical region of the implant for the COATED implant. In contrast, the CTRL implant exhibited stress values ranging from 32.125 MPa to 51.273 MPa. **Table 9** provides

the numerical stress values for both the implants on the bone, considering three points of analysis (A: crestal; B: middle; C: apical).

Table 9. Results of von Mises stress values on the bone at three analysis points (A: crestal; B: middle; C: apical).

Position	D2 Bone		D3 Bone		D4 Bone	
A	15.242	15.365	25.477	28.126	32.125	33.723
B	13.463	14.418	18.136	19.256	42.778	45.336
C	17.526	17.621	42.215	43.369	51.273	51.449
	CTRL	COATED	CTRL	COATED	CTRL	COATED

Von Mises Stress (MPa)



Upon examining the three groups of bone types, it became evident that stress levels increased progressively from the crestal region to the apical region of the implant. Additionally, the average stress values calculated for the three conditions indicated that the application of N2 coating resulted in an increase in bone stress of 2.537% for the D2 bone type, 5.736% for the D3 bone type, and 3.433% for the D4 bone type. This underscored the effectiveness of the collagen coating, particularly for D3 and D4 bone classifications, as it enhanced stress distribution within the bone, thus mitigating stress-related issues that may result in resorption.

Regarding the implant itself, as observed from **Figure 57**, there were no significant differences observed between the COATED and CTRL implants due to the markedly low stiffness of collagen (2.1 MPa) in relation to titanium (110,000 MPa). However, it is noteworthy that the areas experiencing the greatest stress on the implant were located at the Morse cone connection, where stress levels ranged between 18.23 MPa and 35.42 MPa across all three groups. Elevated results were particularly noted when the implant was placed in the D4 bone. Despite this, given titanium's compressive strength of 930 MPa, all configurations analyzed exhibited adequate mechanical resistance, confirming their structural integrity.

Evaluation of Micromovements and ISQ Using FEM Analysis

The numerical results derived from the FEM simulation were systematically

collected, analyzed, and graphically represented using color maps. In **Figure 58**, the distribution of micro-movements, measured in mm, is illustrated for both COATED and CTRL implants in the context of a 100 N load, specifically across bone types D2, D3, and D4.

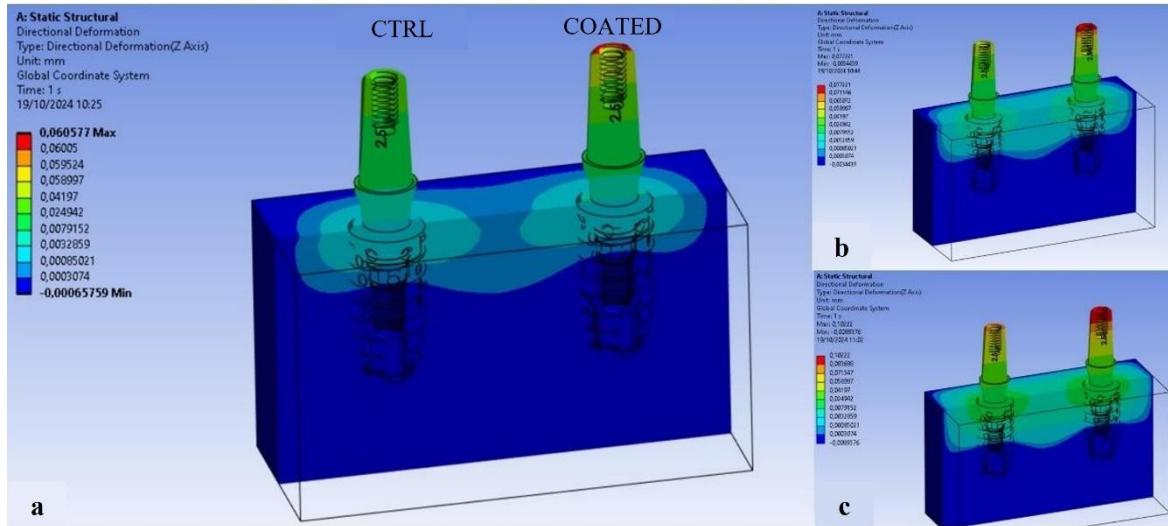


Figure 58. Results of micro-movements of the implants in the horizontal direction under applied load across different bone types: (a) D2 (b) D3, and (c) D4.

As illustrated in **Figure 58**, the application of a COATED implant resulted in higher micro-movements. Specifically, the recorded values were 0.0605 mm for the COATED implant in D2 bone, 0.0772 mm in D3, and 0.1022 mm in D4. In comparison, the CTRL implant exhibited micro-movements of 0.0446 mm for D2, 0.0653 mm for D3, and 0.0831 mm for D4 bone. Furthermore, it is noteworthy that as bone quality decreased from D2 to D4, an increase in the micro-movements of the implant was observed.

Then, utilizing the mathematical relationship that correlates maximum micro-movements expressed in mm (**Figure 56**), it became possible to calculate the ISQ values. These values were subsequently compared with those obtained *in vitro* from the previous study conducted on polyurethane blocks, as presented in **Table 10**.

Table 10. Results of the implant stability quotient (ISQ) values obtained from the *in vitro* study on polyurethane blocks and the finite element analysis (FEA) correlated using the formula proposed by Pagliani et al. [299].

	<i>in vitro</i> TEST		FEA TEST	
	CTRL	COATED	CTRL	COATED
10 PCF/D4	40.800	43.200	41.244	43.457
20 PCF/D3	61.800	62.400	61.422	61.724
30 PCF/D2	69.600	70.200	68.422	69.125

The results indicated no notable differences between the COATED and CTRL implants across any of the examined bone blocks. Notably, higher ISQ values were recorded in the D2 block, intermediate values in the D3 block, and lower values in the D4 block. However, it is important to highlight that a slight enhancement in ISQ was noted for the D4 bone block, where the placement of a COATED implant resulted in a stability increase of 5.882%, in comparison to increases of 0.9709% in the D3 block and 0.8621% in the D2 block.

Utilizing FEA, it was possible to assess ISQ values predicted on the micro-movements calculated according to the formula proposed by Pagliani et al. [299]. The results indicated that the discrepancy between the two methods (FEA and *in vitro* experimentation) remained below 2% across all cases.

4.2.4.3. DISCUSSION

The objective of the current study was to conduct numerical simulations to investigate the mechanical behavior of the N2-coated implant within the surrounding tissues under specified loading conditions utilizing FEA. Following the development of 3D models that accurately represented the implants and bone densities observed in the polyurethane blocks used during the *in vitro* study, the mechanical properties of all materials were established, and it was possible to proceed with the discretization of the models. Two distinct types of forces were subsequently applied to the implants. Initially, an evaluation of stress and strain was performed on both the COATED and CTRL implants, as well as the surrounding bone, employing a perpendicular force of 150 N, which simulated a static masticatory load. Furthermore, a horizontal load of 100 N was applied to assess the micro-movements generated through numerical methods [299], thereby facilitating a correlation with the ISQ measurements obtained from prior *in vitro* assessments conducted on the artificial bone.

The results of the study demonstrated a comparable stress distribution between the two implant types, with increased stress being associated with lower bone density. Notably, elevated stress values were particularly observed when the implants were positioned within the D4 bone, as bone quality is recognized as a significant factor influencing stress distribution [302]. Nevertheless, the COATED implants exhibited an improved and more uniform stress distribution within the surrounding bone, particularly in the D3 (28.126 MPa and 43.369 MPa in the crestal and apical zones, respectively,

compared to 25.477 MPa and 42.215 MPa for the CTRL implants in the same zones) and D4 (33.723 MPa and 51.449 MPa in the crestal and apical zones, respectively, compared to 33.125 MPa and 51.273 MPa for the CTRL implants) bone classifications. The application of the N2 coating resulted in an increase in bone stress of 5.736% for the D3 bone type, 3.433% for the D4 bone type, and 2.537% for the D2 bone type. This finding underscored the effectiveness of the collagen coating, particularly in low-quality bone classifications, in enhancing stress distribution within this type of bone. This enhancement may provide the necessary mechanical stimuli to prevent declines in bone density and structural integrity, which can lead to a reduction in bone volume. Furthermore, it achieved this without surpassing the strength threshold of bone, which could result in stress-related conditions leading to bone resorption [276,303]. Additionally, with regard to the implants, no considerable differences in stress distribution were noted between the COATED and CTRL implants. However, it is significant to observe that the regions experiencing the highest stress on the implant were located at the Morse cone connection across all three groups (18.23-35.42 MPa). Despite these observations, given the compressive strength of titanium (930 MPa), all examined configurations showed sufficient mechanical resistance, thereby affirming their structural integrity. Furthermore, the evaluation of implant stability through FEM indicated an increase in micro-movements of COATED implants correlated with a decrease in bone density. The subsequent ISQ calculations demonstrated an enhancement in stability associated with improved bone quality (69.125 ISQ compared to 68.422 ISQ in the D2 bone type, 61.724 ISQ compared to 61.422 ISQ in the D3 bone type, and 43.457 ISQ compared to 41.244 ISQ in the D4 bone type). Although the findings revealed no notable differences between the COATED and CTRL implants across the examined bone blocks, it is noteworthy to highlight that a slight enhancement in ISQ was recorded for the D4 bone block, where the placement of a COATED implant resulted in a stability increase of 5.882%, in contrast to increases of 0.9709% in the D3 block and 0.8621% in the D2 block. Comparatively, the numerical method yielded results that aligned with the in vitro tests (error < 2%), enabling the conclusion that the N2-coated implant has the potential to reduce stresses and strains on the surrounding bone, particularly within D3 and D4 bone types. Consequently, this may mitigate resorption phenomena while maintaining high implant stability.

The phenomenon of stress-shielding arises when a titanium implant fails to adequately transfer mechanical loads to the surrounding bone, often resulting in bone resorption [304]. The quality and quantity of bone significantly influence how its adaptive

response to mechanical forces, leading to alterations in shape or density that enhance strength. Specifically, a reduction in bone density, as observed in conditions such as bone atrophy or osteoporosis, diminishes bone strength, rendering it more susceptible to fractures and injuries. This scenario necessitates the application of appropriate stimuli to maintain bone integrity [278]. Despite the existing body of FEA studies addressing various implants, there remains a paucity of research focused on the efficacy of collagen-coated implants in mitigating these challenges. Previous FEA investigations have demonstrated that the incorporation of bioactive coatings can substantially reduce induced stress concentrations and overall implant displacements while also exhibiting enhanced bonding strength. Research findings indicated that the application of bioceramics, apatite-based coatings, and ceramic/polymer composites onto metallic implants facilitates secondary fixation and encourages a more gradual transition between the mechanical properties of rigid implants and the surrounding bone. This approach led to a reduction in stress distribution and implant displacements [290-292]. The results from these studies contrasted with the findings of the present investigation. For instance, coating such as hydroxyapatite, poly-lactic acid combined with hydroxyapatite, monticellite, and TiN on titanium implants exhibited substantially higher Young's moduli compared to N2 (with reported values of 71800, 440000, 51000, and 305000 MPa as opposed to 2.1 MPa). This disparity suggested that these coatings reduce stress distribution on surrounding tissues more than N2 collagen, which has been shown to increase stress distribution in surrounding tissue, particularly in cases of poor bone quality. However, this may potentially stimulate bone remodeling, thereby diminishing the stress-shielding phenomenon that could result in progressive bone resorption, in accordance with Frost's theory [276]. Importantly, this occurs without inducing excessive stress that could lead to bone resorption, while also indicating enhanced stability of the implant. Furthermore, the same studies indicated improved BIC and bond strength in comparison to uncoated implants, which aligns with the properties of collagen in promoting the osseointegration process over time [147,215-217].

FEA serves as a critical tool for predicting the behavior of the implant materials and surrounding bone. The FEM can be employed to examine various aspects of dental implant design, particularly the influence of implant thread design on stress distribution. This methodology thus allows for the identification of parameters that significantly affect stress distribution both in the surrounding bone and within the implant itself. In this regard, numerous FEA studies have been conducted to explore the effects of factors such as bone density, implant geometry and materials, implant positioning, and surgical technique on

the distribution of stresses and strains experienced by implants and the surrounding bone [248,274,305-307]. Among them, prior FEA investigations conducted by our research group have demonstrated the efficacy of Maestro implants in substantially mitigating stress distribution in the surrounding bone. This research corroborates the beneficial role of healing chambers in promoting bone decompression, alongside enhancing bone integration and stability [272,308,309].

However, like any modeling and simulation tool, there are some limitations that are important to consider when using FEA to assess stress variations. Among them, we find the accuracy of the input data. In fact, the accuracy of FEA results depends on the quality of the input data used, such as loading conditions and material properties. With respect to this last point, all materials were considered homogeneous, isotropic, and linearly elastic, although the mandibular bone was crosswise isotropic and inhomogeneous. Alternatively, considering the materials as anisotropic, with mechanical properties that vary in different directions, they would require the use of more sophisticated and complex FEA models and the consideration of additional parameters to describe and determine the mechanical behavior of the material. Therefore, simplifying a material as isotropic can be an approximation and may not fully reflect the actual behavior of the material. Consistent with previous literature, this study assumed that bone is homogeneous and isotropic to facilitate the modeling process. Such assumptions, however, may lead to considerable underestimations of stress distributions, thereby potentially compromising the accuracy and reliability of clinical outcomes [293]. Furthermore, a fixed contact between the bone and implant was assumed to simplify the analysis; yet, from a clinical perspective, achieving perfect osseointegration is implausible due to the inherent micro-movements present at the implant interface [310]. Nonetheless, enhanced osseointegration is associated with increased implant stability and minimized bone deformation. The rationale for this assumption is derived from the clinical focus on attaining optimal mechanical stability and effective load transfer from the implant to the adjacent bone. Moreover, it is essential to recognize that FEA studies are numerical analyses, providing approximations of the actual component behavior rather than a true representation. The results obtained through FEA should be carefully assessed by comparing them with clinical outcomes. This approach, for instance, can be utilized to evaluate stress and deformation within the bone, offering advantages over the use of strain gauges that only detect alterations in surface deformation. The acquired data can aid designers in optimizing the implant and offer valuable insights to clinicians regarding insertion techniques. Stress and strain are crucial parameters for

bone maintenance and implant survival, and studying the stresses directly on the patient is impractical. Therefore, the FEM becomes useful in evaluating these parameters on the implant.

In conclusion, to the best of our knowledge, this represented the first FEA study assessing the stresses and stability associated with a collagen-coated dental implant across various bone densities. The findings indicated that N2-collagen coating may effectively modulate stress distribution around the implant, especially in low-density bone, while maintaining high implant stability. By facilitating a better interface and promoting intraosseous reactions, collagen helps distribute mechanical loads more uniformly, which may reduce the likelihood of bone loss and improve the implant's overall stability during functional loading. Additionally, it is essential to recognize that N2 collagen possesses a biological function that may promote superior osseointegration and long-term stability of the implant.

4.3. A COLLAGENATED XENOGRAFT TO IMPROVE ANGIOGENESIS

Vascularization is essential for the health and regeneration of oral bone, particularly in regions such as the alveolar bone, which provides support for teeth and is critical for optimal oral function [311]. Bones within the oral cavity are perpetually undergoing remodeling, growth, and repair, processes that are heavily reliant on a complex network of blood vessels. The vascular system supplies oxygen, nutrients, hormones, and growth factors that are vital for sustaining the structure and function of bone tissue. Specifically, blood vessels facilitate the activity of cells responsible for bone turnover, including osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells), thus ensuring an equilibrium that maintains bone density and strength [312]. The significance of vascularization is particularly pronounced during oral interventions such as tooth extractions, periodontal surgeries, or the placement of dental implants. When bone is injured or lost, the capacity for bone regeneration largely depends on an adequate blood supply. Inadequate vascularization can impede wound healing and bone regeneration, resulting in a slow or incomplete healing process, which poses significant challenges in complex procedures like dental implants or bone grafting [313]. Conditions such as periodontal disease, which induces chronic inflammation and vascular damage, as well as systemic diseases like diabetes, can also compromise vascular health and adversely affect bone regeneration, resulting in diminished bone integrity and an increased risk of complications following dental interventions [314,315].

In light of the pivotal role of vascularization in oral bone health, enhancing vascular supply and promoting angiogenesis—the formation of new blood vessels—have become focal points of interest in dental and oral medicine. Researchers are investigating innovative therapies, including the application of growth factors, such as vascular endothelial growth factor (VEGF) and stem cell treatments, to stimulate the formation of new blood vessels and improve bone regeneration [316]. Additionally, the development of vascularized bone grafts, which integrate blood vessels from adjacent tissues, aims to enhance the efficacy of bone reconstruction surgeries [317]. These advanced therapeutic strategies seek to improve bone healing, prevent bone loss, and elevate the likelihood of success in dental implants and other restorative procedures, ultimately fostering both functional and aesthetic outcomes in oral health treatments. Therefore, the interplay between vascularization and oral bone health remains a critical area of focus for enhancing

long-term oral health and optimizing the outcomes of dental interventions.

The ECM provides a vital framework for angiogenesis, offering both structural support and molecular signals that are essential for all stages of blood vessel formation. This includes vascular sprouting, lumen formation, vessel maturation, and, ultimately, vessel stabilization. The 3D network of the ECM comprises glycosaminoglycans, proteoglycans, collagens, fibronectin, laminin, and elastin. Consequently, the initial stages of sprouting angiogenesis are typically believed to occur within an environment enriched with preexisting interstitial collagens and supplemented by fibrin, vitronectin, and fibronectin sourced from blood plasma. Although the fundamental significance of the ECM and its principal components in facilitating endothelial cell migration, proliferation, survival, and angiogenesis has been extensively studied, the specific role of collagen fibers in supporting these processes remains less defined [318]. Type I collagen fibrils, which are commonly associated with capillaries and larger blood vessels, constitute a predominant element of most tissue stroma. Given that type I collagen is also recognized as an optimal substrate for angiogenesis, it can be regarded as the principal intermediate matrix constituent driving angiogenesis processes throughout the body [319]. However, it is notable that angiogenesis can occur during development in certain tissues that may lack fibrillar collagen or fibrin, such as the brain [320]. This suggests the possible speculation that angiogenesis may not be strictly dependent on the presence of collagen, but rather that collagen may expedite capillary tube formation when rapid revascularization is necessary for tissue healing.

Nonetheless, type I collagen remains one of the most pro-angiogenic molecules known, and collagen-based biomaterials have emerged as promising solutions for enhancing vascularization and angiogenesis in tissue engineering applications [321,322]. Research indicated that collagenated biomaterials not only support the formation of capillary-like structures *in vitro* but also enhance neovascularization *in vivo*, thereby improving the integration of implants within host tissues. This capability is particularly significant in the context of ischemic conditions and tissue regeneration, where adequate blood supply is essential for the survival and functionality of tissues [323,324]. Particularly, collagenated porcine bone grafts are currently recognized as the gold standard for bone regeneration procedures in dentistry, aiming to provide the optimal handling, granulometry, consistency, biocompatibility, chemical composition, gradual resorption, and elevated angiogenic potential to achieve ideal regenerative outcomes [51,325,326].

Over the past decade, the zebrafish model has emerged as a powerful alternative

in vivo model for studying angiogenesis and tissue regeneration [327]. Its optical transparency, high fecundity, potential for genetic manipulation, ease of maintenance, rapid developmental timeline, and exceptional regenerative capabilities have led to its widespread adoption in the field. Although the anatomical and physiological characteristics of zebrafish may appear distant from those of humans, there are significant similarities at the molecular level. Transgenic zebrafish lines, which express fluorescent proteins under specific tissue promoters, facilitate the investigation of the proliferation and migration of cells involved in the formation of new blood vessels *in vivo* [328]. Notably, zebrafish possess the remarkable ability to regenerate a variety of tissues, including the heart, retina, spinal cord, scales, and fins. This model has also proven particularly useful for elucidating the immune response mechanisms and signaling networks during wound healing, as well as for examining vascular regeneration, owing to its simple morphology, relative transparency, accessibility, and virtually unlimited regenerative potential [329].

In the final section of this doctoral project, the angiogenic and regenerative capabilities of a collagen-based bone graft material were explored using the zebrafish model. This study emphasized the critical importance of promoting vascularization, as the establishment of a robust blood vessel network within newly forming tissues is essential for ensuring optimal bone regeneration and long-term implant stability. By elucidating the material's potential to stimulate blood vessel formation, this research provided valuable insights into its broader applications and potential enhancements in regenerative therapies.

4.3.1. MATERIALS AND METHODS

The Collagen-Based Biomaterial

GTO[®] (OsteoBiol[®], TecnoSS[®] s.r.l., Giaveno, Italy) is a heterologous bone grafting material derived from porcine origin, composed of a mix of collagenated cortico-cancellous granules with sizes ranging from 600 to 1000 μm (80%), combined with TSV Gel (20%), which is formulated from heterologous type I and III collagen gel, polyunsaturated fatty acids, and a biocompatible synthetic copolymer, all diluted in an aqueous solution. GTO[®] has demonstrated a gradual resorption process, exhibiting significant osteoconductive properties. Moreover, the preserved collagen matrix inherent to the granules promotes effective blood clotting and facilitates the infiltration of repairing and regenerative cells. These distinctive characteristics ensure a substantial rate of new bone formation, while maintaining adequate graft volume and promoting healthy new bony

tissue, which collectively contribute to successful implant rehabilitation [330,331]. GTO[®] is available as a ready-to-use, pre-hydrated biomaterial, thus eliminating the need for additional hydration with saline or blood at the defect site. The incorporation of TSV Gel provides optimal stickiness of the material, enhancing its adaptability to the recipient site and ensuring stability during application.

Preparation of the Biomaterial for Testing

GTO[®] was evaluated through two methodologies: direct application into the embryos' water (0.2 g/mL; GTO[®] suspension) and as an extract (GTO[®] supernatant), with comparisons made to untreated control groups (CTRL). The preparation of the extracts from the tested material adhered to the ISO 10993-12 standard. Specifically, 0.2 g of the biomaterial were dissolved in 1 mL of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄ in distilled water; Sigma-Aldrich, St. Louis, MO, USA), or in E3 medium supplemented with 5% FBS, or in E3 medium supplemented with 2.5% DMSO. Subsequently, the distinct extracted phases of the material were consolidated and incubated for 3 days at 37 °C with agitation at 180 rpm. Following this extraction phase, the resultant suspension was subjected to centrifugation at 13,000 × g for 15', and the supernatant was meticulously separated from the pellet for immediate use in subsequent *in vivo* experiments utilizing the zebrafish (*Danio rerio*) embryo model.

In Vivo Experiments in the Zebrafish Model

All experiments with zebrafish (*Danio rerio*) embryos were performed in accordance with the European Directive 2010/63/EU and the Ethical Guidelines for the Care and Use of Laboratory Animals of the Institute of Molecular Genetics and Genetic Engineering of the University of Belgrade, Serbia. Wild-type (AB) zebrafish embryos, kindly provided by Dr. Ana Cvejić (Wellcome Trust Sanger Institute, Cambridge, UK), were reared to the adult stage in a temperature- and light-controlled zebrafish facility at 28 °C and a standard 14:10 h light-dark photoperiod. The fish were fed twice daily with commercial dry food (SDS200 and SDS300 granular food, Special Diet Services, Essex, UK; and TetraMinTM flakes, Tetra Melle, Germany) and daily with *Artemia nauplii*.

In Vivo Toxicity Assessment

The toxicity of the GTO[®] formulations was assessed in accordance with the established guidelines set forth by the OECD for the Testing of Chemicals [332]. Embryos generated by pairwise mating were thoroughly washed to eliminate debris and

subsequently distributed at a density of 10 embryos per well in 24-well plates containing 1 mL of E3 medium, which were maintained at a temperature of 28 °C. To evaluate both acute (lethal) and developmental (teratogenic) toxicity, embryos were exposed to a concentration 0.2 g/mL of each tested formulation at 6 h post-fertilization (hpf), an early embryonic stage that ensures heightened sensitivity to the evaluated specimens. Treated embryos were observed daily under a stereomicroscope (Carl Zeiss™ Stemi 508 doc Stereomicroscope, Oberkochen, Germany) for the emergence of apical endpoints (**Table 11**), continuing this evaluation until 120 hpf. Dead embryos were systematically collected and discarded every 24 h. E3 medium served as a negative control. The experiments were conducted in duplicate, involving a total of ten embryos for each experimental condition. At 120 hpf, embryos were anesthetized through the addition of a 0.1% (w/v) Tricaine solution (cat. E10521, Sigma-Aldrich, St. Louis, MO, USA), subsequently photographed, and euthanized by freezing at -20 °C for a minimum of 24 h.

Table 11. Lethal and teratogenic effects observed in zebrafish (*Danio rerio*) embryos at different hours post-fertilization (hpf).

Category	Toxicological parameters	Exposure time (hpf)				
		24	48	72	96	120
Lethal effect	Coagulated eggs ^a	●	●	●	●	●
	Lack of the heart beating	●	●	●	●	●
	Non-detachment of the tail	●	●	●	●	●
	Lack of somite formation	●	●	●	●	●
Teratogenic effect	Malformation of head	●	●	●	●	●
	Malformation of eyes ^b	●	●	●	●	●
	Malformation of sacculi/otoliths ^c	●	●	●	●	●
	Malformation of chorda	●	●	●	●	●
	Malformation of tails ^d	●	●	●	●	●
	Scoliosis	●	●	●	●	●
	Yolk edema	●	●	●	●	●
	Yolk deformation	●	●	●	●	●
	Growth retardation ^e		●	●	●	●
	Hatching			●	●	●
	Swim bladder development					●
	Yolk absorption			●	●	●
Hepatotoxicity	Liver darkening			●	●	●
Cardiotoxicity	Pericardial edema		●	●	●	●
	Heart morphology			●	●	●
	Heart beating rate (beat/minute)				●	●

^aNo clear organ structure is recognized; ^bMalformation of the eye was recorded, including retardation in eye development and abnormality in shape and size; ^cPresence of none, one, or more than two otoliths per sacculus, along with reduction and enlargement of the otic vesicles; ^dTail malformation is recorded when the tail is bent, twisted, or shorter than that

of control embryos, as assessed through optical comparison; °Growth retardation is recorded by comparing the body length after hatching with that of the control embryos.

Determination of Inflammatory and Immunosuppressive In Vivo Responses

To evaluate the potential inflammatory and/or immunosuppressive effects of the tested materials, 6-hpf old embryos from transgenic Tg(*mpx*:GFP)*i*114 zebrafish line, which express green fluorescent protein (GFP) specifically in neutrophils were utilized [333]. These embryos were exposed to GTO® formulations (0.2 g/mL) and incubated at 28 °C. At 72 hpf, the embryos were subjected to imaging using a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA), and the occurrence of neutrophils, quantified as fluorescence intensity, was determined using ImageJ software version 1.52. This methodology allowed for direct visualization of the impact of the tested material on neutrophil occurrence and accumulation within the exposed tissues or internal organs, as previously documented [334]. The experiments were performed in duplicate, employing ten embryos per group. Subsequently, five embryos were randomly selected for imaging and analysis of neutrophil occurrence through ImageJ software version 1.52. The effects of the applied treatments were analyzed in comparison to the CTRL sample.

Wound Healing Assay

The embryos were exposed to GTO® formulations (0.2 g/mL) and incubated at 28 °C. For the purpose of inducing wounds, zebrafish embryos at 72 hpf were anesthetized using 0.02% (w/v) Tricaine solution. A full-thickness wound was created at the intersection of the anterior anal and dorsal fins by a sterile scalpel. The experimental procedure was conducted over a 120-hpf period, with ten embryos per group. Subsequently, five embryos were randomly selected for analysis. The extent of tissue regrowth (μm^3) was quantified using a stereomicroscope and ImageJ software version 1.52. The effect of applied treatments was analyzed in relation to the CTRL sample.

In Vivo Angiogenic Potential

The impact of GTO® on the development of blood vessels was examined in transgenic zebrafish Tg(*fli1*:EGFP)*y* embryos characterized by GFP-expressing endothelial cells [335], as previously described [336]. Briefly, zebrafish embryos at 6 hpf were exposed to GTO® formulations (0.2 g/mL) and incubated at 28 °C. At the 72 hpf stage, the embryos were anesthetized utilizing a 0.02% (w/v) Tricaine solution, followed by imaging under a fluorescence microscope to assess the development of subintestinal vessels (SIVs).

A total of ten embryos were utilized per group. To evaluate the effect of the treatments on new SIV formation within the designated basket, five embryos were randomly selected for imaging, and the number of normally developed SIVs was quantified by ImageJ software version 1.52. The results of the treatments were analyzed in comparison to those of the CTRL group.

Statistical Analysis

Statistical analyses were conducted utilizing GraphPad Prism Software Analysis version 9. The normality of the data distribution was assessed via the Kolmogorov-Smirnov test. Subsequently, the data were analyzed employing one-way or two-way ANOVA with the Bonferroni post-hoc test, or the Kruskal-Wallis test, contingent upon the distribution of the data. The level of statistical significance was established at $p \leq 0.05$. Results are reported as the mean $\pm$ standard deviations.

4.3.2. RESULTS

Toxicity Assessment

To assess the biocompatibility of GTO[®], the toxicity profiles of two distinct formulations of the material—supernatant and suspension—were evaluated using an *in vivo* zebrafish model and compared against a CTRL group. AB zebrafish embryos were exposed to the GTO[®] formulations (0.2 g/mL), and a systematic analysis was conducted to examine various toxicity endpoints (**Table 11**). The *in vivo* data collected from this assay indicated that GTO[®] did not produce any adverse effects in either formulation on the developing embryos during exposure lasting up to 120 hpf. The results demonstrated no significant differences when compared to the CTRL group. All exposed embryos exhibited survival rates without any skeletal malformations, and there were no adverse effects observed on heart rate or blood circulation in the caudal region relative to the CTRL embryos. Additionally, there were no discernable changes in liver coloration or yolk consumption. These findings suggested that the material exhibits a safe profile at the tested concentration and merits further *in vivo* investigation (**Figure 59**).

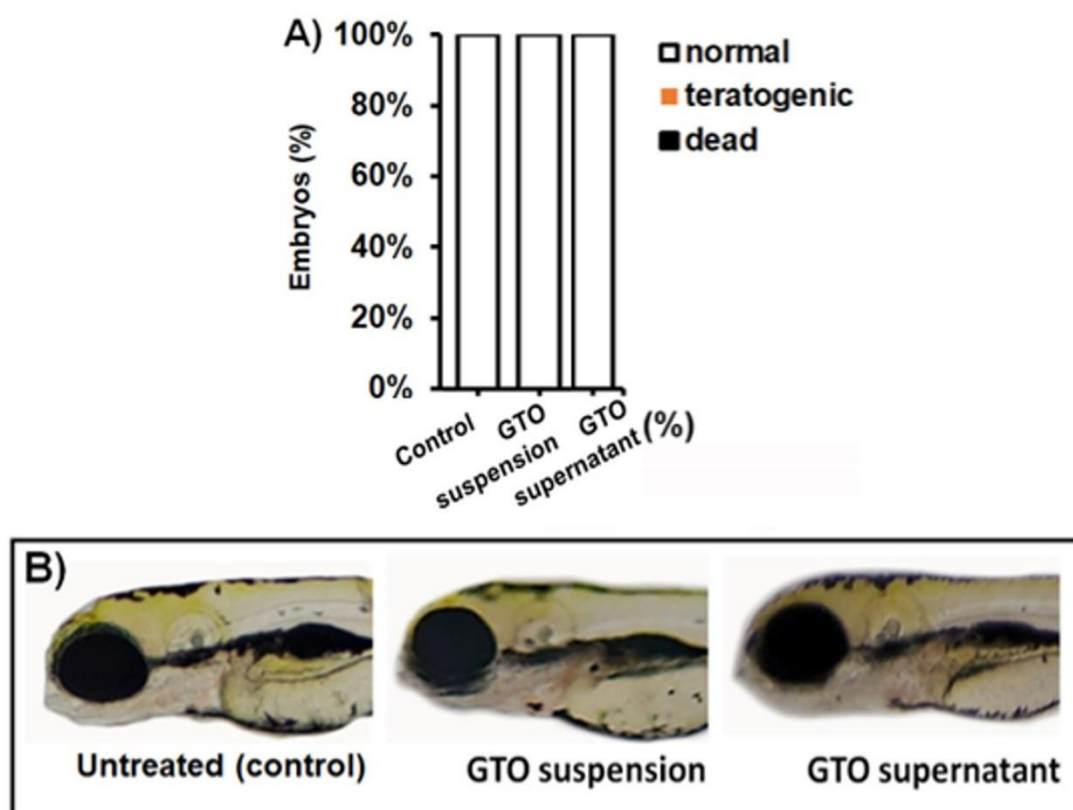


Figure 59. Evaluation of the toxicity of GTO[®] suspension and supernatant (0.2 g/mL) *in vivo* using the zebrafish (*Danio rerio*) model over a period of 120 h post-fertilization (hpf). Both GTO[®] formulations did not adversely affect (a) the survival of the exposed embryos or (b) their morphological characteristics, regardless of the duration of exposure. Magnification: 10 $\times$.

Immunotoxicity Assessment

Inflammation is a prevalent concern associated with dental materials; therefore, we investigated whether both GTO[®] formulations elicited such a biological response *in vivo*. The transgenic Tg(*mpx*:GFP)ⁱ¹¹⁴ zebrafish embryos were utilized and directly exposed to the tested materials for up to 72 hpf, allowing for monitoring the presence of neutrophils within the exposed organism in real-time using fluorescence microscopy. Notably, the data obtained from this assay demonstrated that none of the materials tested induced an inflammatory response or an immunosuppressive reaction (neutropenia) in the treated embryos. The number of neutrophils remained stable, showing no significant increase or decrease when compared to the CTRL group. This indicated that neither the GTO[®] suspension nor the supernatant resulted in tissue inflammation or neutropenia (Figure 60).

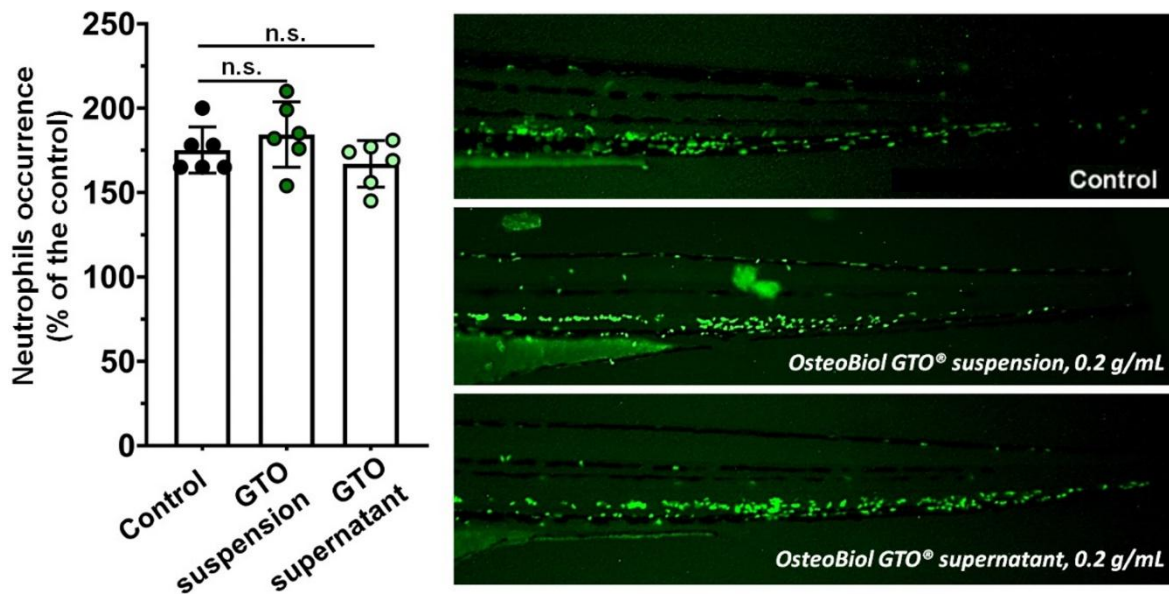


Figure 60. The biocompatibility of GTO[®] suspension and supernatant (0.2 g/mL) evaluated in the Tg(*mpx:GFP*)i114 zebrafish line, utilizing fluorescently labeled neutrophils. The presence of neutrophils is quantified as the percentage (%) of the control group after 72 hpf, and representative images illustrating fluorescence intensity are presented. n.s.: Not statistically significant. Magnification: 20×.

Caudal Fin Regeneration

Zebrafish caudal tail fins were transected at 72 hpf, and images were captured over a 3-day period to assess regeneration using a stereomicroscope. The extent of regeneration in embryos was evaluated immediately following the initial wounding and again 3 days post-wounding. The results indicated that the embryos achieved nearly complete regeneration by the third day post-wounding when treated with GTO[®]. At this time, the regeneration area returned to levels observed prior to injury. Both formulations of GTO[®] displayed a statistically significant enhancement in embryo-tail wound healing compared to the CTRL group ($p < 0.01$), with no significant differences detected between the two GTO[®] formulations (**Figure 61**).

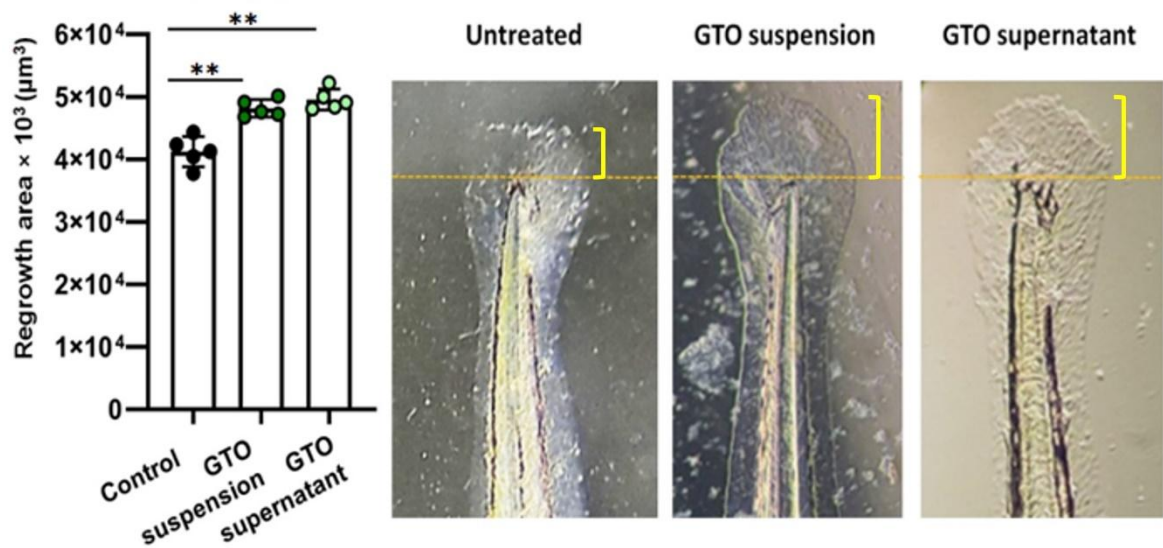


Figure 61. Zebrafish caudal amputation and regeneration test. A full-thickness wound was created at the intersection of the anterior anal and dorsal fins by a sterile scalpel, then the regrowth area (μm^3) was quantified at 3 days post-wounding. $**p < 0.01$. Magnification: 10 $\times$.

Angiogenic Effect

To evaluate the angiogenic potential of GTO[®] suspension and supernatant, the development of new SIVs was analyzed in *Tg(fli-1:EGFP)y* zebrafish embryos, which exhibit GFP expression in their endothelial cells. Observations were conducted using a fluorescent microscope. As illustrated in **Figure 62**, the number of SIVs present in the yolk region of *Tg(fli-1:EGFP)y* embryos in both GTO[®] formulation-treated groups demonstrated a statistically significant enhancement when compared to the CTRL group after 72 hpf.

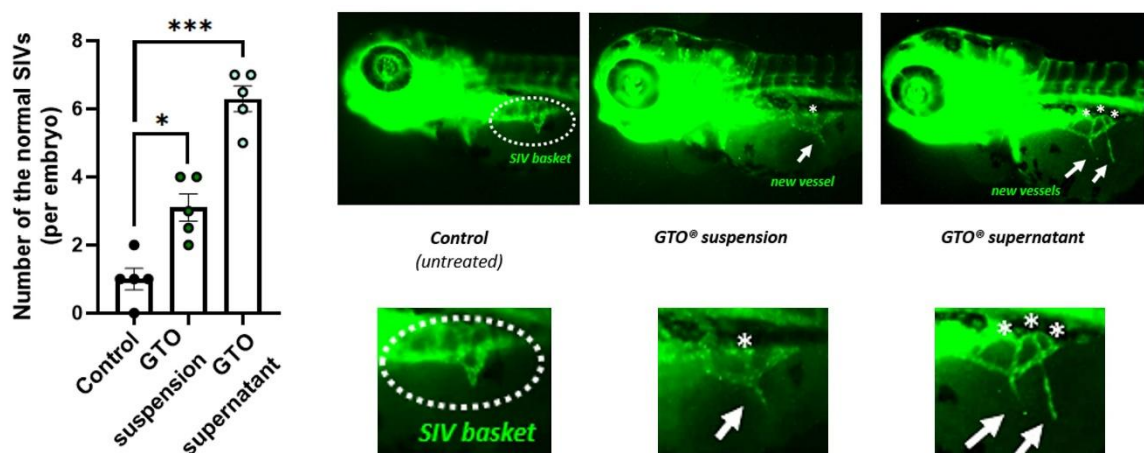


Figure 61. The effect of GTO[®] suspension and supernatant on the formation of new subintestinal vessels (SIVs) in the yolk region (basket) of *Tg(fli-1:EGFP)y* zebrafish

embryos after 72 hpf. The number of normal SIVs was quantified using a fluorescence microscope, which allowed for the counting of intersegmental vessels (asterisk) and new sprouts (arrow). * $p < 0.05$, *** $p < 0.001$. Magnification: 20 $\times$.

Specifically, the GTO[®] suspension showed a significant increase with a $p < 0.05$, whereas the GTO[®] supernatant exhibited an even more significant increase with a $p < 0.001$. No substantial differences were identified between the treated groups; however, embryos in the supernatant group displayed some instances of new vessel branching. Conversely, SIVs in the CTRL group were nearly absent. These findings indicated that GTO[®] has the potential to promote angiogenesis in zebrafish, particularly highlighting the efficacy of the supernatant.

4.3.3. DISCUSSION

The present research aimed to elucidate the role of collagen in facilitating and enhancing vascularization and tissue regeneration. This investigation specifically focused on assessing the toxicity, wound healing capabilities, and neoangiogenesis properties of a novel collagenated xenograft (GTO[®]), employing an *in vivo* preclinical model of zebrafish. The establishment of a robust blood vessel network in newly developing tissues is essential for facilitating optimal bone regeneration and ensuring the long-term stability of implants [29]. By elucidating the potential of the material to stimulate angiogenesis, this study provided valuable insights that may enhance its applicability and effectiveness in regenerative therapies. The zebrafish model is widely acknowledged by regulatory bodies such as the Food and Drug Administration (FDA), National Institutes of Health (NIH), and the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) as an important biotech platform for preclinical investigations concerning biomaterials and pharmaceuticals, owing to its high degree of genomic, molecular, physiological, immunological, and pharmacological similarity to humans. Moreover, it serves as a viable alternative to mammalian models for preclinical experimentation due to its cost-effectiveness, reduced timeframes, ease of maintenance, high reproductive capacity, rapid *ex utero* development, absence of ethical concerns, and the transparency of embryos [337]. The investigated collagenated bone graft material was evaluated both in its suspended form and as a supernatant extract in the culture water of *Danio rerio* embryos. Over a period of 72 to 120 hpf, the findings indicated that neither formulation of the tested biomaterial exhibited toxicological effects on the embryos or

triggered an inflammatory response, as corroborated by the quantification of fluorescent neutrophils. Furthermore, both formulations demonstrated a statistically significant increase in the percentage of regrowth area ($p < 0.01$) following caudal amputation and in the number of new SIVs emerging from the yolk region ($p < 0.05$ for the suspension and $p < 0.001$ for the supernatant) when compared to the untreated groups. Notably, in the latter case, no significant differences were detected between the two formulations, indicating that both the biomaterial in its original form and the released supernatant, derived from the extraction of the organic and inorganic components over a period of 3 days, were capable of exerting similar beneficial outcomes. Consequently, the *in vivo* preclinical data obtained from the exposure of zebrafish embryos to either the suspension or supernatant of the collagenated biomaterial demonstrated the complete biocompatibility of the product, along with its effective promotion of wound healing and new vessel formation.

The zebrafish model has shown promise in the field of dentistry, particularly for preclinical testing of dental implants and materials. A literature review indicated that much of the research in this domain focuses on genetics, craniofacial development, infectious diseases, chemical toxicity, and material safety, although the application of this model remains relatively infrequent [338]. For example, Radunovic et al. [339] demonstrated the biocompatibility and safety of graphene-oxide functionalized titanium surfaces on *Danio rerio* embryos. In a separate investigation of the same group, the angiogenic and non-toxic or immunomodulatory effects of newly synthesized calcium phosphate and acetylsalicylic acid-based cements were evaluated [340]. In this case, the study reported minimal or no inhibitory effects on the development of intersegmental vessels (ISVs) associated with the tested cements. This stands in contrast to the significant collagen-dependent enhancement observed in the formation of SIVs in the current investigation. Further research conducted by Zhu et al. [341] examined the effects of zinc ions released from novel zinc-modified titanium surfaces on angiogenesis and osteogenesis, revealing that both processes were enhanced in zebrafish embryos.

Notably, there is a paucity of studies addressing the application of the zebrafish model for the investigation of collagenated biomaterials used in dentistry. However, a limited number of investigations have explored the role of collagen in wound healing, tissue repair, and vessel formation within zebrafish models. Specifically, Xiong et al. [342] demonstrated that particular collagen peptide mixtures derived from fish promoted caudal wound healing and vessel proliferation while mitigating inflammatory responses. One specific peptide mixture not only facilitated repair and proliferation but also led to the

clearance of cellular debris and apoptotic cells within the cutaneous lesion. These findings appeared to be aligned with the enhancement of wound healing demonstrated in the current research. Additionally, a study by Senk and Djonov [343] corroborated our findings on the angiogenic potential of collagen, illustrating how collagen fibers provide guidance cues for capillary regrowth during the process of regenerative angiogenesis.

The capabilities of GTO® in facilitating vascularization and promoting tissue regeneration have been demonstrated in an *in vitro* study and in numerous clinical investigations, respectively. In the first case, the authors have confirmed enhanced secretion of VEGF and fibroblast growth factor 2 (FGF-2), as well as the recruitment, proliferation, and organization of endothelial cells [344]. In clinical studies, GTO® has shown an excellent rate of new bone formation, effective graft volume preservation, and the presence of healthy new bony tissue, ultimately leading to successful implant rehabilitation. Its adhesive properties make it particularly effective in treating peri-implant lesions and in horizontal augmentation procedures, such as two-wall defects resulting from crest resorption, as well as for socket preservation in cases with compromised buccal plates [330,331,345,346]. However, although the efficacy of this collagenated biomaterial in new bone formation has been extensively investigated, this research represents the first *in vivo* assessment of its angiogenic potential using a zebrafish model.

Overall, the present study provided significant insights into the role of collagenated xenograft GTO® in promoting vascularization, wound healing, and neoangiogenesis, utilizing a zebrafish model to evaluate its potential applications in oral regenerative therapies. While the findings indicated that both the material's suspension and supernatant forms support angiogenesis and demonstrate biocompatibility, several limitations merit consideration. Firstly, the reliance on the zebrafish model, though beneficial due to its physiological similarities to humans, may not fully replicate the complex interactions that occur in mammalian systems. This limitation raises concerns regarding the translational applicability of the results to human clinical settings, as the vasculature and immune responses in zebrafish can differ from those in larger mammals. Moreover, while the study demonstrated significant effects on vascularization and healing, the mechanisms underlying these processes remain only partially elucidated, and further investigation into specific pathways activated by the collagenated biomaterial is warranted. The absence of long-term follow-up in the zebrafish model also limited the ability to assess the durability of the observed outcomes—specifically, whether the angiogenic benefits translate to sustained improvements in tissue regeneration in more complex environments.

Additionally, while the study showed that both the suspended and supernatant forms of the biomaterial yield positive outcomes, it did not explore potential dosimetric effects or the precise concentrations of active components involved, which could influence the efficacy of the treatment. Lastly, the literature highlights a relative scarcity of studies directly linking collagenated biomaterials to effective angiogenesis and tissue regeneration in dental applications using the zebrafish model. While related research exists, the limited scope of comparison underscores the need for more comprehensive evaluations of collagen's effects in oral and maxillofacial contexts. Future studies should address these limitations by incorporating mammalian models to validate findings, exploring the mechanistic pathways involved in collagen-mediated angiogenesis, and considering the long-term effects on tissue integration and regeneration. However, while the current research laid a foundational understanding of the potential of GTO[®] in neovascularization, a thorough exploration of its biological mechanisms and the broader applicability of the findings will be critical for advancing its clinical use.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

This doctoral research project has thoroughly demonstrated the significant potential of various collagen-based biomaterials in advancing dental regenerative therapies. The studies have underscored collagen's intrinsic biocompatibility, osteoconductive properties, and ability to modulate critical biological processes such as cell adhesion, proliferation, differentiation, and vascularization. The development of collagen-coated dental implants and the investigation of innovative collagen-based scaffolds has shown promising results in enhancing osseointegration, soft tissue healing, and angiogenesis, paving the way for more predictable and sustainable outcomes in clinical settings. The findings emphasized the importance of tailoring biomaterial properties to mimic the hierarchical structure of natural ECM components, thus creating an optimal microenvironment for tissue regeneration. Moreover, the *in vitro*, *ex vivo*, and *in vivo* models used in this research have validated the feasibility and efficacy of the proposed biomaterials, offering significant insights into the interaction dynamics between the biomaterials and host tissues.

Building on the findings presented in this doctoral project, several promising avenues could be pursued for future exploration:

- Enhancing the N2 functionalization technique on implant surfaces could significantly improve the stability of the coating and the clinical outcomes related to dental implants;

- Further optimization of the N2 coating properties through the incorporation of bioactive molecules and antimicrobial agents may augment the osteogenic and antibiofilm properties of the collagen-coated implants, thereby reducing infection risks and improving the implants' resilience against bacterial colonization, as well as promoting their long-term stability and maintenance;

- It is imperative to evaluate the *in vitro* soft tissue adhesion and regeneration capabilities of the N2-coated surfaces in relation to the abutment section. This assessment will provide crucial insights into the capacity of the coated implants to integrate with soft tissues, a vital aspect influencing overall implant success;

- Following these *in vitro* assessments, subsequent animal studies should be conducted to rigorously evaluate the overall beneficial potential of collagen-coated implants. Specifically, these investigations will aid in establishing the efficacy of N2 in fostering new bone formation, soft tissue adhesion, and the long-term stability of the

implants in a living organism, thereby bridging the gap between laboratory findings and clinical applications;

- It is important to acknowledge that the biomechanical evaluations conducted during this project predominantly utilized *in vitro* bone models or numerical simulations. While these methodologies provided invaluable preliminary insights, they cannot completely replicate the complexities of *in vivo* environments. Therefore, additional validation through comprehensive *in vivo* studies is essential to confirm the biomechanical performance of the coated implants under actual physiological conditions. This progression towards *in vivo* analysis will be crucial in the development of an innovative implant surface coated with N2, specifically designed to enhance integration with periodontal tissues, ensure an effective antibacterial seal, and confirm biomechanical robustness.

In conclusion, while the current research establishes a strong foundation, the proposed future inquiries hold substantial promise for advancing the field of dental implant technology. By addressing the identified limitations and exploring the enhancement avenues outlined above, future work can lead to the development of superior collagen-coated implants that improve clinical outcomes, enhance patient satisfaction, and ensure long-term implant success. Ultimately, the integration of improved functionalization strategies and comprehensive *in vivo* evaluations will contribute to a new generation of dental implants that are not only effective but also tailored to meet the complex requirements of periodontal health and tissue integration. Through continued innovation and resolution of existing challenges, collagen-based biomaterials have the potential to redefine standards in both dental and regenerative therapies. This doctoral research may significantly contribute to the foundation of this endeavor, setting the stage for future advancements that emphasize both functional and aesthetic outcomes for patients.

6. REFERENCES

1. Ricard-Blum S. The collagen family. *Cold Spring Harb Perspect Biol.* 2011 Jan 1;3(1):a004978. doi: 10.1101/cshperspect.a004978.
2. Bielajew BJ, Hu JC, Athanasiou KA. Collagen: quantification, biomechanics, and role of minor subtypes in cartilage. *Nat Rev Mater.* 2020 Oct;5(10):730-747. doi: 10.1038/s41578-020-0213-1.
3. Elango J, Hou C, Bao B, Wang S, Maté Sánchez de Val JE, Wenhui W. The Molecular Interaction of Collagen with Cell Receptors for Biological Function. *Polymers (Basel).* 2022 Feb 23;14(5):876. doi: 10.3390/polym14050876.
4. D'souza Z, Chettiankandy TJ, Ahire MS, ThakurA, Sonawane SG, Sinha A. Collagen – structure, function and distribution in orodental tissues. *J Global Oral Health.* 2020 Feb;2(2):134-9, doi:10.25259/JGOH_4_2020.
5. Binlateh T, Thammanichanon P, Rittipakorn P, Thinsathid N, Jitprasertwong P. Collagen-Based Biomaterials in Periodontal Regeneration: Current Applications and Future Perspectives of Plant-Based Collagen. *Biomimetics (Basel).* 2022 Mar 24;7(2):34. doi: 10.3390/biomimetics7020034.
6. Singh D, Rai V, Agrawal DK. Regulation of Collagen I and Collagen III in Tissue Injury and Regeneration. *Cardiol Cardiovasc Med.* 2023 Jan;7(1):5-16. doi: 10.26502/fccm.92920302.
7. Iezzi G, Di Lillo F, Furlani M, Degidi M, Piattelli A, Giuliani A. The Symmetric 3D Organization of Connective Tissue around Implant Abutment: A Key-Issue to Prevent Bone Resorption. *Symmetry.* 2021 Apr;13(7):1126. doi: 10.3390/sym13071126.
8. Romanos GE, Schröter-Kermani C, Weingart D, Strub JR. Health human periodontal versus peri-implant gingival tissues: an immunohistochemical differentiation of the extracellular matrix. *Int J Oral Maxillofac Implants.* 1995 Nov-Dec;10(6):750-8. PMID: 8530179.
9. Faegh S, Chou HY, Muftu S. Load Transfer Along the Bone-Implant Interface and Its Effects on Bone Maintenance. *InTech* 2011 Aug; doi: 10.5772/18433.
10. Schierano G, Ramieri G, Cortese M, Aimetti M, Preti G. Organization of the connective tissue barrier around long-term loaded implant abutments in man. *Clin Oral Implants Res.* 2002 Oct;13(5):460-4. doi: 10.1034/j.1600-0501.2002.130503.x.
11. Quaranta A, Piattelli A, Scarano A, Quaranta M, Pompa G, Iezzi G. Light-microscopic evaluation of the dimensions of peri-implant mucosa around immediately loaded and submerged titanium implants in monkeys. *J Periodontol.* 2008 Sep;79(9):1697-703. doi: 10.1902/jop.2008.070631.
12. Traini T, Degidi M, Caputi S, Strocchi R, Di Iorio D, Piattelli A. Collagen fiber orientation in human peri-implant bone around immediately loaded and unloaded titanium dental implants. *J Periodontol.* 2005 Jan;76(1):83-9. doi: 10.1902/jop.2005.76.1.83
13. Traini T, Pecora G, Iezzi G, Piattelli A. Preferred collagen fiber orientation human peri-implant bone after a short- and long-term loading period: a case report. *J Oral Implantol.* 2006;32(4):177-81. doi: 10.1563/285.1.
14. Traini T, Degidi M, Strocchi R, Caputi S, Piattelli A. Collagen fiber orientation near dental implants in human bone: do their organization reflect differences in loading? *J Biomed Mater Res B Appl Biomater.* 2005 Jul;74(1):538-46. doi: 10.1002/jbm.b.30245.
15. Hossain N, Mobarak H, Islam A, Hossain A, Al Mahmud Z, Rayhan T, Chowdhury MA. Recent development of dental implant materials, synthesis process, and failure – A review. *Results in Chemistry.* 2023 Sep;6(11):101136. doi:

- 10.1016/j.rechem.2023.101136.
16. Pandey C, Rokaya D, Bhattarai BP. Contemporary Concepts in Osseointegration of Dental Implants: A Review. *Biomed Res Int.* 2022 Jun 14;2022:6170452. doi: 10.1155/2022/6170452.
 17. Sun TC, Chang TK. Soft tissue management around dental implant in esthetic zone - the current concepts and novel techniques. *J Dent Sci.* 2024 Jul;19(3):1348-1358. doi: 10.1016/j.jds.2024.03.003.
 18. Merin B, Aby MT, Riya S, Girish H. Recent Advances in Implant Biomaterials: A Comprehensive Review. *Kerala Dental Journal* 2024, 47, 99-102. https://doi.org/10.4103/KSDJ.KSDJ_33_24; Merin B, Aby MT, Riya S, Girish H. Recent Advances in Implant Biomaterials: A Comprehensive Review. *Kerala Dental Journal* 2024 Sep-Dec;47(3):99-102. doi: 10.4103/KSDJ.KSDJ_33_24.
 19. Accioni F, Vázquez J, Merinero M, Begines B, Alcudia A. Latest Trends in Surface Modification for Dental Implantology: Innovative Developments and Analytical Applications. *Pharmaceutics.* 2022 Feb 21;14(2):455. doi: 10.3390/pharmaceutics14020455.
 20. Rico-Llanos GA, Borrego-González S, Moncayo-Donoso M, Becerra J, Visser R. Collagen Type I Biomaterials as Scaffolds for Bone Tissue Engineering. *Polymers (Basel).* 2021 Feb 17;13(4):599. doi: 10.3390/polym13040599.
 21. Amirrah IN, Lokanathan Y, Zulkiflee I, Wee MFMR, Motta A, Fauzi MB. A Comprehensive Review on Collagen Type I Development of Biomaterials for Tissue Engineering: From Biosynthesis to Bioscaffold. *Biomedicines.* 2022 Sep 16;10(9):2307. doi: 10.3390/biomedicines10092307.
 22. Wosicka-Frąckowiak H, Poniedziałek K, Woźny S, Kuprianowicz M, Nyga M, Jadach B, Milanowski B. Collagen and Its Derivatives Serving Biomedical Purposes: A Review. *Polymers.* 2024; 16(18):2668. doi:10.3390/polym16182668.
 23. Chen FM, Liu X. Advancing biomaterials of human origin for tissue engineering. *Prog Polym Sci.* 2016 Feb 1;53:86-168. doi: 10.1016/j.progpolymsci.2015.02.004.
 24. Patil VA, Masters KS. Engineered Collagen Matrices. *Bioengineering (Basel).* 2020 Dec 16;7(4):163. doi: 10.3390/bioengineering7040163.
 25. Salvatore L, Gallo N, Natali ML, Terzi A, Sannino A, Madaghiele M. Mimicking the Hierarchical Organization of Natural Collagen: Toward the Development of Ideal Scaffolding Material for Tissue Regeneration. *Front Bioeng Biotechnol.* 2021 Apr 27;9:644595. doi: 10.3389/fbioe.2021.644595.
 26. Casale C, Imperato G, Mazio C, Netti PA, Urciuolo F. Geometrical confinement controls cell, ECM and vascular network alignment during the morphogenesis of 3D bioengineered human connective tissues. *Acta Biomater.* 2021 Sep 1;131:341-354. doi: 10.1016/j.actbio.2021.06.022.
 27. Liu J, Hua F, Zhang H, Hu J. Influence of using collagen on the soft and hard tissue outcomes of immediate dental implant placement: A systematic review and meta-analysis. *J Stomatol Oral Maxillofac Surg.* 2023 Feb;124(1S):101385. doi: 10.1016/j.jormas.2023.101385.
 28. Ru L, Pan B, Zheng J. Signalling pathways in the osteogenic differentiation of periodontal ligament stem cells. *Open Life Sci.* 2023 Sep 11;18(1):20220706. doi: 10.1515/biol-2022-0706.
 29. Grosso A, Burger MG, Lunger A, Schaefer DJ, Banfi A, Di Maggio N. It Takes Two to Tango: Coupling of Angiogenesis and Osteogenesis for Bone Regeneration. *Front Bioeng Biotechnol.* 2017 Nov 3;5:68. doi: 10.3389/fbioe.2017.00068.
 30. Patino MG, Neiders ME, Andreana S, Noble B, Cohen RE. Collagen as an implantable material in medicine and dentistry. *J Oral Implantol.* 2002;28(5):220-5. doi:

- 10.1563/1548-1336(2002)028<0220:CAAIMI>2.3.CO;2.
31. Zimmermann G, Moghaddam A. Allograft bone matrix versus synthetic bone graft substitutes. *Injury*. 2011 Sep;42 Suppl 2:S16-21. doi: 10.1016/j.injury.2011.06.199.
 32. Zhao R, Yang R, Cooper PR, Khurshid Z, Shavandi A, Ratnayake J. Bone Grafts and Substitutes in Dentistry: A Review of Current Trends and Developments. *Molecules*. 2021 May 18;26(10):3007. doi: 10.3390/molecules26103007.
 33. Li Y, Liu Y, Li R, Bai H, Zhu Z, Zhu L, Zhu C, Che Z, Liu H, Wang J, Huang L. Collagen-based biomaterials for bone tissue engineering. *Materials & Design*. 2021 Nov; 210:110049. doi: 10.1016/j.matdes.2021.110049.
 34. Tahmasebi E, Alam M, Yazdani M, Tebyanian H, Yazdani A, Seifalian A, Mosaddad SA. Current biocompatible materials in oral regeneration: a comprehensive overview of composite materials. *J Mat Res Tech*. 2020 Se-Oct;9(5):11731-11755. doi: 10.1016/j.jmrt.2020.08.042.
 35. Salvatore L, Natali ML, Brunetti C, Sannino A, Gallo N. An Update on the Clinical Efficacy and Safety of Collagen Injectables for Aesthetic and Regenerative Medicine Applications. *Polymers (Basel)*. 2023 Feb 17;15(4):1020. doi: 10.3390/polym15041020.
 36. Gill S, Prakash M, Forghany M, Vaderhobli RM. An ethical perspective to using bone grafts in dentistry. *J Am Dent Assoc*. 2022 Jan;153(1):88-91. doi: 10.1016/j.adaj.2021.09.011.
 37. Fan L, Ren Y, Emmert S, Vučković I, Stojanovic S, Najman S, Schnettler R, Barbeck M, Schenke-Layland K, Xiong X. The Use of Collagen-Based Materials in Bone Tissue Engineering. *Int J Mol Sci*. 2023 Feb 13;24(4):3744. doi: 10.3390/ijms24043744.
 38. Guo X, Ma Y, Wang H, Yin H, Shi X, Chen Y, Gao G, Sun L, Wang J, Wang Y, Fan D. Status and developmental trends in recombinant collagen preparation technology. *Regen Biomater*. 2023 Nov 29;11:rbad106. doi: 10.1093/rb/rbad106.
 39. Davison-Kotler E, Marshall WS, García-Gareta E. Sources of Collagen for Biomaterials in Skin Wound Healing. *Bioengineering (Basel)*. 2019 Jun 30;6(3):56. doi: 10.3390/bioengineering6030056.
 40. Silvipriya KS, Kumar K, Bhat A, Kumar B, John A, Lakshmanan P. Collagen: animal sources and biomedical application. *J Appl Pharm Sci*. 2015;5:123–127. doi: 10.7324/japs.2015.50322.
 41. Avila Rodríguez MI, Rodríguez Barroso LG, Sánchez ML. Collagen: A review on its sources and potential cosmetic applications. *J Cosmet Dermatol*. 2018 Feb;17(1):20-26. doi: 10.1111/jocd.12450.
 42. Salvatore L, Gallo N, Natali ML, Campa L, Lunetti P, Madaghiele M, Blasi FS, Corallo A, Capobianco L, Sannino A. Marine collagen and its derivatives: Versatile and sustainable bio-resources for healthcare. *Mater Sci Eng C Mater Biol Appl*. 2020 Aug;113:110963. doi: 10.1016/j.msec.2020.110963.
 43. Salvatore L, Gallo N, Aiello D, Lunetti P, Barca A, Blasi L, Madaghiele M, Bettini S, Giancane G, Hasan M, Borovkov V, Natali ML, Campa L, Valli L, Capobianco L, Napoli A, Sannino A. An insight on type I collagen from horse tendon for the manufacture of implantable devices. *Int J Biol Macromol*. 2020 Jul 1;154:291-306. doi: 10.1016/j.ijbiomac.2020.03.082.
 44. Gallo N, Natali ML, Sannino A, Salvatore L. An Overview of the Use of Equine Collagen as Emerging Material for Biomedical Applications. *J Funct Biomater*. 2020 Nov 1;11(4):79. doi: 10.3390/jfb11040079.
 45. Meyer M. Processing of collagen based biomaterials and the resulting materials properties. *BioMed Eng OnLine*. 2019 Mar;18:24. doi: 10.1186/s12938-019-0647-0.

46. Mandatori D, D'Amico E, Romasco T, Gatto ML, Notarangelo MP, Mangano C, Furlani M, Penolazzi L. A 3D in vitro model of biphasic calcium phosphate (BCP) scaffold combined with human osteoblasts, osteoclasts, and endothelial cells as a platform to mimic the oral microenvironment for tissue regeneration. *J Dent*. 2024 Dec;151:105411. doi: 10.1016/j.jdent.2024.105411.
47. Kämmerer PW, Scholz M, Baudisch M, Liese J, Wegner K, Frerich B, Lang H. Guided Bone Regeneration Using Collagen Scaffolds, Growth Factors, and Periodontal Ligament Stem Cells for Treatment of Peri-Implant Bone Defects In Vivo. *Stem Cells Int*. 2017;2017:3548435. doi: 10.1155/2017/3548435.
48. Yang Z, Wu C, Shi H, Luo X, Sun H, Wang Q, Zhang D. Advances in Barrier Membranes for Guided Bone Regeneration Techniques. *Front Bioeng Biotechnol*. 2022 Jun 22;10:921576. doi: 10.3389/fbioe.2022.921576.
49. Parvin N, Kumar V, Joo SW, Mandal TK. Cutting-Edge Hydrogel Technologies in Tissue Engineering and Biosensing: An Updated Review. *Materials (Basel)*. 2024 Sep 29;17(19):4792. doi: 10.3390/ma17194792.
50. Alqahtani AM. Guided Tissue and Bone Regeneration Membranes: A Review of Biomaterials and Techniques for Periodontal Treatments. *Polymers (Basel)*. 2023 Aug 10;15(16):3355. doi: 10.3390/polym15163355.
51. Romasco T, Tumedei M, Inchingolo F, Pignatelli P, Montesani L, Iezzi G, Petrini M, Piattelli A, Di Pietro N. A Narrative Review on the Effectiveness of Bone Regeneration Procedures with OsteoBiol® Collagenated Porcine Grafts: The Translational Research Experience over 20 Years. *J Funct Biomater*. 2022 Aug 18;13(3):121. doi: 10.3390/jfb13030121.
52. Zhu J, Li Z, Zou Y, Lu G, Ronca A, D'Amora U, Liang J, Fan Y, Zhang Z, Sun Y. Advanced application of collagen-based biomaterials in tissue repair and restoration. *J Leather Sci Eng*. 2022 Nov;4:30. doi: 10.1186/s42825-022-00102-6.
53. el Blidi O, el Omari N, Balahbib A, Ghchime R, el Menyiy N, Ibrahimi A, Kaddour K, Bouyahya A, Chokairi O, Barkiyou M. Extraction Methods, Characterization and Biomedical Applications of Collagen: A Review. *Biointerface Res. Appl. Chem* 2021;11:13587–13613. doi: 10.33263/BRIAC115.1358713613.
54. Terzi A, Gallo N, Bettini S, Sibillano T, Altamura D, Madaghiele M, De Caro L, Valli L, Salvatore L, Sannino A, Giannini C. Sub- and Supramolecular X-Ray Characterization of Engineered Tissues from Equine Tendon, Bovine Dermis, and Fish Skin Type-I Collagen. *Macromol Biosci*. 2020 May;20(5):e2000017. doi: 10.1002/mabi.202000017.
55. Salvatore L, Russo F, Natali ML, Rajabimashhadi Z, Bagheri S, Mele C, Lionetto F, Sannino A, Gallo N. On the effect of pepsin incubation on type I collagen from horse tendon: Fine tuning of its physico-chemical and rheological properties. *Int J Biol Macromol*. 2024 Jan;256(Pt 2):128489. doi: 10.1016/j.ijbiomac.2023.128489.
56. Romasco T, Mandrillo PM, Morsut E, Tumedei M, Mandatori D, Petrini M, Curia MC, De Angelis F, D'Arcangelo C, Piattelli A, Di Pietro N. Morpho-Functional Effect of a New Collagen-Based Medical Device on Human Gingival Fibroblasts: An In Vitro Study. *Biomedicines*. 2023 Mar 5;11(3):786. doi: 10.3390/biomedicines11030786.
57. Brito C, Tenenbaum HC, Wong BK, Schmitt C, Nogueira-Filho G. Is keratinized mucosa indispensable to maintain peri-implant health? A systematic review of the literature. *J Biomed Mater Res B Appl Biomater*. 2014 Apr;102(3):643-50. doi: 10.1002/jbm.b.33042.
58. Toma AI, Fuller JM, Willett NJ, Goudy SL. Oral wound healing models and emerging regenerative therapies. *Transl Res*. 2021 Oct;236:17-34. doi: 10.1016/j.trsl.2021.06.003.

59. Diller RB, Tabor AJ. The Role of the Extracellular Matrix (ECM) in Wound Healing: A Review. *Biomimetics* (Basel). 2022 Jul 1;7(3):87. doi: 10.3390/biomimetics7030087.
60. desJardins-Park HE, Foster DS, Longaker MT. Fibroblasts and wound healing: an update. *Regen Med*. 2018 Jul 1;13(5):491-495. doi: 10.2217/rme-2018-0073.
61. Rajshankar D, Wang B, Worndl E, Menezes S, Wang Y, McCulloch CA. Focal adhesion kinase regulates tractional collagen remodeling, matrix metalloproteinase expression, and collagen structure, which in turn affects matrix-induced signaling. *J Cell Physiol*. 2020 Mar;235(3):3096-3111. doi: 10.1002/jcp.29215.
62. Piccolo S, Dupont S, Cordenonsi M. The biology of YAP/TAZ: hippo signaling and beyond. *Physiol Rev*. 2014 Oct;94(4):1287-312. doi: 10.1152/physrev.00005.2014.
63. Rajshankar D, Wang Y, McCulloch CA. Osteogenesis requires FAK-dependent collagen synthesis by fibroblasts and osteoblasts. *FASEB J*. 2017 Mar;31(3):937-953. doi: 10.1096/fj.201600645R.
64. Sharma S, Rai VK, Narang RK, Markandeywar TS. Collagen-based formulations for wound healing: A literature review. *Life Sci*. 2022 Feb 1;290:120096. doi: 10.1016/j.lfs.2021.120096. Epub 2021 Oct 26. Erratum in: *Life Sci*. 2022 May 15;297:120436. doi: 10.1016/j.lfs.2022.120436.
65. Alfonso García SL, Parada-Sanchez MT, Arboleda Toro D. The phenotype of gingival fibroblasts and their potential use in advanced therapies. *Eur J Cell Biol*. 2020 Sep;99(7):151123. doi: 10.1016/j.ejcb.2020.151123.
66. Randelli F, Menon A, Giaì Via A, Mazzoleni MG, Sciancalepore F, Brioschi M, Gagliano N. Effect of a Collagen-Based Compound on Morpho-Functional Properties of Cultured Human Tenocytes. *Cells*. 2018 Dec 6;7(12):246. doi: 10.3390/cells7120246.
67. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc*. 2007;2(2):329-33. doi: 10.1038/nprot.2007.30.
68. Jonkman JE, Cathcart JA, Xu F, Bartolini ME, Amon JE, Stevens KM, Colarusso P. An introduction to the wound healing assay using live-cell microscopy. *Cell Adh Migr*. 2014;8(5):440-51. doi: 10.4161/cam.36224.
69. De Angelis F, Mandatori D, Schiavone V, Melito FP, Valentinuzzi S, Vadini M, Di Tomo P, Vanini L, Pelusi L, Pipino C, Del Boccio P, D'Arcangelo C, Pandolfi A. Cytotoxic and Genotoxic Effects of Composite Resins on Cultured Human Gingival Fibroblasts. *Materials* (Basel). 2021 Sep 11;14(18):5225. doi: 10.3390/ma14185225.
70. Ramshaw JA. Biomedical applications of collagens. *J Biomed Mater Res B Appl Biomater*. 2016 May;104(4):665-75. doi: 10.1002/jbm.b.33541.
71. Randelli F, Sartori P, Carlomagno C, Bedoni M, Menon A, Vezzoli E, Sommariva M, Gagliano N. The Collagen-Based Medical Device MD-Tissue Acts as a Mechanical Scaffold Influencing Morpho-Functional Properties of Cultured Human Tenocytes. *Cells*. 2020 Dec 8;9(12):2641. doi: 10.3390/cells9122641.
72. Hashimoto K, Yamashita K, Enoyoshi K, Dahan X, Takeuchi T, Kori H, Gotoh M. The effects of coating culture dishes with collagen on fibroblast cell shape and swirling pattern formation. *J Biol Phys*. 2020 Dec;46(4):351-369. doi: 10.1007/s10867-020-09556-3.
73. Rhee S, Grinnell F. Fibroblast mechanics in 3D collagen matrices. *Adv Drug Deliv Rev*. 2007 Nov 10;59(13):1299-305. doi: 10.1016/j.addr.2007.08.006.
74. Talebi Ardakani MR, Hajizadeh F, Yadegari Z. Comparison of Attachment and Proliferation of Human Gingival Fibroblasts on Different Collagen Membranes. *Ann Maxillofac Surg*. 2018 Jul-Dec;8(2):218-223. doi: 10.4103/ams.ams_150_17.

75. Fujioka-Kobayashi M, Ülgür II, Katagiri H, Vuignier S, Schaller B. In vitro observation of macrophage polarization and gingival fibroblast behavior on three-dimensional xenogeneic collagen matrixes. *J Biomed Mater Res A*. 2020 Jun;108(6):1408-1418. doi: 10.1002/jbm.a.36911.
76. Gajbhiye S, Wairkar S. Collagen fabricated delivery systems for wound healing: A new roadmap. *Biomater Adv*. 2022 Nov;142:213152. doi: 10.1016/j.bioadv.2022.213152.
77. Smith PC, Martínez C, Martínez J, McCulloch CA. Role of Fibroblast Populations in Periodontal Wound Healing and Tissue Remodeling. *Front Physiol*. 2019 Apr 24;10:270. doi: 10.3389/fphys.2019.00270.
78. Kuehlmann B, Bonham CA, Zucal I, Prantl L, Gurtner GC. Mechanotransduction in Wound Healing and Fibrosis. *J Clin Med*. 2020 May 11;9(5):1423. doi: 10.3390/jcm9051423.
79. Dieterle MP, Husari A, Steinberg T, Wang X, Ramminger I, Tomakidi P. From the Matrix to the Nucleus and Back: Mechanobiology in the Light of Health, Pathologies, and Regeneration of Oral Periodontal Tissues. *Biomolecules*. 2021 May 31;11(6):824. doi: 10.3390/biom11060824.
80. Ilić D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*. 1995 Oct 12;377(6549):539-44. doi: 10.1038/377539a0.
81. Klingberg F, Chow ML, Koehler A, Boo S, Buscemi L, Quinn TM, Costell M, Alman BA, Genot E, Hinz B. Prestress in the extracellular matrix sensitizes latent TGF- β 1 for activation. *J Cell Biol*. 2014 Oct 27;207(2):283-97. doi: 10.1083/jcb.201402006.
82. Chen J, Cheng J, Zhao C, Zhao B, Mi J, Li W. The Hippo pathway: a renewed insight in the craniofacial diseases and hard tissue remodeling. *Int J Biol Sci*. 2021 Sep 24;17(14):4060-4072. doi: 10.7150/ijbs.63305.
83. Umezaki Y, Hashimoto Y, Nishishita N, Kawamata S, Baba S. Human Gingival Integration-Free iPSCs; a Source for MSC-Like Cells. *Int J Mol Sci*. 2015 Jun 15;16(6):13633-48. doi: 10.3390/ijms160613633.
84. Corrado B, Bonini I, Alessio Chirico V, Rosano N, Gisonni P. Use of injectable collagen in partial-thickness tears of the supraspinatus tendon: a case report. *Oxf Med Case Reports*. 2020 Nov 24;2020(11):omaa103. doi: 10.1093/omcr/omaa103.
85. Martin LS, Massafra U, Bizzi E, Migliore A. A double blind randomized active-controlled clinical trial on the intra-articular use of Md-Knee versus sodium hyaluronate in patients with knee osteoarthritis ("Joint"). *BMC Musculoskelet Disord*. 2016 Feb 22;17:94. doi: 10.1186/s12891-016-0948-4.
86. Perussolo J, Donos N. Maintenance of peri-implant health in general dental practice. *Br Dent J*. 2024 May;236(10):781-789. doi: 10.1038/s41415-024-7406-8.
87. Alva H, Prasad KD, Prasad AD. Bioseal: The physiological and biological barrier for osseointegrated supported prosthesis. *J Dent Implants* 2013 Jul-Dec;3(2):148-152. doi: 10.4103/0974-6781.118855.
88. Silva RCS, Agrelli A, Andrade AN, Mendes-Marques CL, Arruda IRS, Santos LRL, Vasconcelos NF, Machado G. Titanium Dental Implants: An Overview of Applied Nanobiotechnology to Improve Biocompatibility and Prevent Infections. *Materials (Basel)*. 2022 Apr 27;15(9):3150. doi: 10.3390/ma15093150.
89. Covani U, Giammarinaro E, Di Pietro N, Boncompagni S, Rastelli G, Romasco T, Velasco-Ortega E, Jimenez-Guerra A, Iezzi G, Piattelli A, Marconcini S. Electron Microscopy (EM) Analysis of Collagen Fibers in the Peri-Implant Soft Tissues around Two Different Abutments. *J Funct Biomater*. 2023 Aug 29;14(9):445. doi:

- 10.3390/jfb14090445.
90. Wang Y, Zhang Y, Miron RJ. Health, Maintenance, and Recovery of Soft Tissues around Implants. *Clin Implant Dent Relat Res*. 2016 Jun;18(3):618-34. doi: 10.1111/cid.12343.
 91. Atsuta I, Ayukawa Y, Kondo R, Oshiro W, Matsuura Y, Furuhashi A, Tsukiyama Y, Koyano K. Soft tissue sealing around dental implants based on histological interpretation. *J Prosthodont Res*. 2016 Jan;60(1):3-11. doi: 10.1016/j.jpor.2015.07.001.
 92. Pesce P, Menini M, Tommasato G, Patini R, Canullo L. Influence of modified titanium abutment surface on peri-implant soft tissue behaviour: A systematic review of histological findings. *Int J Oral Implantol (Berl)*. 2019;12(4):419-429. PMID: 31781697.
 93. Roehling S, Schlegel KA, Woelfler H, Gahlert M. Zirconia compared to titanium dental implants in preclinical studies-A systematic review and meta-analysis. *Clin Oral Implants Res*. 2019 May;30(5):365-395. doi: 10.1111/clr.13425.
 94. Blázquez-Hinarejos M, Ayuso-Montero R, Jané-Salas E, López-López J. Influence of surface modified dental implant abutments on connective tissue attachment: A systematic review. *Arch Oral Biol*. 2017 Aug;80:185-192. doi: 10.1016/j.archoralbio.2017.04.020.
 95. Mattheos N, Vergoullis I, Janda M, Miseli A. The Implant Supracrestal Complex and Its Significance for Long-Term Successful Clinical Outcomes. *Int J Prosthodont*. 2021 Jan-Feb;34(1):88-100. doi: 10.11607/ijp.7201.
 96. Nelson CM. Geometric control of tissue morphogenesis. *Biochim Biophys Acta*. 2009 May;1793(5):903-10. doi: 10.1016/j.bbamcr.2008.12.014.
 97. Delgado-Ruiz RA, Calvo-Guirado JL, Abboud M, Ramirez-Fernandez MP, Maté-Sánchez de Val JE, Negri B, Gomez-Moreno G, Markovic A. Connective Tissue Characteristics around Healing Abutments of Different Geometries: New Methodological Technique under Circularly Polarized Light. *Clin Implant Dent Relat Res*. 2015 Aug;17(4):667-80. doi: 10.1111/cid.12161.
 98. Kim S, Oh KC, Han DH, Heo SJ, Ryu IC, Kwon JH, Han CH. Influence of transmucosal designs of three one-piece implant systems on early tissue responses: a histometric study in beagle dogs. *Int J Oral Maxillofac Implants*. 2010 Mar-Apr;25(2):309-14. PMID: 20369089.
 99. Bolle C, Gustin MP, Fau D, Exbrayat P, Boivin G, Grosgeat B. Early Periimplant Tissue Healing on 1-Piece Implants With a Concave Transmucosal Design: A Histomorphometric Study in Dogs. *Implant Dent*. 2015 Oct;24(5):598-606. doi: 10.1097/ID.0000000000000301.
 100. Valente NA, Wu M, Toti P, Derchi G, Barone A. Impact of Concave/Convergent vs Parallel/ Divergent Implant Transmucosal Profiles on Hard and Soft Peri-implant Tissues: A Systematic Review with Meta-Analyses. *Int J Prosthodont*. 2020 Sep/Oct;33(5):553-564. doi: 10.11607/ijp.6726.
 101. Rompen E, Raepsaet N, Domken O, Touati B, Van Dooren E. Soft tissue stability at the facial aspect of gingivally converging abutments in the esthetic zone: a pilot clinical study. *J Prosthet Dent*. 2007 Jun;97(6 Suppl):S119-25. doi: 10.1016/S0022-3913(07)60015-8. Erratum in: *J Prosthet Dent*. 2008 Mar;99(3):167. PMID: 17618926.
 102. Huh JB, Rhee GB, Kim YS, Jeong CM, Lee JY, Shin SW. Influence of Implant transmucosal design on early peri-implant tissue responses in beagle dogs. *Clin Oral Implants Res*. 2014 Aug;25(8):962-8. doi: 10.1111/clr.12179.
 103. Chien HH, Schroering RL, Prasad HS, Tatakis DN. Effects of a new implant abutment

- design on peri-implant soft tissues. *J Oral Implantol.* 2014 Oct;40(5):581-8. doi: 10.1563/AAID-JOI-D-12-00313.
104. Scarano A, Piattelli M, Vrespa G, Caputi S, Piattelli A. Bacterial adhesion on titanium nitride-coated and uncoated implants: an in vivo human study. *J Oral Implantol.* 2003;29(2):80-5. doi: 10.1563/1548-1336(2003)029<0080:BAOTNA>2.3.CO;2.
 105. Petrini M, Pierfelice TV, D'Amico E, Di Pietro N, Pandolfi A, D'Arcangelo C, De Angelis F, Mandatori D, Schiavone V, Piattelli A, Iezzi G. Influence of Nano, Micro, and Macro Topography of Dental Implant Surfaces on Human Gingival Fibroblasts. *Int J Mol Sci.* 2021 Sep 13;22(18):9871. doi: 10.3390/ijms22189871.
 106. Bacakova L, Filova E, Parizek M, Ruml T, Svorcik V. Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants. *Biotechnol Adv.* 2011 Nov-Dec;29(6):739-67. doi: 10.1016/j.biotechadv.2011.06.004.
 107. Stanford CM. Surface modification of biomedical and dental implants and the processes of inflammation, wound healing and bone formation. *Int J Mol Sci.* 2010 Jan 25;11(1):354-69. doi: 10.3390/ijms11010354.
 108. Cruz MB, Silva N, Marques JF, Mata A, Silva FS, Caramês J. Biomimetic Implant Surfaces and Their Role in Biological Integration-A Concise Review. *Biomimetics (Basel).* 2022 Jun 6;7(2):74. doi: 10.3390/biomimetics7020074.
 109. Sterzenbach T, Helbig R, Hannig C, Hannig M. Bioadhesion in the oral cavity and approaches for biofilm management by surface modifications. *Clin Oral Investig.* 2020 Dec;24(12):4237-4260. doi: 10.1007/s00784-020-03646-1.
 110. Reimers SNM, Es-Souni M, Şen S. Investigating adhesion of primary human gingival fibroblasts and osteoblasts to orthodontic mini-implants by scanning electron microscopy. *Sci Rep.* 2024 Jul 30;14(1):17475. doi: 10.1038/s41598-024-68486-5.
 111. Webb K, Hlady V, Tresco PA. Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblast attachment, spreading, and cytoskeletal organization. *J Biomed Mater Res.* 1998 Sep 5;41(3):422-30. doi: 10.1002/(sici)1097-4636(19980905)41:3<422::aid-jbm12>3.0.co;2-k.
 112. Osman MA, Alamoush RA, Kushnerev E, Seymour KG, Watts DC, Yates JM. Biological response of epithelial and connective tissue cells to titanium surfaces with different ranges of roughness: An in-vitro study. *Dent Mater.* 2022 Nov;38(11):1777-1788. doi: 10.1016/j.dental.2022.09.010.
 113. Yildiz H, Sen E, Dalcik H, Meseli SE. Evaluation of cell morphology and adhesion capacity of human gingival fibroblasts on titanium discs with different roughened surfaces: an in vitro scanning electron microscope analysis and cell culture study. *Folia Morphol (Warsz).* 2023;82(1):63-71. doi: 10.5603/FM.a2022.0072.
 114. Kim YS, Ko Y, Kye SB, Yang SM. Human gingival fibroblast (HGF-1) attachment and proliferation on several abutment materials with various colors. *Int J Oral Maxillofac Implants.* 2014 Jul-Aug;29(4):969-75. doi: 10.11607/jomi.3704.
 115. Nardone G, Oliver-De La Cruz J, Vrbsky J, Martini C, Pribyl J, Skládal P, Pešl M, Caluori G, Pagliari S, Martino F, Maceckova Z, Hajduch M, Sanz-Garcia A, Pugno NM, Stokin GB, Forte G. YAP regulates cell mechanics by controlling focal adhesion assembly. *Nat Commun.* 2017 May 15;8:15321. doi: 10.1038/ncomms15321.
 116. Yamano S, Ma AK, Shanti RM, Kim SW, Wada K, Sukotjo C. The influence of different implant materials on human gingival fibroblast morphology, proliferation, and gene expression. *Int J Oral Maxillofac Implants.* 2011 Nov-Dec;26(6):1247-55. PMID: 22167430.
 117. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M, Garner P, Holgate ST, Howells DW, Karp NA, Lazic SE, Lidster K, MacCallum CJ, Macleod M, Pearl EJ, Petersen OH, Rawle F,

- Reynolds P, Rooney K, Sena ES, Silberberg SD, Steckler T, Würbel H. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biol.* 2020 Jul 14;18(7):e3000410. doi: 10.1371/journal.pbio.3000410.
118. Boncompagni S, Rossi AE, Micaroni M, Beznoussenko GV, Polishchuk RS, Dirksen RT, Protasi F. Mitochondria are linked to calcium stores in striated muscle by developmentally regulated tethering structures. *Mol Biol Cell.* 2009 Feb;20(3):1058-67. doi: 10.1091/mbc.e08-07-0783.
 119. Berglundh T, Lindhe J. Dimension of the periimplant mucosa. Biological width revisited. *J Clin Periodontol.* 1996 Oct;23(10):971-3. doi: 10.1111/j.1600-051x.1996.tb00520.x.
 120. Abrahamsson I, Berglundh T, Moon IS, Lindhe J. Peri-implant tissues at submerged and non-submerged titanium implants. *J Clin Periodontol.* 1999 Sep;26(9):600-7. doi: 10.1034/j.1600-051x.1999.260907.x.
 121. Abrahamsson I, Berglundh T, Wennström J, Lindhe J. The peri-implant hard and soft tissues at different implant systems. A comparative study in the dog. *Clin Oral Implants Res.* 1996 Sep;7(3):212-9. doi: 10.1034/j.1600-0501.1996.070303.x.
 122. Romanos GE, Traini T, Johansson CB, Piattelli A. Biologic width and morphologic characteristics of soft tissues around immediately loaded implants: studies performed on human autopsy specimens. *J Periodontol.* 2010 Jan;81(1):70-8. doi: 10.1902/jop.2009.090364.
 123. Ruggeri A, Franchi M, Marini N, Trisi P, Piattelli A. Supracrestal circular collagen fiber network around osseointegrated nonsubmerged titanium implants. *Clin Oral Implants Res.* 1992 Dec;3(4):169-75. doi: 10.1034/j.1600-0501.1992.030403.x.
 124. Bolle C, Gustin MP, Fau D, Boivin G, Exbrayat P, Grosogoeat B. Soft Tissue and Marginal Bone Adaptation on Platform-Switched Implants with a Morse Cone Connection: A Histomorphometric Study in Dogs. *Int J Periodontics Restorative Dent.* 2016 Mar-Apr;36(2):221-8. doi: 10.11607/prd.2254.
 125. Tetè S, Mastrangelo F, Bianchi A, Zizzari V, Scarano A. Collagen fiber orientation around machined titanium and zirconia dental implant necks: an animal study. *Int J Oral Maxillofac Implants.* 2009 Jan-Feb;24(1):52-8. PMID: 19344025.
 126. Mangano C, Piattelli A, Scarano A, Raspanti M, Shibli JA, Mangano FG, Perrotti V, Iezzi G. A light and scanning electron microscopy study of human direct laser metal forming dental implants. *Int J Periodontics Restorative Dent.* 2014 Jan-Feb;34(1):e9-17. doi: 10.11607/prd.1213.
 127. Buehler MJ. Nature designs tough collagen: explaining the nanostructure of collagen fibrils. *Proc Natl Acad Sci U S A.* 2006 Aug 15;103(33):12285-90. doi: 10.1073/pnas.0603216103.
 128. Rodríguez X, Vela X, Calvo-Guirado JL, Nart J, Stappert CF. Effect of platform switching on collagen fiber orientation and bone resorption around dental implants: a preliminary histologic animal study. *Int J Oral Maxillofac Implants.* 2012 Sep-Oct;27(5):1116-22. PMID: 23057024.
 129. Rodríguez X, Navajas A, Vela X, Fortuño A, Jimenez J, Nevins M. Arrangement of Peri-implant Connective Tissue Fibers Around Platform-Switching Implants with Conical Abutments and Its Relationship to the Underlying Bone: A Human Histologic Study. *Int J Periodontics Restorative Dent.* 2016 Jul-Aug;36(4):533-40. doi: 10.11607/prd.2580.
 130. Lin J, Shi Y, Men Y, Wang X, Ye J, Zhang C. Mechanical Roles in Formation of Oriented Collagen Fibers. *Tissue Eng Part B Rev.* 2020 Apr;26(2):116-128. doi: 10.1089/ten.TEB.2019.0243.
 131. Sun B. The mechanics of fibrillar collagen extracellular matrix. *Cell Rep Phys Sci.*

- 2021 Aug 18;2(8):100515. doi: 10.1016/j.xcrp.2021.100515.
132. Birk DE, Nurminskaya MV, Zycband EI. Collagen fibrillogenesis in situ: fibril segments undergo post-depositional modifications resulting in linear and lateral growth during matrix development. *Dev Dyn*. 1995 Mar;202(3):229-43. doi: 10.1002/aja.1002020303.
 133. Yang G, Mahadik B, Choi JY, Fisher JP. Vascularization in tissue engineering: fundamentals and state-of-art. *Prog Biomed Eng (Bristol)*. 2020 Jan;2(1):012002. doi: 10.1088/2516-1091/ab5637.
 134. Saeidi N, Karmelek KP, Paten JA, Zareian R, DiMasi E, Ruberti JW. Molecular crowding of collagen: a pathway to produce highly-organized collagenous structures. *Biomaterials*. 2012 Oct;33(30):7366-74. doi: 10.1016/j.biomaterials.2012.06.041.
 135. Alamoudi A. Nanoengineering and Surface Modifications of Dental Implants. *Cureus*. 2024 Jan 2;16(1):e51526. doi: 10.7759/cureus.51526.
 136. Giner L, Mercadé M, Torrent S, Punset M, Pérez RA, Delgado LM, Gil FJ. Double acid etching treatment of dental implants for enhanced biological properties. *J Appl Biomater Funct Mater*. 2018 Apr;16(2):83-89. doi: 10.5301/jabfm.5000376.
 137. Grassi S, Piattelli A, Ferrari DS, Figueiredo LC, Feres M, Iezzi G, Shibli JA. Histologic evaluation of human bone integration on machined and sandblasted acid-etched titanium surfaces in type IV bone. *J Oral Implantol*. 2007;33(1):8-12. doi: 10.1563/0-791.1.
 138. Cervino G, Fiorillo L, Iannello G, Santonocito D, Risitano G, Cicciù M. Sandblasted and Acid Etched Titanium Dental Implant Surfaces Systematic Review and Confocal Microscopy Evaluation. *Materials (Basel)*. 2019 May 30;12(11):1763. doi: 10.3390/ma12111763.
 139. Narasimha Rao G, Pampana S, Yarram A, Suresh Sajjan MC, Ramaraju AV, Bheemalingeswara Rao D. Surface Modifications of Dental Implants: An Overview. *Int J Dental Mat*. 2019;1(1):17-24. doi: 10.37983/IJDM.2019.1103.
 140. Harshika G, Gitanjali Y, Rajesh B. Implant surface modification as a basis of osseointegration: A narrative review. *J Dent Def Sect*. 2022 Jul-Dec;16(2):139-145. doi: 10.4103/jodd.jodd_13_21.
 141. Ballarre J, Aydemir T, Liverani L, Roether JA, Goldmann WH, Boccaccini AR. Versatile bioactive and antibacterial coating system based on silica, gentamicin, and chitosan: Improving early stage performance of titanium implants. *Surf Coat Tech*. 2020 Jan 15;381:125138. doi: 10.1016/j.surfcoat.2019.125138.
 142. Abu Alfaraj T, Al-Madani S, Alqahtani NS, Almohammadi AA, Alqahtani AM, AlQabbani HS, Bajunaid MK, Alharthy BA, Aljalfan N. Optimizing Osseointegration in Dental Implantology: A Cross-Disciplinary Review of Current and Emerging Strategies. *Cureus*. 2023 Oct 30;15(10):e47943. doi: 10.7759/cureus.47943.
 143. Albrektsson T, Wennerberg A. Oral implant surfaces: Part 1--review focusing on topographic and chemical properties of different surfaces and in vivo responses to them. *Int J Prosthodont*. 2004 Sep-Oct;17(5):536-43. PMID: 15543910.
 144. Kim JC, Lee M, Yeo IL. Three interfaces of the dental implant system and their clinical effects on hard and soft tissues. *Mater Horiz*. 2022 May 10;9(5):1387-1411. doi: 10.1039/d1mh01621k.
 145. Damiati L, Eales MG, Nobbs AH, Su B, Tsimbouri PM, Salmeron-Sanchez M, Dalby MJ. Impact of surface topography and coating on osteogenesis and bacterial attachment on titanium implants. *J Tissue Eng*. 2018 Aug 2;9:2041731418790694. doi: 10.1177/2041731418790694.
 146. Emam SM, Moussa N. Signaling pathways of dental implants' osseointegration: a narrative review on two of the most relevant; NF-κB and Wnt pathways. *BDJ Open*

- 2024 Apr 5;10:29. doi: 10.1038/s41405-024-00211-w.
147. Ao HY, Xie YT, Yang SB, Wu XD, Li K, Zheng XB, Tang TT. Covalently immobilised type I collagen facilitates osteoconduction and osseointegration of titanium coated implants. *J Orthop Translat.* 2015 Sep 26;5:16-25. doi: 10.1016/j.jot.2015.08.005.
 148. de Almeida TCS, Valverde TM, da Mata Martins TM, de Paula Oliveira F, da Silva Cunha P, Boense Tavares MA, Marchezini Rodrigues E, Silva Albergaria GD, Maia Vieira G, Assis Gomes D, Lana Gastelois P, Lopes de Souza R, de Góes AM, Kitten GT, Delany Martins M. Enhanced osteogenic response by collagen type I coating on surface-modified titanium bone implants. *Mater Today Commun.* 2024 Mar;39(3):108535. doi: 10.1016/j.mtcomm.2024.108535.
 149. Ersanli C, Tzora A, Skoufos I, Voudarou CC, Zeugolis DI. Recent Advances in Collagen Antimicrobial Biomaterials for Tissue Engineering Applications: A Review. *Int J Mol Sci.* 2023 Apr 25;24(9):7808. doi: 10.3390/ijms24097808.
 150. Bronk JK, Russell BH, Rivera JJ, Pasqualini R, Arap W, Höök M, Barbu EM. A multifunctional streptococcal collagen-mimetic protein coating prevents bacterial adhesion and promotes osteoid formation on titanium. *Acta Biomater.* 2014 Jul;10(7):3354-62. doi: 10.1016/j.actbio.2014.04.005.
 151. Raphael J, Holodniy M, Goodman SB, Heilshorn SC. Multifunctional coatings to simultaneously promote osseointegration and prevent infection of orthopaedic implants. *Biomaterials.* 2016 Apr;84:301-314. doi: 10.1016/j.biomaterials.2016.01.016.
 152. Di Stefano DA, Piattelli A, Zaniol T, Iezzi G. Implant and Prosthetic Success Following Peri-implant Guided Bone Regeneration in the Esthetic Zone Using an Equine Cortical Bone Membrane and an Equine Enzyme-Treated Bone Graft: A Retrospective Study with 9-year Follow-Up. *Int J Oral Maxillofac Implants.* 2020 Jul/Aug;35(4):824-832. doi: 10.11607/jomi.7906.
 153. Rajabimashhadi Z, Gallo N, Russo F, Ghiyami S, Mele C, Giordano ME, Lionetto MG, Salvatore L, Lionetto F. Production and physico-chemical characterization of nano-sized collagen from equine tendon. *Int J Biol Macromol.* 2024 Oct;277(Pt 2):134220. doi: 10.1016/j.ijbiomac.2024.134220.
 154. Di Stefano DA, Andreasi Bassi M, Ludovichetti M, Pagnutti S. Maxillary sinus lift with a collagenic equine heterologous bone substitute. *Histomorphometric analysis. Ital. Oral Surg.* 2012 Dec;11(5):56–62. doi: 10.1016/j.ios.2011.05.008.
 155. Schnutenhaus S, Doering I, Dreyhaupt J, Rudolph H, Luthardt RG. Alveolar ridge preservation with a collagen material: a randomized controlled trial. *J Periodontal Implant Sci.* 2018 Aug;48(4):236-250. doi: 10.5051/jpis.2018.48.4.236.
 156. Tizzoni R, Tizzoni M. How do GTR and GBR Differ? A Periodontitis Case Treated Using an Equine-derived, Enzyme-deantigenic, Collagenpreserving Bone Graft, and Collagen Membranes. *J Contemp Dent Pract.* 2019 May 1;20(5):639-644. PMID: 31316031.
 157. Tizzoni R, Tizzoni M. How do GTR and GBR Differ? A Periodontitis Raja SG. Local application of gentamicin-containing collagen implant in the prophylaxis and treatment of surgical site infection following cardiac surgery. *Int J Surg.* 2012;10 Suppl 1:S10-4. doi: 10.1016/j.ijisu.2012.05.018.
 158. D'Ambrosi R, Valli F, De Luca P, Ursino N, Uselli FG. MaioRegen Osteochondral Substitute for the Treatment of Knee Defects: A Systematic Review of the Literature. *J Clin Med.* 2019 Jun 1;8(6):783. doi: 10.3390/jcm8060783.
 159. Gallo N, Natali ML, Curci C, Picerno A, Gallone A, Vulpi M, Vitarelli A, Ditunno P, Cascione M, Sallustio F, Rinaldi R, Sannino A, Salvatore L. Analysis of the Physico-Chemical, Mechanical and Biological Properties of Crosslinked Type-I Collagen from

- Horse Tendon: Towards the Development of Ideal Scaffolding Material for Urethral Regeneration. *Materials* (Basel). 2021 Dec 12;14(24):7648. doi: 10.3390/ma14247648.
160. Sahin M. The role of topical Genta Fleece HD and gentamicin spray in prevention of sternum wound infections after open heart surgery: a comparative study. *Arch Med Sci Atheroscler Dis*. 2018 Feb 15;3:e29-e34. doi: 10.5114/amsad.2018.73526.
 161. Maternini M, Guttadauro A, Mascagni D, Milito G, Stuto A, Renzi A, Ripamonti L, Bottini C, Nudo R, Del Re L, Gabrielli F. Non cross-linked equine collagen (Salvecoll-E gel) for treatment of complex ano-rectal fistula. *Asian J. Surg*. 2019 Jul;43(2):401–404. doi: 10.1016/j.asjsur.2019.06.009.
 162. Simonovic J, Toljic B, Nikolic N, Peric M, Vujin J, Panajotovic R, Gajic R, Bekyarova E, Cataldi A, Parpura V, Milasin J. Differentiation of stem cells from apical papilla into neural lineage using graphene dispersion and single walled carbon nanotubes. *J Biomed Mater Res A*. 2018 Oct;106(10):2653-2661. doi: 10.1002/jbm.a.36461.
 163. Elshahat S, Elgendy AA, Elsewify T. Osteogenic Differentiation and Proliferation of Apical Papilla Stem Cells Using Chitosan-Coated Nanohydroxyapatite and Bioactive Glass Nanoparticles. *Eur J Dent*. 2024 May;18(2):665-671. doi: 10.1055/s-0043-1777044.
 164. Terzi A, Gallo N, Bettini S, Sibillano T, Altamura D, Campa L, Natali ML, Salvatore L, Madaghiele M, De Caro L, Valli L, Sannino A, Giannini C. Investigations of Processing-Induced Structural Changes in Horse Type-I Collagen at Sub and Supramolecular Levels. *Front Bioeng Biotechnol*. 2019 Aug 26;7:203. doi: 10.3389/fbioe.2019.00203.
 165. Gallo N, Lunetti P, Bettini S, Barca A, Madaghiele M, Valli L, Capobianco L, Sannino A, Salvatore L. Assessment of physico-chemical and biological properties of sericin-collagen substrates for PNS regeneration. *Int J Polym Mater Polym Biomater*. 2020;70(6):403–413. doi: 10.1080/00914037.2020.1725755.
 166. Prontera CT, Gallo N, Giannuzzi R, Pugliese M, Primiceri V, Mariano F, Maggiore A, Gigli G, Sannino A, Salvatore L, Maiorano V. Collagen Membrane as Water-Based Gel Electrolyte for Electrochromic Devices. *Gels*. 2023 Apr 6;9(4):310. doi: 10.3390/gels9040310.
 167. Nitti P, Gallo N, Palazzo B, Sannino A, Polini A, Verri T, Barca A, Gervaso F. Effect of L-Arginine treatment on the in vitro stability of electrospun aligned chitosan nanofiber mats. *Poly Test*. 2020 Nov;91:106758. doi: 10.1016/j.polymertesting.2020.106758.
 168. Simonović J, Toljić B, Rašković B, Jovanović V, Lazarević M, Milošević M, Nikolić N, Panajotović R, Milašin J. Raman microspectroscopy: toward a better distinction and profiling of different populations of dental stem cells. *Croat Med J*. 2019 Apr 30;60(2):78-86. doi: 10.3325/CroatMedJ_60_0078.
 169. Bakopoulou A, Leyhausen G, Volk J, Koidis P, Geurtsen W. Comparative characterization of STRO-1(neg)/CD146(pos) and STRO-1(pos)/CD146(pos) apical papilla stem cells enriched with flow cytometry. *Arch Oral Biol*. 2013 Oct;58(10):1556-68. doi: 10.1016/j.archoralbio.2013.06.018.
 170. Böhm S, Strauß C, Stoiber S, Kasper C, Charwat V. Impact of Source and Manufacturing of Collagen Matrices on Fibroblast Cell Growth and Platelet Aggregation. *Materials* (Basel). 2017 Sep 15;10(9):1086. doi: 10.3390/ma10091086.
 171. Urbanczyk M, Layland SL, Schenke-Layland K. The role of extracellular matrix in biomechanics and its impact on bioengineering of cells and 3D tissues. *Matrix Biol*. 2020 Jan;85-86:1-14. doi: 10.1016/j.matbio.2019.11.005.
 172. Viridi JK, Pethe P. Biomaterials Regulate Mechanosensors YAP/TAZ in Stem Cell

- Growth and Differentiation. *Tissue Eng Regen Med*. 2021 Apr;18(2):199-215. doi: 10.1007/s13770-020-00301-4.
173. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006 Aug 25;126(4):677-89. doi: 10.1016/j.cell.2006.06.044.
 174. Kleinheinz J, Stratmann U, Joos U, Wiesmann HP. VEGF-activated angiogenesis during bone regeneration. *J Oral Maxillofac Surg*. 2005 Sep;63(9):1310-6. doi: 10.1016/j.joms.2005.05.303.
 175. Weinzierl K, Hemprich A, Frerich B. Bone engineering with adipose tissue derived stromal cells. *J Craniomaxillofac Surg*. 2006 Dec;34(8):466-71. doi: 10.1016/j.jcms.2006.07.860.
 176. Kleinheinz J, Jung S, Wermker K, Fischer C, Joos U. Release kinetics of VEGF165 from a collagen matrix and structural matrix changes in a circulation model. *Head Face Med*. 2010 Jul 19;6:17. doi: 10.1186/1746-160X-6-17.
 177. Badieyan ZS, Berezhanskyy T, Utzinger M, Aneja MK, Emrich D, Erben R, Schüller C, Altpeter P, Ferizi M, Hasenpusch G, Rudolph C, Plank C. Transcript-activated collagen matrix as sustained mRNA delivery system for bone regeneration. *J Control Release*. 2016 Oct 10;239:137-48. doi: 10.1016/j.jconrel.2016.08.037.
 178. Barrino F, Vassallo V, Cammarota M, Lepore M, Portaccio M, Schiraldi C, La Gatta A. A comprehensive in vitro characterization of non-crosslinked, diverse tissue-derived collagen-based membranes intended for assisting bone regeneration. *PLoS One*. 2024 Jul 15;19(7):e0298280. doi: 10.1371/journal.pone.0298280.
 179. Alqutub MN, Mukhtar AH, Alali Y, Vohra F, Abduljabbar T. Osteogenic Differentiation of Periodontal Ligament Stem Cells Seeded on Equine-Derived Xenograft in Osteogenic Growth Media. *Medicina (Kaunas)*. 2022 Oct 25;58(11):1518. doi: 10.3390/medicina58111518.
 180. Romito GA, Fonseca MA, Soares HH, de Oliveira Lazarin R, Sapata VM, Nishyama R, Conde MC, Hammerle CHF, Schwarz F, Villar CC. Clinical outcomes following atrophic alveolar ridge reconstruction using collagenated xenogeneic bone block or autogenous bone block: One-year follow-up of a randomized controlled clinical. *J Clin Periodontol*. 2024 Jan;51(1):14-23. doi: 10.1111/jcpe.13891.
 181. Grasso G, Mummolo S, Bernardi S, Pietropaoli D, D'Ambrosio G, Iezzi G, Piattelli A, Bianchi S, Marchetti E. Histological and Histomorphometric Evaluation of New Bone Formation after Maxillary Sinus Augmentation with Two Different Osteoconductive Materials: A Randomized, Parallel, Double-Blind Clinical Trial. *Materials (Basel)*. 2020 Dec 3;13(23):5520. doi: 10.3390/ma13235520.
 182. Tetè S, Zizzari VL, Vinci R, Zara S, Di Tore U, Manica M, Cataldi A, Mortellaro C, Piattelli A, Gherlone E. Equine and porcine bone substitutes in maxillary sinus augmentation: a histological and immunohistochemical analysis of VEGF expression. *J Craniofac Surg*. 2014 May;25(3):835-9. doi: 10.1097/SCS.0000000000000679.
 183. Tumedei M, Mancinelli R, Di Filippo ES, Marrone M, Iezzi G, Piattelli A, Fulle S. Osteogenic Potential of Human Dental Pulp Stem Cells Co-Cultured with Equine Bone Substitute Combined with Melatonin. *Int J Periodontics Restorative Dent*. 2022 Jan-Feb;42(1):75-81. doi: 10.11607/prd.5430.
 184. De Castilho T, Rosa GDS, Stievani FC, Apolônio EVP, Pfeifer JPH, Altheman VG, Paliologo V, Santos NJD, Fonseca-Alves CE, Alves ALG. Biocompatibility of hydrogel derived from equine tendon extracellular matrix in horses subcutaneous tissue. *Front Bioeng Biotechnol*. 2024 Jan 8;11:1296743. doi: 10.3389/fbioe.2023.1296743.
 185. Kucera T, Ryskova L, Soukup T, Malakova J, Cermakova E, Mericka P, Suchanek J, Sponer P. Elution kinetics of vancomycin and gentamicin from carriers and their

- effects on mesenchymal stem cell proliferation: an in vitro study. *BMC Musculoskeletal Disord.* 2017 Sep 2;18(1):381. doi: 10.1186/s12891-017-1737-4.
186. Amado S, Rodrigues JM, Luís AL, Armada-da-Silva PA, Vieira M, Gartner A, Simões MJ, Veloso AP, Fornaro M, Raimondo S, Varejão AS, Geuna S, Maurício AC. Effects of collagen membranes enriched with in vitro-differentiated N1E-115 cells on rat sciatic nerve regeneration after end-to-end repair. *J Neuroeng Rehabil.* 2010 Feb 11;7:7. doi: 10.1186/1743-0003-7-7.
 187. Zhao Q, Ren H, Wang N, Yuan X, Zhao Y, Wen Q. NOTUM plays a bidirectionally modulatory role in the odontoblastic differentiation of human stem cells from the apical papilla through the WNT/ β -catenin signaling pathway. *Arch Oral Biol.* 2024 Apr;160:105896. doi: 10.1016/j.archoralbio.2024.105896.
 188. Touya N, Devun M, Handschin C, Casenave S, Ahmed Omar N, Gaubert A, Dusserre N, De Oliveira H, Kérourédan O, Devillard R. In vitro and in vivo characterization of a novel tricalcium silicate-based ink for bone regeneration using laser-assisted bioprinting. *Biofabrication.* 2022 Mar 9;14(2). doi: 10.1088/1758-5090/ac584b.
 189. Devillard R, Rémy M, Kalisky J, Bourget JM, Kérourédan O, Siadous R, Bareille R, Amédée-Vilamitjana J, Chassande O, Fricain JC. In vitro assessment of a collagen/alginate composite scaffold for regenerative endodontics. *Int Endod J.* 2017 Jan;50(1):48-57. doi: 10.1111/iej.12591.
 190. Leite ML, Soares DG, Anovazzi G, Anselmi C, Hebling J, de Souza Costa CA. Fibronectin-loaded Collagen/Gelatin Hydrogel Is a Potent Signaling Biomaterial for Dental Pulp Regeneration. *J Endod.* 2021 Jul;47(7):1110-1117. doi: 10.1016/j.joen.2021.04.009.
 191. Noohi P, Abdekhodaie MJ, Saadatmand M, Nekoofar MH, Dummer PMH. The development of a dental light curable PRFe-loaded hydrogel as a potential scaffold for pulp-dentine complex regeneration: An in vitro study. *Int Endod J.* 2023 Apr;56(4):447-464. doi: 10.1111/iej.13882.
 192. Leite ML, de Oliveira Ribeiro RA, Soares DG, Hebling J, de Souza Costa CA. Poly(caprolactone)-aligned nanofibers associated with fibronectin-loaded collagen hydrogel as a potent bioactive scaffold for cell-free regenerative endodontics. *Int Endod J.* 2022 Dec;55(12):1359-1371. doi: 10.1111/iej.13823.
 193. Liu JY, Chen X, Yue L, Huang GT, Zou XY. CXC Chemokine Receptor 4 Is Expressed Paravascularly in Apical Papilla and Coordinates with Stromal Cell-derived Factor-1 α during Transmigration of Stem Cells from Apical Papilla. *J Endod.* 2015 Sep;41(9):1430-6. doi: 10.1016/j.joen.2015.04.006.
 194. Leite ML, Soares DG, Anovazzi G, Filipe Koon Wu M, Bordini EAF, Hebling J, DE Souza Costa CA. Bioactivity effects of extracellular matrix proteins on apical papilla cells. *J Appl Oral Sci.* 2021 Sep 3;29:e20210038. doi: 10.1590/1678-7757-2021-0038.
 195. Zhang H, Li L, Sun X, Hou B, Luo C. Research and development of microenvironment's influence on stem cells from the apical papilla - construction of novel research microdevices: tooth-on-a-chip. *Biomed Microdevices.* 2024 Jul 18;26(3):33. doi: 10.1007/s10544-024-00715-0.
 196. Pan H, Yang Y, Xu H, Jin A, Huang X, Gao X, Sun S, Liu Y, Liu J, Lu T, Wang X, Zhu Y, Jiang L. The odontoblastic differentiation of dental mesenchymal stem cells: molecular regulation mechanism and related genetic syndromes. *Front Cell Dev Biol.* 2023 Sep 25;11:1174579. doi: 10.3389/fcell.2023.1174579.
 197. Park BW, Hah YS, Choi MJ, Ryu YM, Lee SG, Kim DR, Kim JR, Byun JH. In vitro osteogenic differentiation of cultured human dental papilla-derived cells. *J Oral Maxillofac Surg.* 2009 Mar;67(3):507-14. doi: 10.1016/j.joms.2008.08.037.
 198. Baas J, Jakobsen T, Elmengaard B, Bechtold JE, Soballe K. The effect of adding an

- equine bone matrix protein lyophilisate on fixation and osseointegration of HA-coated Ti implants. *J Biomed Mater Res A*. 2012 Jan;100(1):188-94. doi: 10.1002/jbm.a.33253.
199. Bellone G, Vizio B, Scirelli T, Emanuelli G. A Xenogenic Bone Derivative as a Potential Adjuvant for Bone Regeneration and Implant Osseointegration: An In Vitro Study. *Tissue Eng Regen Med*. 2017 Apr 7;14(3):243-251. doi: 10.1007/s13770-017-0029-2.
 200. ASTM F67-13(2017); Standard Specification for Unalloyed Titanium, for Surgical Implant Applications (UNS R50250, UNS R50400, UNS R50550, UNS R50700). American Society for Testing and Materials (ASTM), F04.12, 2017, 13.01, 1-6. DOI: 10.1520/F0067-13R17.
 201. Gehrke SA, Pérez-Díaz L, Mazón P, De Aza PN. Biomechanical Effects of a New Macrogeometry Design of Dental Implants: An In Vitro Experimental Analysis. *J Funct Biomater*. 2019 Oct 25;10(4):47. doi: 10.3390/jfb10040047.
 202. Simonovic J, Toljic B, Lazarevic M, Markovic MM, Peric M, Vujin J, Panajotovic R, Milasin J. The Effect of Liquid-Phase Exfoliated Graphene Film on Neurodifferentiation of Stem Cells from Apical Papilla. *Nanomaterials (Basel)*. 2022 Sep 8;12(18):3116. doi: 10.3390/nano12183116.
 203. Lazarevic M, Petrovic S, Pierfelice TV, Ignjatovic N, Piattelli A, Vlajic Tovilovic T, Radunovic M. Antimicrobial and Osteogenic Effects of Collagen Membrane Decorated with Chitosan-Nano-Hydroxyapatite. *Biomolecules*. 2023 Mar 23;13(4):579. doi: 10.3390/biom13040579.
 204. Tschernitschek H, Borchers L, Geurtsen W. Nonalloyed titanium as a bioinert metal--a review. *Quintessence Int*. 2005 Jul-Aug;36(7-8):523-30. PMID: 15997933.
 205. Abdulghafor MA, Mahmood MK, Tassery H, Tardivo D, Falguiere A, Lan R. Biomimetic Coatings in Implant Dentistry: A Quick Update. *J Funct Biomater*. 2023 Dec 30;15(1):15. doi: 10.3390/jfb15010015.
 206. Wang QQ, Li W, Yang BC. Regulation on the biocompatibility of bioactive titanium metals by type I collagen. *J Biomed Mater Res A*. 2011 Oct;99(1):125-34. doi: 10.1002/jbm.a.33142.
 207. Naomi R, Ridzuan PM, Bahari H. Current Insights into Collagen Type I. *Polymers (Basel)*. 2021 Aug 9;13(16):2642. doi: 10.3390/polym13162642.
 208. Rupp F, Scheideler L, Olshanska N, de Wild M, Wieland M, Geis-Gerstorfer J. Enhancing surface free energy and hydrophilicity through chemical modification of microstructured titanium implant surfaces. *J Biomed Mater Res A*. 2006 Feb;76(2):323-34. doi: 10.1002/jbm.a.30518.
 209. Barfeie A, Wilson J, Rees J. Implant surface characteristics and their effect on osseointegration. *Br Dent J*. 2015 Mar 13;218(5):E9. doi: 10.1038/sj.bdj.2015.171.
 210. Goldman M, Juodzbaly G, Vilkinis V. Titanium surfaces with nanostructures influence on osteoblasts proliferation: a systematic review. *J Oral Maxillofac Res*. 2014 Oct 1;5(3):e1. doi: 10.5037/jomr.2014.5301.
 211. Li D., Ferguson S.J., Beutler T., Cochran D.L., Sittig C., Hirt H.P., Buser D. Biomechanical comparison of the sandblasted and acid-etched and the machined and acid-etched titanium surface for dental implants. *J. Biomed. Mater. Res*. 2002;60:325–332. doi: 10.1002/jbm.10063.
 212. Kligman S, Ren Z, Chung CH, Perillo MA, Chang YC, Koo H, Zheng Z, Li C. The Impact of Dental Implant Surface Modifications on Osseointegration and Biofilm Formation. *J Clin Med*. 2021 Apr 12;10(8):1641. doi: 10.3390/jcm10081641.
 213. DE Colli M, Radunovic M, Zizzari VL, DI Giacomo V, DI Nisio C, Piattelli A, Calvo Guirado JL, Zavan B, Cataldi A, Zara S. Osteoblastic differentiating potential of dental

- pulp stem cells in vitro cultured on a chemically modified microrough titanium surface. *Dent Mater J*. 2018 Mar 30;37(2):197-205. doi: 10.4012/dmj.2016-418.
214. Morra M, Cassinelli C, Cascardo G, Bollati D, Baena RR. Gene expression of markers of osteogenic differentiation of human mesenchymal cells on collagen I-modified microrough titanium surfaces. *J Biomed Mater Res A*. 2011 Feb;96(2):449-55. doi: 10.1002/jbm.a.32948.
 215. Sverzut AT, Crippa GE, Morra M, de Oliveira PT, Beloti MM, Rosa AL. Effects of type I collagen coating on titanium osseointegration: histomorphometric, cellular and molecular analyses. *Biomed Mater*. 2012 Jun;7(3):035007. doi: 10.1088/1748-6041/7/3/035007.
 216. Shao J, Weng L, Li J, Lin H, Wang H, Lin J. Regulation of Macrophage Polarization by Mineralized Collagen Coating to Accelerate the Osteogenic Differentiation of Mesenchymal Stem Cells. *ACS Biomater Sci Eng*. 2022 Feb 14;8(2):610-619. doi: 10.1021/acsbiomaterials.1c00834.
 217. Costa DG, Ferraz EP, Abuna RPF, de Oliveira PT, Morra M, Beloti MM, Rosa AL. The effect of collagen coating on titanium with nanotopography on in vitro osteogenesis. *J Biomed Mater Res A*. 2017 Oct;105(10):2783-2788. doi: 10.1002/jbm.a.36140.
 218. Cecconi S, Mattioli-Belmonte M, Manzotti S, Orciani M, Piccioli A, Gigante A. Bone-derived titanium coating improves in vivo implant osseointegration in an experimental animal model. *J Biomed Mater Res B Appl Biomater*. 2014 Feb;102(2):303-10. doi: 10.1002/jbm.b.33008.
 219. Kunert-Keil C., Gredes T., Heinemann F., Dominiak M., Botzenhart U., Gedrange T. Socket augmentation using a commercial collagen-based product—An animal study in pigs. *Mater. Sci. Eng. C*. 2015;46:177–183. doi: 10.1016/j.msec.2014.10.033.
 220. Mozzati M, Gallezio G, Staiti G, Iezzi G, Piattelli A, Mortellaro C. Socket Preservation Using a Biomimetic Nanostructured Matrix and Atraumatic Surgical Extraction Technique. *J Craniofac Surg*. 2017 Jun;28(4):1042-1045. doi: 10.1097/SCS.0000000000003496.
 221. Lu Y, Cai WJ, Ren Z, Han P. The Role of Staphylococcal Biofilm on the Surface of Implants in Orthopedic Infection. *Microorganisms*. 2022 Sep 26;10(10):1909. doi: 10.3390/microorganisms10101909.
 222. Munnix IC, Gilio K, Siljander PR, Raynal N, Feijge MA, Hackeng TM, Deckmyn H, Smethurst PA, Farndale RW, Heemskerk JW. Collagen-mimetic peptides mediate flow-dependent thrombus formation by high- or low-affinity binding of integrin $\alpha 2\beta 1$ and glycoprotein VI. *J Thromb Haemost*. 2008 Dec;6(12):2132-42. doi: 10.1111/j.1538-7836.2008.03167.x.
 223. Sundar G, Joseph JCP, John A, Abraham A. Natural collagen bioscaffolds for skin tissue engineering strategies in burns: a critical review. *Int J Polym Mater Polym Biomater*. 2020;70(9):593–604. doi: 10.1080/00914037.2020.1740991.
 224. Sarikaya A, Record R, Wu CC, Tullius B, Badylak S, Ladisch M. Antimicrobial activity associated with extracellular matrices. *Tissue Eng*. 2002 Feb;8(1):63-71. doi: 10.1089/107632702753503063.
 225. Brennan EP, Reing J, Chew D, Myers-Irvin JM, Young EJ, Badylak SF. Antibacterial activity within degradation products of biological scaffolds composed of extracellular matrix. *Tissue Eng*. 2006 Oct;12(10):2949-55. doi: 10.1089/ten.2006.12.2949.
 226. Yuan P, Chen M, Lu X, Yang H, Wang L, Bai T, Zhou W, Liu T, Yu S. Application of advanced surface modification techniques in titanium-based implants: latest strategies for enhanced antibacterial properties and osseointegration. *J Mater Chem B*. 2024 Oct 23;12(41):10516-10549. doi: 10.1039/d4tb01714e.

227. Zhao S, Xu Y, Xu W, Weng Z, Cao F, Wan X, Cui T, Yu Y, Liao L, Wang X. Tremella-Like ZnO@Col-I-Decorated Titanium Surfaces with Dual-Light-Defined Broad-Spectrum Antibacterial and Triple Osteogenic Properties. *ACS Appl Mater Interfaces*. 2020 Jul 8;12(27):30044-30051. doi: 10.1021/acsami.0c05413.
228. Zhang L, Xue Y, Gopalakrishnan S, Li K, Han Y, Rotello VM. Antimicrobial Peptide-Loaded Pectolite Nanorods for Enhancing Wound-Healing and Biocidal Activity of Titanium. *ACS Appl Mater Interfaces*. 2021 Jun 23;13(24):28764-28773. doi: 10.1021/acsami.1c04895.
229. Marín-Pareja N, Salvagni E, Guillem-Marti J, Aparicio C, Ginebra MP. Collagen-functionalised titanium surfaces for biological sealing of dental implants: effect of immobilisation process on fibroblasts response. *Colloids Surf B Biointerfaces*. 2014 Oct 1;122:601-610. doi: 10.1016/j.colsurfb.2014.07.038.
230. Iwanami-Kadowaki K, Uchikoshi T, Uezono M, Kikuchi M, Moriyama K. Development of novel bone-like nanocomposite coating of hydroxyapatite/collagen on titanium by modified electrophoretic deposition. *J Biomed Mater Res A*. 2021 Oct;109(10):1905-1911. doi: 10.1002/jbm.a.37182.
231. Cho WT, Kim SY, Jung SI, Kang SS, Kim SE, Hwang SH, Jeong CM, Huh JB. Effects of Gamma Radiation-Induced Crosslinking of Collagen Type I Coated Dental Titanium Implants on Osseointegration and Bone Regeneration. *Materials (Basel)*. 2021 Jun 13;14(12):3268. doi: 10.3390/ma14123268.
232. Marín-Pareja N, Cantini M, González-García C, Salvagni E, Salmerón-Sánchez M, Ginebra MP. Different Organization of Type I Collagen Immobilized on Silanized and Nonsilanized Titanium Surfaces Affects Fibroblast Adhesion and Fibronectin Secretion. *ACS Appl Mater Interfaces*. 2015 Sep 23;7(37):20667-77. doi: 10.1021/acsami.5b05420.
233. Liu CF, Chang KC, Sun YS, Nguyen DT, Huang HH. Combining Sandblasting, Alkaline Etching, and Collagen Immobilization to Promote Cell Growth on Biomedical Titanium Implants. *Polymers (Basel)*. 2021 Jul 31;13(15):2550. doi: 10.3390/polym13152550.
234. Korn P, Schulz MC, Hintze V, Range U, Mai R, Eckelt U, Schnabelrauch M, Möller S, Becher J, Scharnweber D, Stadlinger B. Chondroitin sulfate and sulfated hyaluronan-containing collagen coatings of titanium implants influence peri-implant bone formation in a minipig model. *J Biomed Mater Res A*. 2014 Jul;102(7):2334-44. doi: 10.1002/jbm.a.34913.
235. Albrektsson T, Albrektsson B. Osseointegration of bone implants. A review of an alternative mode of fixation. *Acta Orthop Scand*. 1987 Oct;58(5):567-77. doi: 10.3109/17453678709146401.
236. Javed F, Ahmed HB, Crespi R, Romanos GE. Role of primary stability for successful osseointegration of dental implants: Factors of influence and evaluation. *Interv Med Appl Sci*. 2013 Dec;5(4):162-7. doi: 10.1556/IMAS.5.2013.4.3.
237. Tettamanti L, Andrisani C, Bassi MA, Vinci R, Silvestre-Rangil J, Tagliabue A. Immediate loading implants: review of the critical aspects. *Oral Implantol (Rome)*. 2017 Sep 27;10(2):129-139. doi: 10.11138/orl/2017.10.2.129.
238. Junker R, Dimakis A, Thoneick M, Jansen JA. Effects of implant surface coatings and composition on bone integration: a systematic review. *Clin Oral Implants Res*. 2009 Sep;20 Suppl 4:185-206. doi: 10.1111/j.1600-0501.2009.01777.x.
239. Ferguson SJ, Langhoff JD, Voelter K, von Rechenberg B, Scharnweber D, Bierbaum S, Schnabelrauch M, Kautz AR, Frauchiger VM, Mueller TL, van Lenthe GH, Schlottig F. Biomechanical comparison of different surface modifications for dental implants. *Int J Oral Maxillofac Implants*. 2008 Nov-Dec;23(6):1037-46. PMID:

- 19216272.
- 240.Stadlinger B, Hintze V, Bierbaum S, Möller S, Schulz MC, Mai R, Kuhlisch E, Heinemann S, Scharnweber D, Schnabelrauch M, Eckelt U. Biological functionalization of dental implants with collagen and glycosaminoglycans-A comparative study. *J Biomed Mater Res B Appl Biomater*. 2012 Feb;100(2):331-41. doi: 10.1002/jbm.b.31953.
 - 241.Bose S, Li S, Mele E, Silberschmidt VV. Exploring the Mechanical Properties and Performance of Type-I Collagen at Various Length Scales: A Progress Report. *Materials (Basel)*. 2022 Apr 8;15(8):2753. doi: 10.3390/ma15082753.
 - 242.Bae EB, Yoo JH, Jeong SI, Kim MS, Lim YM, Ahn JJ, Lee JJ, Lee SH, Kim HJ, Huh JB. Effect of Titanium Implants Coated with Radiation-Crosslinked Collagen on Stability and Osseointegration in Rat Tibia. *Materials (Basel)*. 2018 Dec 11;11(12):2520. doi: 10.3390/ma11122520.
 - 243.Morra M, Cassinelli C, Meda L, Fini M, Giavaresi G, Giardino R. Surface analysis and effects on interfacial bone microhardness of collagen-coated titanium implants: a rabbit model. *Int J Oral Maxillofac Implants*. 2005 Jan-Feb;20(1):23-30. PMID: 15747670.
 - 244.Misch CE. Bone density: A key determinant for clinical success. *Contemp. Implant Dent*. 1999;8:109–118.
 - 245.Misch CE, Judy KW. Classification of partially edentulous arches for implant dentistry. *Int J Oral Implantol*. 1987;4:7–13.
 - 246.Tabrizi A, Rizzo P, Ochs MW. Electromechanical impedance method to assess dental implant stability. *Smart Mater Struct*. 2012;21:115022. doi: 10.1088/0964-1726/21/11/115022.
 - 247.Jar C, Archibald A, Gibson M, Westover L. An analytical model to measure dental implant stability with the Advanced System for Implant Stability Testing (ASIST). *J Mech Behav Biomed Mater*. 2024 Feb;150:106238. doi: 10.1016/j.jmbbm.2023.106238.
 - 248.Comuzzi L, Ceddia M, Di Pietro N, Inchingolo F, Inchingolo AM, Romasco T, Tumedei M, Specchiulli A, Piattelli A, Trentadue B. Crestal and Subcrestal Placement of Morse Cone Implant-Abutment Connection Implants: An In Vitro Finite Element Analysis (FEA) Study. *Biomedicines*. 2023 Nov 16;11(11):3077. doi: 10.3390/biomedicines11113077.
 - 249.Comuzzi L, Romasco T, Piattelli A, Inchingolo F, Mourão CF, Di Pietro N. Comparative Evaluation of Primary Stability in Truncated Cone Implants with Different Macro-Geometries in Low-Density Polyurethane Blocks Simulating Maxillary Sinus Rehabilitations. *Prosthesis*. 2024;6(4):923-938. doi: 10.3390/prosthesis6040067.
 - 250.ASTM F-1839-08; Standard Specification for Rigid Polyurethane Foam for Use as a Standard Material for Test Orthopedic Devices for Instruments. American Society for Testing and Materials (ASTM): West Conshohocken, PA, USA, 2008.
 - 251.do Vale Souza JP, de Moraes Melo Neto CL, Piacenza LT, Freitas da Silva EV, de Melo Moreno AL, Penitente PA, Brunetto JL, Dos Santos DM, Goiato MC. Relation Between Insertion Torque and Implant Stability Quotient: A Clinical Study. *Eur J Dent*. 2021 Oct;15(4):618-623. doi: 10.1055/s-0041-1725575.
 - 252.Romasco T, Pignatelli P, Tumedei M, Hossein HHS, Cipollina A, Piattelli A, Inchingolo F, Di Pietro N. The influence of truncated-conical implant length on primary stability in maxillary and mandibular regions: an in vitro study using polyurethane blocks. *Clin Oral Investig*. 2023 Dec 26;28(1):28. doi: 10.1007/s00784-023-05444-x.

- 253.Comuzzi L, Tumedei M, Covani U, Romasco T, Petrini M, Montesani L, Piattelli A, Di Pietro N. Primary Stability Assessment of Conical Implants in Under-Prepared Sites: An In Vitro Study in Low-Density Polyurethane Foams. *Appl Sci*. 2023;13(10):6041. doi: 10.3390/app13106041.
- 254.Greenstein G, Cavallaro J. Implant Insertion Torque: Its Role in Achieving Primary Stability of Restorable Dental Implants. *Compend Contin Educ Dent*. 2017 Feb;38(2):88-95; quiz 96. PMID: 28156122.
- 255.Huang YC, Huang YC, Ding SJ. Primary stability of implant placement and loading related to dental implant materials and designs: A literature review. *J Dent Sci*. 2023 Oct;18(4):1467-1476. doi: 10.1016/j.jds.2023.06.010.
- 256.Ramesh R, Sasi A, Mohamed SC, Joseph SP. "Compression Necrosis" - A Cause of Concern for Early Implant Failure? Case Report and Review of Literature. *Clin Cosmet Investig Dent*. 2024 Mar 7;16:43-52. doi: 10.2147/CCIDE.S453798.
- 257.Brancacci E, García González S, Galve-Huertas A, Bennani A, Hernández Alfaro F, Aboul-Hosn Centenero S. Influence of Insertion Torques on the Surface Integrity in Different Dental Implants: An Ex Vivo Descriptive Study. *Materials (Basel)*. 2023 Mar 14;16(6):2330. doi: 10.3390/ma16062330.
- 258.Simeone SG, Rios M, Simonpietri J. "Reverse torque of 30 Ncm applied to dental implants as test for osseointegration"-a human observational study. *Int J Implant Dent*. 2016 Dec;2(1):26. doi: 10.1186/s40729-016-0060-4.
- 259.Javed F, Romanos GE. The role of primary stability for successful immediate loading of dental implants. A literature review. *J Dent*. 2010 Aug;38(8):612-20. doi: 10.1016/j.jdent.2010.05.013.
- 260.Sennerby L, Meredith N. Resonance frequency analysis: measuring implant stability and osseointegration. *Compend Contin Educ Dent*. 1998 May;19(5):493-8, 500, 502; quiz 504. PMID: 9693511.
- 261.Gehrke SA, Cortellari GC, de Oliveira Fernandes GV, Scarano A, Martins RG, Cançado RM, Mesquita AMM. Randomized Clinical Trial Comparing Insertion Torque and Implant Stability of Two Different Implant Macrogeometries in the Initial Periods of Osseointegration. *Medicina (Kaunas)*. 2023 Jan 14;59(1):168. doi: 10.3390/medicina59010168.
- 262.Gehrke SA, Eliers Treichel TL, Pérez-Díaz L, Calvo-Guirado JL, Aramburú Júnior J, Mazón P, de Aza PN. Impact of Different Titanium Implant Thread Designs on Bone Healing: A Biomechanical and Histometric Study with an Animal Model. *J Clin Med*. 2019 May 31;8(6):777. doi: 10.3390/jcm8060777.
- 263.Morra M, Cassinelli C, Cascardo G, Cahalan P, Cahalan L, Fini M, Giardino R. Surface engineering of titanium by collagen immobilization. Surface characterization and in vitro and in vivo studies. *Biomaterials*. 2003 Nov;24(25):4639-54. doi: 10.1016/s0142-9612(03)00360-0.
- 264.Calvert KL, Trumble KP, Webster TJ, Kirkpatrick LA. Characterization of commercial rigid polyurethane foams used as bone analogs for implant testing. *J Mater Sci Mater Med*. 2010 May;21(5):1453-61. doi: 10.1007/s10856-010-4024-6.
- 265.Berglundh T, Armitage G, Araujo MG, Avila-Ortiz G, Blanco J, Camargo PM, Chen S, Cochran D, Derks J, Figuero E, Hämmerle CHF, Heitz-Mayfield LJA, Huynh-Ba G, Iacono V, Koo KT, Lambert F, McCauley L, Quirynen M, Renvert S, Salvi GE, Schwarz F, Tarnow D, Tomasi C, Wang HL, Zitzmann N. Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Clin Periodontol*. 2018 Jun;45 Suppl 20:S286-S291. doi: 10.1111/jcpe.12957.
- 266.Gehrke SA, Júnior JA, Eirles Treichel TL, Dedavid BA. Biomechanical and

- histological evaluation of four different implant macrogeometries in the early osseointegration process: An in vivo animal study. *J Mech Behav Biomed Mater*. 2022 Jan;125:104935. doi: 10.1016/j.jmbbm.2021.104935.
267. Gehrke SA, Júnior JA, Treichel TLE, do Prado TD, Dedavid BA, de Aza PN. Effects of insertion torque values on the marginal bone loss of dental implants installed in sheep mandibles. *Sci Rep*. 2022 Jan 11;12(1):538. doi: 10.1038/s41598-021-04313-5.
 268. Moghadasi K, Mohd Isa MS, Ariffin MA, Mohd jamil MZ, Raja S, Wu B, Yamani M, Bin Muhamad MR, Yusof F, Fadzil Jamaludin M, bin Ab Karim MS, Abdul Razak BB, Yusoff NB. A review on biomedical implant materials and the effect of friction stir based techniques on their mechanical and tribological properties. *J Mater Res Technol*. 2022 Mar-Apr;17:1054-1121. <https://doi.org/10.1016/j.jmrt.2022.01.050>.
 269. Rammelt S, Heck C, Bernhardt R, Bierbaum S, Scharnweber D, Goebbels J, Ziegler J, Biewener A, Zwipp H. In vivo effects of coating loaded and unloaded Ti implants with collagen, chondroitin sulfate, and hydroxyapatite in the sheep tibia. *J Orthop Res*. 2007 Aug;25(8):1052-61. doi: 10.1002/jor.20403.
 270. Schliephake H, Aref A, Scharnweber D, Bierbaum S, Roessler S, Sewing A. Effect of immobilized bone morphogenic protein 2 coating of titanium implants on peri-implant bone formation. *Clin Oral Implants Res*. 2005 Oct;16(5):563-9. doi: 10.1111/j.1600-0501.2005.01143.x.
 271. Lages FS, Douglas-de Oliveira DW, Costa FO. Relationship between implant stability measurements obtained by insertion torque and resonance frequency analysis: A systematic review. *Clin Implant Dent Relat Res*. 2018 Feb;20(1):26-33. doi: 10.1111/cid.12565.
 272. Romasco T, De Bortoli N Jr, Paulo De Bortoli J, Jorge Jayme S, Piattelli A, Di Pietro N. Primary stability evaluation of different morse cone implants in low-density artificial bone blocks: A comparison between high-and low-speed drilling. *Heliyon*. 2024 Jul 27;10(15):e35225. doi: 10.1016/j.heliyon.2024.e35225.
 273. Mello BF, De Carvalho Formiga M, Bianchini MA, Borges I Jr., Coura G, Tumedei M, Fuller R, Petrini M, Romasco T, Vaz P, et al. Insertion Torque (IT) and Implant Stability Quotient (ISQ) Assessment in Dental Implants with and without Healing Chambers: A Polyurethane In Vitro Study. *Appl Sci*. 2023;13(18):10215. doi: 10.3390/app131810215.
 274. Cipollina A, Ceddia M, Di Pietro N, Inchingolo F, Tumedei M, Romasco T, Piattelli A, Specchiulli A, Trentadue B. Finite Element Analysis (FEA) of a Premaxillary Device: A New Type of Subperiosteal Implant to Treat Severe Atrophy of the Maxilla. *Biomimetics*. 2023;8(4):336. doi: 10.3390/biomimetics8040336.
 275. Huang HM, Lee SY, Yeh CY, Lin CT. Resonance frequency assessment of dental implant stability with various bone qualities: a numerical approach. *Clin Oral Implants Res*. 2002 Feb;13(1):65-74. doi: 10.1034/j.1600-0501.2002.130108.x.
 276. Frost HM. A 2003 update of bone physiology and Wolff's Law for clinicians. *Angle Orthod*. 2004 Feb;74(1):3-15. doi: 10.1043/0003-3219(2004)074<0003:AUBPA>2.0.CO;2.
 277. Misch CE. Bone density: a key determinant for treatment planning. *Contemp Implant Dent*. 2007;3rd ed:130-146.
 278. Al-Ekrish AA, Widmann G, Alfadda SA. Revised, Computed Tomography-Based Lekholm and Zarb Jawbone Quality Classification. *Int J Prosthodont*. 2018 Jul/Aug;31(4):342-345. doi: 10.11607/ijp.5714.
 279. Huang YC, Huang YC, Ding SJ. Primary stability of implant placement and loading related to dental implant materials and designs: A literature review. *J Dent Sci*. 2023 Oct;18(4):1467-1476. doi: 10.1016/j.jds.2023.06.010.

280. Shayeb MA, Elfadil S, Abutayyem H, Shqaidef A, Marrapodi MM, Cicciù M, Minervini G. Bioactive surface modifications on dental implants: a systematic review and meta-analysis of osseointegration and longevity. *Clin Oral Investig*. 2024 Oct 11;28(11):592. doi: 10.1007/s00784-024-05958-y.
281. Fajri H, Putri R, Gunawarman G, Tjong D, Manjas M. Collagen Coating on Titanium Alloy (TNTZ) with Dip Coating Method as Prosthetic Devices in Biomedical Applications. *Journal Of Subsea And Offshore -Science And Engineering- (JSOse)* 2018;15(1): 1-5.
282. Huiskes R, Weinans H, van Rietbergen B. The relationship between stress shielding and bone resorption around total hip stems and the effects of flexible materials. *Clin Orthop Relat Res*. 1992 Jan;(274):124-34.
283. Raaj G, Manimaran P, Kumar CD, Sadan DS, Abirami M. Comparative Evaluation of Implant Designs: Influence of Diameter, Length, and Taper on Stress and Strain in the Mandibular Segment-A Three-Dimensional Finite Element Analysis. *J Pharm Bioallied Sci*. 2019 May;11(Suppl 2):S347-S354. doi: 10.4103/JPBS.JPBS_29_19.
284. Chang Y, Tambe AA, Maeda Y, Wada M, Gonda T. Finite element analysis of dental implants with validation: to what extent can we expect the model to predict biological phenomena? A literature review and proposal for classification of a validation process. *Int J Implant Dent*. 2018 Mar 8;4(1):7. doi: 10.1186/s40729-018-0119-5.
285. Zhang C, Zeng C, Wang Z, Zeng T, Wang Y. Optimization of stress distribution of bone-implant interface (BII). *Biomater Adv*. 2023 Apr;147:213342. doi: 10.1016/j.bioadv.2023.213342.
286. Uto Y, Kuroshima S, Nakano T, Ishimoto T, Inaba N, Uchida Y, Sawase T. Effects of mechanical repetitive load on bone quality around implants in rat maxillae. *PLoS One*. 2017 Dec 15;12(12):e0189893. doi: 10.1371/journal.pone.0189893.
287. Frost HM. Perspectives: bone's mechanical usage windows. *Bone Miner*. 1992 Dec;19(3):257-71. doi: 10.1016/0169-6009(92)90875-e.
288. Azcarate-Velázquez F, Castillo-Oyagüe R, Oliveros-López LG, Torres-Lagares D, Martínez-González ÁJ, Pérez-Velasco A, Lynch CD, Gutiérrez-Pérez JL, Serrera-Figallo MÁ. Influence of bone quality on the mechanical interaction between implant and bone: A finite element analysis. *J Dent*. 2019 Sep;88:103161. doi: 10.1016/j.jdent.2019.06.008.
289. Qian L, Todo M, Matsushita Y, Koyano K. Effects of implant diameter, insertion depth, and loading angle on stress/strain fields in implant/jawbone systems: finite element analysis. *Int J Oral Maxillofac Implants*. 2009 Sep-Oct;24(5):877-86.
290. Chamrad J, Marcián P, Cizek J. Beneficial osseointegration effect of hydroxyapatite coating on cranial implant - FEM investigation. *PLoS One*. 2021 Jul 19;16(7):e0254837. doi: 10.1371/journal.pone.0254837.
291. Mas Ayu H, Mustaqieem MM, Daud R, Shah A, Arafat A, Dambatta MS. Effect of Polylactic Acid/Hydroxyapatite Coating on Dental Implant Using Finite Element Method. *MSF* 2020;995:103–8. doi: 10.4028/www.scientific.net/msf.995.103.
292. Dommeti VK, Pramanik S, Roy S. Design of customized coated dental implants using finite element analysis. *Dent Med Probl*. 2023 Jul-Sep;60(3):385-392. doi: 10.17219/dmp/142447.
293. Macedo JP, Pereira J, Faria J, Souza JCM, Alves JL, López-López J, Henriques B. Finite element analysis of peri-implant bone volume affected by stresses around Morse taper implants: effects of implant positioning to the bone crest. *Comput Methods Biomech Biomed Engin*. 2018 Sep;21(12):655-662. doi: 10.1080/10255842.2018.1507025.
294. Chen X, Mao B, Zhu Z, Yu J, Lu Y, Zhang Q, Yue L, Yu H. A three-dimensional

- finite element analysis of mechanical function for 4 removable partial denture designs with 3 framework materials: CoCr, Ti-6Al-4V alloy and PEEK. *Sci Rep*. 2019 Sep 27;9(1):13975. doi: 10.1038/s41598-019-50363-1.
- 295.Comuzzi L, Tumedei M, Di Pietro N, Romasco T, Heydari Sheikh Hossein H, Montesani L, Inchingolo F, Piattelli A, Covani U. A Comparison of Conical and Cylindrical Implants Inserted in an In Vitro Post-Extraction Model Using Low-Density Polyurethane Foam Blocks. *Materials*. 2023;16(14):5064. doi: 10.3390/ma16145064.
 - 296.Kim GT, Jin J, Mangal U, Lee KJ, Kim KM, Choi SH, Kwon JS. Primary Stability of Orthodontic Titanium Miniscrews due to Cortical Bone Density and Re-Insertion. *Materials (Basel)*. 2020 Oct 5;13(19):4433. doi: 10.3390/ma13194433.
 - 297.Kang N, Wu YY, Gong P, Yue L, Ou GM. A study of force distribution of loading stresses on implant-bone interface on short implant length using 3-dimensional finite element analysis. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2014 Nov;118(5):519-23. doi: 10.1016/j.oooo.2014.05.021.
 - 298.Morita J, Wada M, Mameno T, Maeda Y, Ikebe K. Ideal placement of an implant considering the positional relationship to an opposing tooth in the first molar region: a three-dimensional finite element analysis. *Int J Implant Dent*. 2020 Jul 1;6(1):31. doi: 10.1186/s40729-020-00223-9.
 - 299.Pagliani L, Sennerby L, Petersson A, Verrocchi D, Volpe S, Andersson P. The relationship between resonance frequency analysis (RFA) and lateral displacement of dental implants: an in vitro study. *J Oral Rehabil*. 2013 Mar;40(3):221-7. doi: 10.1111/joor.12024.
 - 300.Fiorillo L, Cicciù M, D'Amico C, Mauceri R, Oteri G, Cervino G. Finite Element Method and Von Mises Investigation on Bone Response to Dynamic Stress with a Novel Conical Dental Implant Connection. *Biomed Res Int*. 2020 Oct 7;2020:2976067. doi: 10.1155/2020/2976067.
 - 301.Baggi L, Di Girolamo M, Vairo G, Sannino G. Comparative evaluation of osseointegrated dental implants based on platform-switching concept: influence of diameter, length, thread shape, and in-bone positioning depth on stress-based performance. *Comput Math Methods Med*. 2013;2013:250929. doi: 10.1155/2013/250929. Epub 2013 Jun 19. Erratum in: *Comput Math Methods Med*. 2014;2014:467358. Baggi, Luigi [added]; Di Girolamo, Michele [added].
 - 302.Yang Y, Liu Y, Yuan X, Ren M, Chen X, Luo L, Zheng L, Liu Y. Three-dimensional finite element analysis of stress distribution on short implants with different bone conditions and osseointegration rates. *BMC Oral Health*. 2023 Apr 15;23(1):220. doi: 10.1186/s12903-023-02945-9.
 - 303.Esposito M, Grusovin MG, Maghaireh H, Worthington HV. Interventions for replacing missing teeth: different times for loading dental implants. *Cochrane Database Syst Rev*. 2013 Mar 28;2013(3):CD003878. doi: 10.1002/14651858.CD003878.pub5.
 - 304.Hériveaux Y, Le Cann S, Fraulob M, Vennat E, Nguyen VH, Haïat G. Mechanical micromodeling of stress-shielding at the bone-implant interphase under shear loading. *Med Biol Eng Comput*. 2022 Nov;60(11):3281-3293. doi: 10.1007/s11517-022-02657-2.
 - 305.Ceddia M, Romasco T, Comuzzi L, Cipollina A, Piattelli A, Dipalma G, Inchingolo AM, Inchingolo F, Di Pietro N, Trentadue B. The Influence of Cement Thickness within the Cap on Stress Distribution for Dental Implants. *J Funct Biomater*. 2024 Jul 21;15(7):199. doi: 10.3390/jfb15070199.
 - 306.Ceddia M, Romasco T, Comuzzi L, Specchiulli A, Piattelli A, Lamberti L, Di Pietro N, Trentadue B. Finite-Element Analysis Study Comparing Titanium and Polyetheretherketone Caps in a Conometric Connection between Implant and

- Prosthesis. *Adv Eng Mater.* 2024 Apr;26(12):2400198. doi: 10.1002/adem.202400198.
- 307.Ceddia M, Comuzzi L, Di Pietro N, Romasco T, Specchiulli A, Piattelli A, Trentadue B. Finite Element Analysis (FEA) for the Evaluation of Retention in a Conometric Connection for Implant and Prosthesis. *Osteology.* 2023; 3(4):140-156. doi: 10.3390/osteology3040015.
 - 308.Ceddia M, Romasco T, De Bortoli N Jr., Mello BF, Piattelli A, Mijiritsky E, Di Pietro N, Trentadue B. Biomechanical Finite Element Analysis of Two Types of Short-Angled Implants Across Various Bone Classifications. *Materials.* 2024; 17(23):5680. doi: 10.3390/ma17235680.
 - 309.Di Pietro N, Ceddia M, Romasco T, De Bortoli Junior N, Mello BF, Tumedei M, Specchiulli A, Piattelli A, Trentadue B. Finite Element Analysis (FEA) of the Stress and Strain Distribution in Cone-Morse Implant–Abutment Connection Implants Placed Equicrestally and Subcrestally. *App Sci.* 2023; 13(14):8147. doi: 10.3390/app13148147.
 - 310.Száva DT, Száva A, Száva J, Gálfi B, Vlase S. Dental Implant and Natural Tooth Micro-Movements during Mastication-In Vivo Study with 3D VIC Method. *J Pers Med.* 2022 Oct 10;12(10):1690. doi: 10.3390/jpm12101690.
 - 311.Wu V, Schulten EAJM, Helder MN, Ten Bruggenkate CM, Bravenboer N, Klein-Nulend J. Bone vitality and vascularization of mandibular and maxillary bone grafts in maxillary sinus floor elevation: A retrospective cohort study. *Clin Implant Dent Relat Res.* 2023 Feb;25(1):141-151. doi: 10.1111/cid.13142.
 - 312.Watson EC, Adams RH. *Biology of Bone: The Vasculature of the Skeletal System.* Cold Spring Harb Perspect Med. 2018 Jul 2;8(7):a031559. doi: 10.1101/cshperspect.a031559.
 - 313.Saghiri MA, Asatourian A, Garcia-Godoy F, Sheibani N. The role of angiogenesis in implant dentistry part I: Review of titanium alloys, surface characteristics and treatments. *Med Oral Patol Oral Cir Bucal.* 2016 Jul 1;21(4):e514-525. doi: 10.4317/medoral.21199.
 - 314.Filipowska J, Tomaszewski KA, Niedźwiedzki Ł, Walocha JA, Niedźwiedzki T. The role of vasculature in bone development, regeneration and proper systemic functioning. *Angiogenesis.* 2017 Aug;20(3):291-302. doi: 10.1007/s10456-017-9541-1.
 - 315.Carulli C, Innocenti M, Brandi ML. Bone vascularization in normal and disease conditions. *Front Endocrinol (Lausanne).* 2013 Aug 26;4:106. doi: 10.3389/fendo.2013.00106.
 - 316.Jang H-J, Yoon J-K. The Role of Vasculature and Angiogenic Strategies in Bone Regeneration. *Biomimetics.* 2024; 9(2):75. doi: 10.3390/biomimetics9020075.
 - 317.Allsopp BJ, Hunter-Smith DJ, Rozen WM. Vascularized versus Nonvascularized Bone Grafts: What Is the Evidence? *Clin Orthop Relat Res.* 2016 May;474(5):1319-27. doi: 10.1007/s11999-016-4769-4.
 - 318.Senger DR, Davis GE. Angiogenesis. *Cold Spring Harb Perspect Biol.* 2011 Aug 1;3(8):a005090. doi: 10.1101/cshperspect.a005090.
 - 319.Sottile J. Regulation of angiogenesis by extracellular matrix. *Biochim Biophys Acta.* 2004 Mar 4;1654(1):13-22. doi: 10.1016/j.bbcan.2003.07.002.
 - 320.Walia A, Yang JF, Huang YH, Rosenblatt MI, Chang JH, Azar DT. Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. *Biochim Biophys Acta.* 2015 Dec;1850(12):2422-38. doi: 10.1016/j.bbagen.2015.09.007.
 - 321.Saberianpour S, Heidarzadeh M, Geranmayeh MH, Hosseinkhani H, Rahbarghazi R, Nouri Mohammad. Tissue engineering strategies for the induction of angiogenesis using biomaterials. *J Biol Eng.* 2018 Dec;12:36. doi: 10.1186/s13036-018-0133-4.

- 322.Ngo MT, Harley BAC. Angiogenic biomaterials to promote therapeutic regeneration and investigate disease progression. *Biomaterials*. 2020 Oct;255:120207. doi: 10.1016/j.biomaterials.2020.120207.
- 323.Ellermann E, Meyer N, Cameron RE, Best SM. In vitro angiogenesis in response to biomaterial properties for bone tissue engineering: a review of the state of the art. *Regen Biomater*. 2023 Mar 27;10:rbad027. doi: 10.1093/rb/rbad027.
- 324.Zhao W, Zhu J, Hang J, Zeng W. Biomaterials to promote vascularization in tissue engineering organs and ischemic fibrotic diseases. *MedComm - Biomater Appl*. 2022 Sep; 1(1):e16. doi:10.1002/mba2.16.
- 325.Nannmark U, Sennerby L. The bone tissue responses to prehydrated and collagenated cortico-cancellous porcine bone grafts: a study in rabbit maxillary defects. *Clin Implant Dent Relat Res*. 2008 Dec;10(4):264-70. doi: 10.1111/j.1708-8208.2007.00080.x.
- 326.Rombouts C, Jeanneau C, Camilleri J, Laurent P, About I. Characterization and angiogenic potential of xenogeneic bone grafting materials: Role of periodontal ligament cells. *Dent Mater J*. 2016 Dec 1;35(6):900-907. doi: 10.4012/dmj.2016-005.
- 327.Zhu WQ, Li K, Su S, Chen W, Liu Y, Qiu J. Effects of Zinc Ions Released From Ti-NW-Zn Surface on Osteogenesis and Angiogenesis In Vitro and in an In Vivo Zebrafish Model. *Front Bioeng Biotechnol*. 2022 Apr 21;10:848769. doi: 10.3389/fbioe.2022.848769.
- 328.Chávez MN, Aedo G, Fierro FA, Allende ML, Egaña JT. Zebrafish as an Emerging Model Organism to Study Angiogenesis in Development and Regeneration. *Front Physiol*. 2016 Mar 8;7:56. doi: 10.3389/fphys.2016.00056.
- 329.Poss KD, Keating MT, Nechiporuk A. Tales of regeneration in zebrafish. *Dev Dyn*. 2003 Feb;226(2):202-10. doi: 10.1002/dvdy.10220.
- 330.Saleh MHA, Sabri H, Di Pietro N, Comuzzi L, Geurs NC, Bou Semaan L, Piattelli A. Clinical Indications and Outcomes of Sinus Floor Augmentation With Bone Substitutes: An Evidence-Based Review. *Clin Implant Dent Relat Res*. 2024 Oct 17. doi: 10.1111/cid.13400.
- 331.Comuzzi L, Tumedei M, Piattelli A, Tartaglia G, Del Fabbro M. Radiographic Analysis of Graft Dimensional Changes in Transcrestal Maxillary Sinus Augmentation: A Retrospective Study. *Materials (Basel)*. 2022 Apr 19;15(9):2964. doi: 10.3390/ma15092964.
- 332.OECD. TN. 236: Fish Embryo Acute Toxicity (FET) Test. In *OECD Guidelines for the Testing of Chemicals*; OECD: Paris, France. 2013; 2:1–22.
- 333.Renshaw SA, Loynes CA, Trushell DM, Elworthy S, Ingham PW, Whyte MK. A transgenic zebrafish model of neutrophilic inflammation. *Blood*. 2006 Dec 15;108(13):3976-8. doi: 10.1182/blood-2006-05-024075.
- 334.Delasoie J, Pavic A, Voutier N, Vojnovic S, Crochet A, Nikodinovic-Runic J, Zobi F. Identification of novel potent and non-toxic anticancer, anti-angiogenic and antimetastatic rhenium complexes against colorectal carcinoma. *Eur J Med Chem*. 2020 Oct 15;204:112583. doi: 10.1016/j.ejmech.2020.112583.
- 335.Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol*. 2002 Aug 15;248(2):307-18. doi: 10.1006/dbio.2002.0711.
- 336.Pavic A, Glišić BĐ, Vojnovic S, Warzajtis B, Savić ND, Antić M, Radenković S, Janjić GV, Nikodinovic-Runic J, Rychlewska U, Djuran MI. Mononuclear gold(III) complexes with phenanthroline ligands as efficient inhibitors of angiogenesis: A comparative study with auranofin and sunitinib. *J Inorg Biochem*. 2017 Sep;174:156-168. doi: 10.1016/j.jinorgbio.2017.06.009.
- 337.MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. *Nat Rev Drug*

- Discov. 2015 Oct;14(10):721-31. doi: 10.1038/nrd4627.
- 338.Ohashi ASC, de Souza Schacher HR, Pizzato CS, Vianna MRMR, de Menezes LM. Zebrafish as model for studies in dentistry. *J Orthod Sci.* 2022 Oct 13;11:46. doi: 10.4103/jos.jos_41_22.
 - 339.Radunovic M, Pavic A, Ivanovic V, Milivojevic M, Radovic I, Di Carlo R, Pilato S, Fontana A, Piattelli A, Petrovic S. Biocompatibility and antibiofilm activity of graphene-oxide functionalized titanium discs and collagen membranes. *Dent Mater.* 2022 Jul;38(7):1117-1127. doi: 10.1016/j.dental.2022.04.024.
 - 340.Vlajić Tovilović T, Petrović S, Lazarević M, Pavić A, Plačkić N, Milovanović A, Milošević M, Miletic V, Veljović D, Radunović M. Effect of Acetylsalicylic Acid on Biological Properties of Novel Cement Based on Calcium Phosphate Doped with Ions of Strontium, Copper, and Zinc. *Int J Mol Sci.* 2024 Jul 20;25(14):7940. doi: 10.3390/ijms25147940.
 - 341.Zhu WQ, Li K, Su S, Chen W, Liu Y, Qiu J. Effects of Zinc Ions Released From Ti-NW-Zn Surface on Osteogenesis and Angiogenesis In Vitro and in an In Vivo Zebrafish Model. *Front Bioeng Biotechnol.* 2022 Apr 21;10:848769. doi: 10.3389/fbioe.2022.848769.
 - 342.Xiong XY, Liu Y, Shan LT, Xu YQ, Liang J, Lai YH, Hsiao CD. Evaluation of collagen mixture on promoting skin wound healing in zebrafish caused by acetic acid administration. *Biochem Biophys Res Commun.* 2018 Oct 28;505(2):516-522. doi: 10.1016/j.bbrc.2018.09.148.
 - 343.Senk A, Djonov V. Collagen fibers provide guidance cues for capillary regrowth during regenerative angiogenesis in zebrafish. *Sci Rep.* 2021 Sep 30;11(1):19520. doi: 10.1038/s41598-021-98852-6.
 - 344.Jeanneau C, Catherine JH, Giraud T, Lan R, About I. The Added Value of a Collagenated Thermosensitive Bone Substitute as a Scaffold for Bone Regeneration. *Materials (Basel).* 2024 Jan 27;17(3):625. doi: 10.3390/ma17030625.
 - 345.Lopez MA, Passarelli PC, Netti A, Marianelli F, Wychowański P, Garcia-Godoy F, D'Addona A. A new technique of alveolar bone augmentation: "Pack into Bone". A retrospective case-series study. *Am J Dent.* 2024 Sept;37(SIA):41A-44A. PMID: 39374512.
 - 346.Passarelli PC, Lopez MA, Netti A, Felicetti A, Wychowański P, Garcia-Godoy F, Chiapasco M, D'Addona A. Alveolar regeneration of the post-extraction site with cortical deficiency using the Lamina Socket Sealing technique: Retrospective study with histomorphometric analysis of regenerated bone and evaluation of soft tissue changes. Part 2/2. *Am J Dent.* 2024 Sept;37(SIA):9A-12A. PMID: 39374504.

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