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YEDITEPE UNIVERSITY

INSTITUTE OF OF HEALTH SCIENCES

DEPARTMENT OF ORAL AND MAXILLOFACIAL SURGERY

**AN IN VITRO STUDY OF THE CYTOTOXICITY
OF GRADE IV COMERCIAALLY PURE TITANIUM
AND GRADE V (TI-6Al-4V) ALLOY USED IN
DENTAL IMPLANTS**

DOCTOR OF PHILOSOPHY THESIS

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DECLARATION

I hereby declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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29 / 07 /2024



DEDICATION

I dedicate this work to my:

- Mother: Jawhra Ramadan Esslami.
- Father: Mokhtar Abdallah Esslami.
- Wonderful Wife: Nagla Mokhtar Marwan.
- Lovely Children: Malak, Mutaz, Moad and Muawiyah.

I could not have done this without you all!



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LIST OF SYMBOLS AND ABBREVIATIONS

Al_2O_3	: Aluminum Oxide Coating
Al_2O_3	: Aluminum Oxide
ALP	: Alkaline Phosphatase Assay
ASTM	: American Society of Testing and Materials
ATCC	: American Type Culture Collection
ATP Assay	: Adenosine Triphosphate Assay
BSA	: Bovine Serum Albumin
$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	: Calcium Hydroxyapatite
Ca_2P	: Calcium phosphide
CCL5	: Chemokine (C-C motif) ligand 5
Co-Cr Alloy	: Cobalt-Chromium Alloy
Co-Cr-Mo	: Cobalt-Chromium-Molybdenum
Cp	: Commercial pure
CpTi	: Commercial Pure Titanium
Cr	: Chromium
Cu	: Copper
DAPI	: 4'6-Diamidino-2-Phenylindole
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
ECM	: Extracellular Matrix
EGF	: Epidermal Growth Factor
FBS	: Fetal Bovine Serum
FDA	: Food and Drug Administration
FGF	: Fibroblast Growth Factor

GPa	: Giga-Pascal
HA	: Hydroxyapatite
HCT 115	: Human Colon Carcinoma Cells
HeLa	: Henrietta Lacks Cells (Human cervical carcinoma Cells)
HGFs	: Human gingival fibroblasts
hMSCs	: Human Mesenchymal Stem Cells
HRC	: Hardness Rockwell C Scale
IGF	: Insulin-like Growth Factor
ISO	: International Standard Organization
KCL	: Potassium chloride
L929	: Mouse Fibroblast Cells
MC3T3-E1cells	: Mus musculus (mouse) calvaria cells
Min	: Minute.
L132	: Human embryonic lung cell line
MTIs	: Mini Transitional Implants
MMPs	: Metalloprotease
Mpa	: Mega-Pascal
Ms	: Macrophages
MSCs	: Mesenchymal Stem Cells
MTT	: (3-(4,5-Dimethylthiazolyl-2)- 2,5diphenyltetrazolium bromide
NaCL	: Sodium Chloride
NaF	: Sodium Fluoride
Ni-Cr	: Nickel-Chromium Alloy
Ni-Cr-Be	: Nickel-Chromium-Beryllium Alloy
NGF	: Nerve Growth Factor

NI	: Nickel
NITI	: Nickel Titanium Alloy
NPs	: Nanoparticles
OB	: Optimal Density
PBS	: Phosphate Buffered Saline
PDGF	: Platelet-Derived Growth Factor
PEEK	: Polyether Ether Ketone
PO ₄ ³⁻	: Phosphate ion or Orthophosphate
RPMI	: Roswell Park Memorial Institute medium
SA	: Sandblastings
SaOs-2	: Bone Osteosarcoma Cells
SaOs-2	: Osteosarcoma Cell Line
SDH	: Succinic Dehydrogenase Activity
SEM	: Scanning Electron Microscope
SLA	: Sandblasting large grit, Acid-etch.
SS	: Stainless Steel
Ta	: Tantalum
TCP	: Tricalcium Phosphate
TEM	: Transmission Electron Microscopy
Ti	: Titanium
Ti Al	: Titanium Aluminide.
Ti6Al4V	: 90% Titanium, 6% Aluminum, 4% Vanadium
Ti-Nb alloys	: Titanium-Niobium alloys
TiO	: Titanium Oxide
TiO ₂	: Titanium Dioxide
TiO ₃	: Dtitanium Trioxide

UV / VIS	: Ultraviolet / Visible
WTNT1	: Wingless-Related Integration Site
ZrO ₂	: Dioxide of Zirconium
μm	: Micrometer
α	: Alpha
α-SMA	: Alpha -Smooth Action
β	: Beta
BALB/c-3T3	:3-day transfer, inoculum 3×10 ⁵ mouse cells



ABSTRACT

Essllami A.(2024) . An in vitro study of the cytotoxicity of grade IV commercially pure titanium and grade V (Ti-6Al-4V) alloy used in dental implants. Yeditepe University Institute of Health Science, Department of Oral and Maxillofacial Surgery, PhD. Istanbul.

This study set out to employ the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to investigate the cytotoxic effects of commercially pure titanium (Grade IV) and its alloy Ti-6Al-4V (Grade V) on the function of mice gingival fibroblasts (L 929 fibroblast) in cell culture. A total of thirty-two (n= 32) commercially pure titanium grade IV (ASTM F67) (No=16) and titanium grade V (Ti6Al4V) (no=16) were submitted to laboratory examination using a test setup like that recommended by International standard ISO 7405 guidelines. The grade IV specimen disks had a diameter of 6 mm and a thickness of 2 mm, but the grade V disks had a diameter of 8 mm and a thickness of 2 mm. The samples were placed in an incubator for 24, 48, and 72 hours, using a mixture of Roswell Park Memorial Institute (RPMI) medium, 10% fetal bovine serum (FBS), and 1% antibiotic. After each incubation period, the toxicity of the extracts on cultured mouse fibroblasts (L929) was assessed using the MTT test. The photometric experiments were performed using a high-quality UV/VIS spectrophotometer (Multiskan GO.USA) To evaluate cell viability, we desire to investigate the optimal circumstances for cell viability among these metals. The data were subjected to statistical analysis using a one-way repeated measures ANOVA test. Post-hoc tests were conducted at a significance threshold of $p < 0.05$ to examine the impact of time on cell viability percentage in each titanium grade type. The results show that after 24 hours, grade IV titanium had a 10% higher cell viability ($80.3 \pm 5.1\%$) than grade V titanium (70.3 ± 4.1) ($p < 0.001$). After 48 hours, it rose by 9.9% and 5.4% ($p < 0.001$). Additionally, grade IV titanium boosted cell viability by 5.4% after 72 hours, compared to grade V titanium ($p < 0.001$). As a conclusion, grade IV commercially pure titanium had superior cell viability than grade V titanium alloy throughout incubation. Both grade IV titanium and grade V titanium alloy are suitable for dental usage.

Keywords: cytotoxicity, gingival fibroblast, L929; MTT assay, Ti-6Al-4V, Grade IV titanium alloy.

ÖZET

Essllami A . (2024). Dental implantlar için ticari saf titanyumun (Grade IV) ve titanyumun alaşımı (Grade-V-Ti-6Al-4V) nın sitotoksitesi üzerine in V vitro Bir çalışma. Yeditepe Üniversitesi, Sağlık bilimler Enstitüsü, Oral Ve Maksillofacial Cerrahi Bölümü, Doktora çalışması. İstanbul.

Bu çalışmanın amacı, ticari saf titanyumun (Sınıf IV) ve Ti-6Al-4V alaşımının (Sınıf V) sıçan gingival fibroblast hücrelerindeki sitotoksitesini 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür (MTT) testi kullanarak değerlendirmektir. ASTM F67 standardına uygun olarak toplamda otuz iki adet sınıf IV ticari saf titanyum (N=16) ve Sınıf V titanyum alaşımı (Ti-6Al-4V) (N=16) örneği laboratuvar incelemesine tabi tutulmuştur. Sınıf IV için kullanılan örnek çapı 6 mm, kalınlığı 2 mm iken, sınıf V için kullanılan diskleri çapı 8 mm, kalınlığı 2 dir. Örnekler, Roswell Park Memorial Institute (RPMI)de %10 FBS ve %1 antibiyotik içeren ortamda 24, 48 ve 72 saat boyunca inkübe edilmiştir. Her inkübasyon süresi sonrasında, sıçan fibroblast hücreleri (L929) üzerindeki sitotoksitesi MTT testi ile ölçülmüştür. Hücre büyümesi ve canlılığı analiz etmek için yüksek kaliteli monokromatör tabanlı UV/VIS spektrofotometre (Multiskan GO) kullanılarak fotometrik analizler gerçekleştirilmiştir. Veriler, tek yönlü tekrarlı ölçümler ANOVA testi ve $p < 0,05$ anlamlılık düzeyindeki post-hoc testlerle istatistiksel olarak analiz edilmiştir. Sonuçlar, sınıf IV titanyumun, sınıf V titanyumdan üçüncü günde (72 saat) anlamlı derecede daha yüksek hücre canlılığı sergilediğini göstermiştir. 24 saat sonra, sınıf IV titanyumun hücre canlılığı $80,3 \pm 5,1$ iken, sınıf V titanyumun hücre canlılığı $70,3 \pm 4,1$ olarak belirlenmiştir ($p < 0,001$). Bu fark, 48 ve 72 saat sonra sırasıyla %9,9 ve %5,4'e kadar artmıştır ($p < 0,001$). Sınıf V titanyumun daha düşük hücre canlılığı, alaşımda bulunan alüminyum ve vanadyumun varlığından kaynaklanmaktadır. Sonuç olarak, ticari saf sınıf IV titanyum, inkübasyon süreleri boyunca sınıf V titanyum alaşımına göre daha iyi hücre canlılığı sergilemektedir. Hem sınıf IV titanyum hem de sınıf V titanyum alaşımı diş hekimliğinde kullanıma uygundur.

Anahtar Kelimeler: Grade V titanyum alaşımı (Ti-6Al-4V), Grade 4 saf titanyum, Sitotoksite, gingival fibroblast, L929; MTT testi.

1. INTRODUCTION

1.1 Background and significance of the study

Biomaterials are natural materials or can be made through a variety of physical and chemical reactions that produce metals and metallic alloys, composites, and ceramics. It can be used for diagnostic or therapeutic purposes, to restore lost organ or tissue function, to improve cosmetic appearance, or to aid in healing (1).

Among the most popular uses of biomaterials in the modern medical field is as medical implants that can restore, support, or enhance lost tissue function. Implants that can remain in the body for a limited time, such as fixation screws and plates used to repair broken bones, as well as implants that require long-term retention in the body, such as hip or hearth prostheses and dental implants (1).

Ever since their debut in 1981, titanium alloys have emerged as the foremost choice among metallic substances for biomedical applications. The adoption of titanium dental implants for the restoration of missing dentition in patients has witnessed a remarkable surge in prevalence (1).

This heightened favorability is attributable to their unparalleled mechanical and physical characteristics, encompassing exemplary resistance to corrosion and an elevated modulus of elasticity in tension. In addition, their outstanding biocompatibility has firmly established titanium that is commercially pure and its alloy counterparts as ubiquitous constituents in the production of orthopedic and dental implant devices (2).

Moreover, the elevated modulus of elasticity exhibited by CpTi, coupled with the inherent challenge of enhancing its mechanical attributes without jeopardizing biocompatibility, has been acknowledged as intrinsic features imposing constraints on its utility as a dental implant material (3). A titanium alloy can be used to increase this strength; for example, a strength of 930 MPa is achieved by combining titanium, aluminum, and vanadium (4).

The improved capacity to insert narrower or shorter implants without heightening the likelihood of implant fracture during use, obviating the necessity for intricate vertical bone augmentation in specific scenarios, and offering a dependable alternative in challenging locations such as narrow ridge defects are all benefits of a more robust dental implant (5).

In orthopedics, grade V titanium is commonly used (2). Its greater strength and higher Young's modulus are the causes of this. Its use in dentistry has been shown to be physiologically acceptable. On the other hand, this alloy emits both aluminum and vanadium, both of which can cause biological problems (2).

It has been discovered that aluminum inhibits the process of bone maturation (3), resulting in inadequacies in structure. It was also found that vanadium possesses cytotoxic capabilities and is able to cause type IV (allergic) responses. The presence of both components in the tissues at appropriate concentrations is required for these negative effects to manifest. It is noteworthy, therefore, that the amounts emitted by this alloy are well below the cutoff point necessary to have any harmful consequences (6).

Metals can undergo biodegradation in the oral cavity due to a combination of factors such as solubility in saliva, chemical and physical destruction, wear, and erosion induced by food, chewing, bacterial activity, humidity, temperature variations, and pH changes (9,10).

Particle concentrations in dental peri-implant mucosa are shown to be elevated in areas affected by peri-implantitis, as compared to areas unaffected by peri-implantitis. Particles were observed in the epithelial cells, macrophages, and the bone and soft tissue of affected regions (9).

By sealing the abutment with soft tissue, gingival fibroblasts preserve connective tissue and the implant (12,9). The extracellular matrix, synthesized by gingival fibroblasts, the most common cell type in peri-implant mucosal tissues, ensures attachment, wound healing, and epithelial downgrowth. For stability and maintenance, dental implants must interact with their surrounding cells (11).

These functions need the attachment and growth of human gingival fibroblasts (HGFs) on the implant's surface, as well as the continuous generation and replacement of the extracellular matrix (ECM) to ensure the synthesis and integrity of gingival connective tissue (12).

In vitro, the biocompatibility of a substance may be assessed using direct, indirect, and extraction test methods. The method chosen is affected by the material's physical and chemical properties (7).

The standard known as ISO 10993 is a standardization method aimed at maximizing the safety of the material because of research in biomaterials. This guideline includes methods for selecting test samples, appropriate extraction methods, and illustrations of control groups (8).

The ISO 10993 standard was developed to investigate the biocompatibility of the studied material on the relevant cells in a controllable in vitro environment before moving on to the in vivo research phase in biocompatibility studies (8).

ISO 7405 (19) as an international standard is entitled " "Preclinical analysis to determine the compatibility of medical equipment with living organisms." The ISO document was created in partnership with the World Dental Federation. The subject matter pertains to the preclinical evaluation of dental materials, in addition to their compliance with ISO 10993 (9).

Permanently established cell lines, such as genetically modified mouse fibroblasts (specifically clone L-929), are commonly accessible and offer a reliable method for consistent cytotoxicity testing across many research facilities (16).

To the best of our knowledge, there have been a few reviews of the impact of commercially pure titanium (grade IV) and Ti-6Al-4 (grade V) alloy on fibroblasts, a crucial cell type involved in the integration of dental implants. If the Ti-6Al-4 grade V titanium alloy has cytotoxic effects on fibroblasts, it may contribute to a failure mechanism (13).

The current research is to study cytotoxicity characteristics of commercially available grade IV pure titanium and its alloy, Ti-6Al-4V (grade V), on gingival fibroblasts. The assessment will be conducted employing an MTT test to measure the activity of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

The primary objective is to determine the viability and cytotoxicity potential of grade IV titanium and grade V titanium alloy on fibroblast cells through the implementation of the MTT assay. This assay relies on the enzymatic activity of the cells to convert MTT into a formazan product, which can be quantified spectrophotometrically to provide information regarding cell viability and metabolic activity.

Furthermore, the study aims to compare the cellular response to Ti-6Al-4V (grade V) with commercially pure titanium (grade IV). By assessing the differential effects of these materials on the fibroblast cells, the research intends to generate substantial data and evidence-based recommendations concerning the biocompatibility of grade IV titanium and grade V titanium alloy specifically in the context of dental implant applications. These recommendations will be derived from the results obtained through the utilization of the MTT assay and can contribute to informed decision-making in dental implantology practice.

2. REVIEW OF LITERATURE

2.1 Soft tissue implant contact

2.1.1 Tooth anatomy

The teeth are diminutive masticatory structures composed of four distinct types of tissue: two outer layers (enamel and cement) and two inner layers (dentin and pulp) (Figure 2.1). The enamel layer encases the exposed and prominent portion of the gum around the tooth, known as the crown (17). The cement covers the root, which is invisible and has a pointed shape. The root is housed in the dental alveoli, which is a cavity in the maxillary bone. Both the cement and the root are supported by the dentin.

The pulp is in a central cavity that extends from the crown to the root of a tooth. This cavity is connected to the periodontium through an opening at the tip of the root. The neck serves as a boundary between the crown and root. The pulp cavity of the crown and root canal contains highly vascularized and innervated connective tissue. Dental pulp, rich in collagen, and odontoblast (dentin cells that create intercellular material) distinguish it from other connective tissues (20). Nutrition, sensory, and dentin support are the key functions (10).

Enamel is a non-vascular and non-innervated tissue that originates from epithelial cells. Ameloblasts, which are responsible for producing enamel, become atrophic as a person ages, resulting in the degeneration of this acellular tissue. Enamel is exceptionally hard, being the toughest substance in the human body, mostly because of its high mineral content. Approximately 96% of the inorganic material in enamel is composed of hydroxyapatite crystals, which are arranged into enamel prisms. The remaining 3-4% is water (19).

Dentin, while comparatively less rigid than enamel, maintains a hardness level exceeding that of bone. It is mostly made up of minerals (about 70%), organic matter (20%), and water (10%) by weight. The crystals predominantly consist of calcium hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, with a minor presence of carbonate and fluoride. Dentin serves as a protective layer for the pulp and exhibits remarkable tensile strength (20).

Cement is a mineralized substance that forms a protective layer around the root. Due to its lack of blood vessels, it is classified as an avascular tissue and therefore not classified as bone tissue. Cement is formed of a combination of inorganic substances and mineralized organic matter; it has a structure and composition comparable to bone, including cementocytes that fill in spaces called lacunae.

It covers the root, which is an invisible and sharply shaped part that is within the dental alveoli (cavity of the maxillary bone) and is covered by dentin (20,21).

Enamel, dentin, and pulp are the actual tooth tissues from an embryological and functional perspective. however, cement, periodontal ligament and gingiva constitute the periodontium (11).

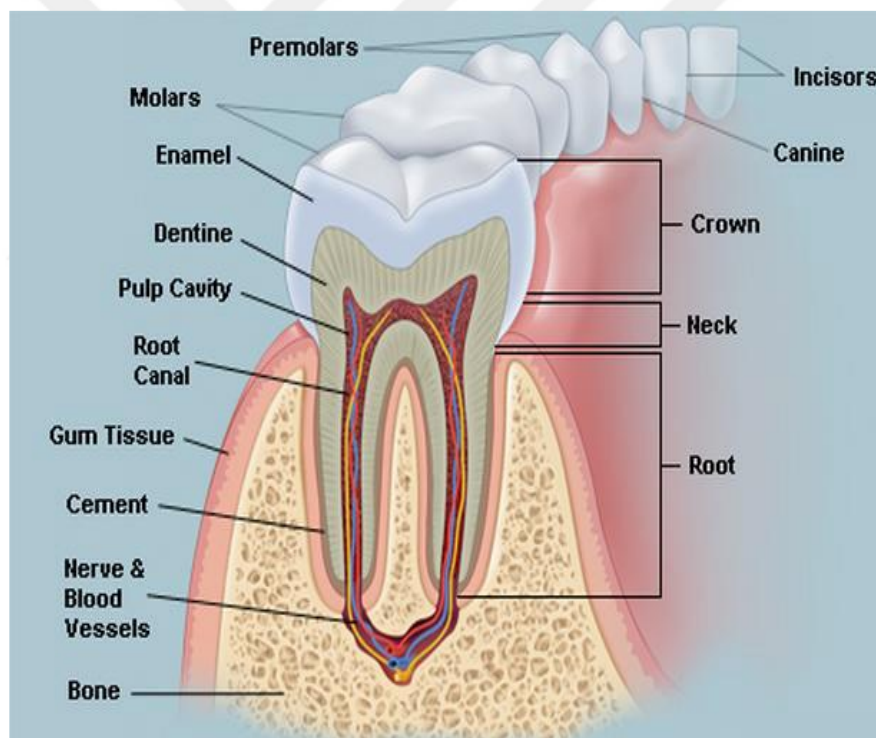


Figure.2.1.Tooth anatomy (12)

2.1.2 Periodontal anatomy

The periodontium refers to the structures that support the tooth, which include:

- Gingiva.
- Paradoxical ligament.
- Root cementum and alveolar bone

The gingiva is a mucous membrane that encircles the neck and overlays the alveolar processes, extending between the individual teeth to create the gingival papilla. The alveolar mucosa, located at the apex of the teeth, can be distinguished from the fixed gingiva by its vibrant red hue. The dento-gingival junction in the normal dentition is composed of keratinized gingival epithelium, non-keratinized sulcular or crevicular epithelium, and junctional epithelium (JE), all of which are supported by collagen fiber bundles in a connective tissue (19).

The periodontal ligament is located in the area between the surface of the tooth root and the wall of the alveolar cavity. It is adjacent to the gingiva at the top and the pulp at the bottom. The primary role of the ligament is to connect the collagen fibers, which are arranged in bundles, to both the cement and alveolar bone. These fibers contain blood vessels and nerves, making them responsive to pressure. Consequently, the ligament serves to safeguard the tooth by offering sufficient protection against such stressors. This tissue contains many types of cells, including cementoblasts, osteoblasts, and fibroblasts (19).

Gingiva is mostly composed of connective tissue, which is generally comprised of collagen fibers (65%), fibroblasts (5%), and blood vessels and nerves (35%) embedded in an amorphous matrix (19). The connective tissue fibers establish a connection between the tooth cementum and the adjacent alveolar bone and gingiva. Cell-mediated hemidesmosomes connect the junctional epithelium (JE) to natural tooth surfaces through a basal lamina at the electron microscopic level (22) (Figure.2.2).

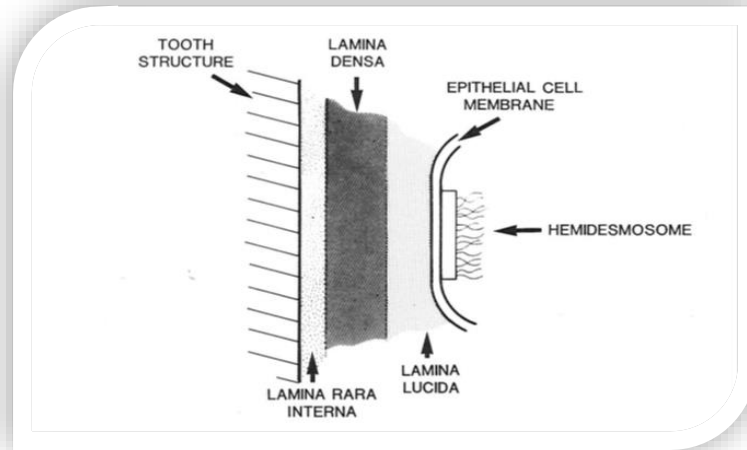


Figure.2. 2. Cell-mediated hemidesmosome attach the JE to natural tooth surfaces (13).

2.1.3 Fibroblasts

Fibroblasts are cells that have an elongated shape with prolonged cell processes, giving them a fusiform or spindle-like appearance when viewed from the side. Fibroblasts have a wide distribution and can be easily cultured in a laboratory setting. They are commonly recognized by their elongated appearance, their capacity to stick to plastic surfaces, and their absence of markers that signify different cell types (14).

Fibroblasts were first identified in the late 19th century primarily for their spatial distribution and microscopic morphology (Figure.2.3) (23). Ernst Ziegler originally used the word "fibroblast" to characterize cells that generate new connective tissue during the healing process (26).

Fibroblasts represent the archetypal and more abundant cellular constituents of connective tissue, demonstrating a remarkable capacity for synthesizing the extracellular matrix and collagen. They are non-vascular, non-epithelial and non-inflammatory. Their pivotal role in wound healing is well-established. Morphologically, Fibroblasts are identified by their cytoplasm that has branches and surrounds an oval-shaped nucleus. Their diameters normally vary between approximately 15 and 20 μm . (15) .

The connective tissue's fibrous components are the handiwork of fibroblasts, forming a structural scaffold for the extracellular matrix while also serving as connectors that link the matrix's constituents to the cells (15).

The gingival connective tissue is highly organized to tightly encircle the tooth's neck, thereby preserving the integrity of the adhesion between the dentin and the gingiva. Collagen fibers specifically strengthen the interdental papilla, provide flexibility to the gingiva, and maintain its anatomical shape (15).

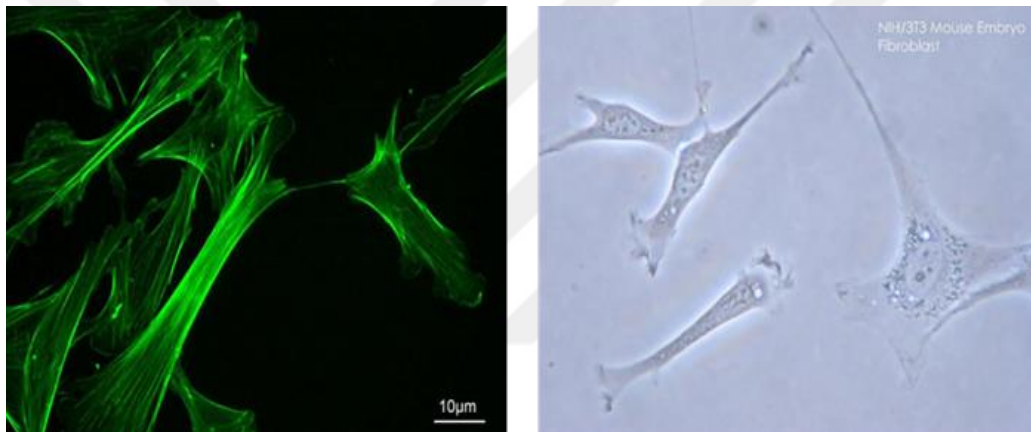


Figure.2. 3.Fibroblasts (14)

The fibrous tissue in the gingiva serves several assignments (24):

- Assistance for the functioning of nerves, blood vessels, and lymphatics.
- The detachment of the epithelium from the underlying tissues.
- Assistance for the temporary cellular population.
- assistance for the enduring cellular population of the immune system

Under normal physiological circumstances, fibroblasts are dormant cells that have a long lifespan, and typically do not undergo growth or death (25). It produces the primary building blocks of the extracellular matrix (ECM), including type I, II, and IV collagen and fibronectin. Additionally, it aids in the creation of basal membranes by releasing type IV collagen and laminin. Additionally, they play a crucial role in the production of proteases, specifically metalloprotease (MMPs), that are vital for the breakdown and regeneration of the matrix (24, 25).

Fibroblasts have a significant part in the process of normal tissue repair (25). During these processes, the fibroblast undergoes a transformation from a dormant cell to an activated state, where it expresses α -smooth-actin cushions (α -SMA) known as myofibroblasts. This transformation is marked by increased cell division and the release of large amounts of extracellular matrix (ECM) protein.

Activated fibroblasts secrete elevated levels of MMPs, which promote a quick turnover of the ECM. This can result in alterations in its composition. Additionally, activated fibroblasts exhibit heightened production of growth factors such as HGF, IGF, NGF, EGF, FGF2, and WNT1. These growth factors stimulate the proliferation of neighboring epithelial cells (16).

The process of fibroblast accumulation typically commences within the timeframe of 3 to 5 days following an injury can endure for up to 14 days. Upon implantation of biomaterials, fibroblasts undergo a transformation referred to as "activation," characterized by the conversion of quiescent cells into a phenotype resembling myofibroblasts (17).

The initiation of fibroblast activation is primarily elicited by stimuli originating from macrophages. The responses exhibited by fibroblasts upon activation encompass cellular proliferation, fibrinogenesis, and the secretion of cytokines and proteolytic enzymes (17).

During the healing process, there is an observable increase in the population of fibroblasts, concurrent with a decline in the number of inflammatory cells. This increased fibroblast population surrounding the implant is mostly explained by local proliferation triggered by fibronectin, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (17).

Fibroblast cells exhibit proliferation when in contact with titanium, niobium, zirconium, and tantalum, but not in close proximity to molybdenum, copper, or vanadium. When soft tissue comes into touch with implanted metals, these reactions are characterized by toxicity or sequestration (18).

Myofibroblasts can also influence the immune response following tissue injury by the release of cytokines and chemokines (16). Upon activation, myofibroblasts infiltrate the location of the tissue injury, generate extracellular matrix (ECM) that provides structural support for other cell types, and possess cytoskeletal components that facilitate contraction during the process of wound healing (16).

2.1.4 Osteoblasts

Mesenchymal stem cells (MSCs), also referred to as marrow stroma cells, are a type of undifferentiated tissue cells that come from the bone marrow stroma. These versatile cells possess the capability to undergo cellular differentiation into many cell types, including adipocytes, chondrocytes, muscle cells, or osteoblasts, contingent upon the surrounding environmental cues. MSCs are particularly alluring subjects of study in bone regeneration research due to their capacity for both proliferation and differentiation into osteogenic lineage cells (18).

Osteoblasts, recognized as the architects of bone formation, are cuboidal cells with a distinct role in the process (19). These cells are situated adjacent to the bone's surface (20) and are characterized by an abundance of rough endoplasmic reticulum, a prominent Golgi apparatus, and secretory vesicles, similar to cells engaged in protein synthesis. Osteoblasts play a pivotal role in bone formation by secreting osteoid at sites dedicated to bone construction (21).

In contrast, osteoclasts are specialized in the breakdown of the bone matrix. This enzymatic resorption process is succeeded through the appearance of newly formed osteoblasts, which then restore the bone tissue that has been absorbed. The newly formed bone undergoes maturation and persists until the initiation of a fresh remodeling cycle.

Following implantation, osteoblasts attach to the surface of the implant and facilitate its secure attachment through a process known as osseointegration. This process establishes a direct solid bond between healthy bone tissue and the surface of an implant that bears weight, ensuring both structural and functional integration (19).

Experiential investigations on the bone reaction to machined cp-Ti and Ti6Al4V reveal comparable osseointegration. According to the available literature, it can be inferred that both commercially pure titanium (Cp-Ti) and Ti6Al4V exhibit analogous outcomes in terms of osseointegration and biomechanical anchorage (22).

A study revealed similar results when examining the adhesion of *S. epidermidis* to Ti6Al4V alloy compared to pure titanium, aluminum, and vanadium metal surfaces. The number of bacterial cells that adhered to pure titanium and Ti6Al4V was equivalent (15).

2.1.5 Summary of the classical stages of wound repair

The haemostatic phase: is triggered by tissue damage, such as those occurring after periodontal surgery (23). The defect location is promptly sealed by the developing blood clot that resulted from the process of blood coagulation.

The inflammatory phase: like hemostatic. The complement system, chemokines, and fibrinogen-degrading peptides attract neutrophils (24). Endothelium controls cell extravasation and migration into surrounding tissue (25).

Damaged areas have neutrophils and monocytes within one and 24 hours. The wound is cleaned, and bacteria and proteases are phagocytosed. Catabolic inflammation is transient but necessary for the anabolic phase, which creates new tissue (26).

The process of new tissue formation begins with the development of "granulation tissue," which refers to a highly vascularized tissue composed of fibroblast cells and an extracellular matrix. The transition from the catabolic to the anabolic phase necessitates the activation of an intricate mechanism that involves a minimum of three types of cells: endothelial cells, fibroblasts, and epithelial cells (27).

The phase of remodeling that occurs over an extended prolonged duration: Fibrosis, often known as scar tissue, initiates its formation during the remodeling phase. Apoptosis affects most of the myofibroblasts, fibroblasts, endothelial cells, and macrophages, resulting in the survival of only a small number of cells in the extracellular matrix that is rich in collagen (39). The diagram in (Figure 2.4) provides a concise overview of the conventional phases involved in the healing process of soft tissues.

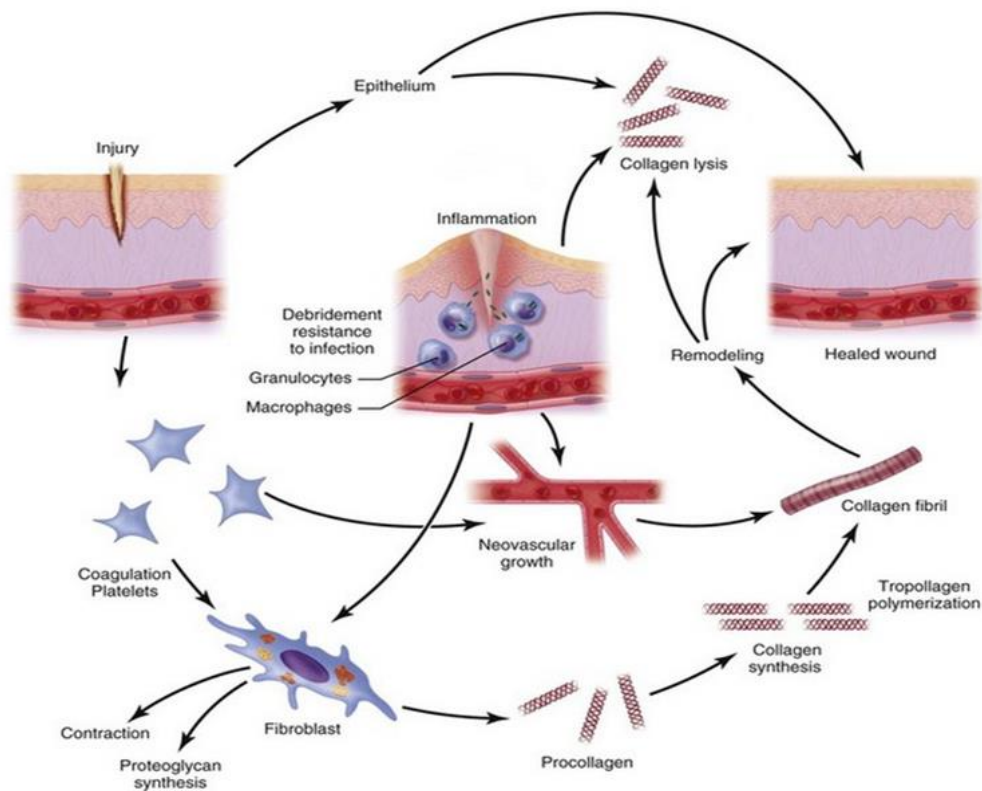


Figure.2. 4.Summary of the soft tissue healing process (28).

2.1.6 Soft tissue healing around implants.

The soft tissue around the implant consists of oral epithelium, junctional epithelium, and the underlying fibrous connective tissue, just like natural teeth. Nevertheless, notable distinctions exist between them: it has been documented that the epithelium and connective tissues around the implant exhibit a lower standard of attachment compared to that of natural teeth (18) .

The success of dental implant therapy relies on three key components: osseointegration, epithelial seal, and connective tissue attachment. An effective seal between the peri-implant epithelium and implant surface can restrict the formation of epithelial tissue, inhibit bacterial colonization, and enhance the protective function of the implant (30). The structure of the soft tissues around dental implants, including both epithelium and connective tissues, has been extensively studied and shown to closely resemble that of natural teeth.

A pivotal component of this interface is the junctional epithelium, which forms a hemidesmosomal attachment to both dental implants and natural teeth, with an approximate length of 2 mm.

Nevertheless, it is crucial to acknowledge that the connective tissue interface differs based on the geometry and type of attachment. In the case of teeth, the connective tissue zone spans approximately 1 mm. In contrast, around dental implants, this connective tissue zone extends to around 2 mm. Collectively, the combination of the junctional and connective tissue attachments is commonly referred to as the "soft tissue seal." (23).

The connective tissue around dental implants is distinguished by collagen fibers that are oriented parallel to the vertical axis of the implant body (24). The role of gingival fibroblasts in creating collagen, glycoproteins, and extracellular matrix is crucial in determining the success of implant treatment. Their initial attachment and response to the implant surface play a significant role in this result (25).

Dental implants are anchored in the jawbone through a direct connection between the bone and the implant. This biological seal protects against the development of inflammatory preimplant illnesses (peri-implant mucositis and peri-implantitis). Therefore, the presence of a soft tissue seal around implants facilitates favorable conditions, ensures dependable osseointegration, and consequently contributes to the long-term durability of the implant. The soft tissue around the implant is referred to as "peri-implant mucosa" (44).

The biocompatibility of dental implants is determined by both the surface qualities of the materials and the growth behavior of cells on the material surface when they are implanted into host bone. The early interaction between cells and materials will directly impact the cell's ability to multiply and specialize (26).

Therefore, osteoblasts, epithelial cells, and gingival fibroblasts are the primary focus of research when it comes to developing biofunctional dental materials. The initial cellular response of three distinct cell types, including adhesion, morphology, and proliferation, to titanium surfaces can alter according to the surface topography (25).

The fusion of the hard and soft tissue with the implant occurs during a wound healing process that spans over several weeks. The characteristics of the peri-implant mucosa are determined throughout the course of wound healing.

Various biomaterials and surgical parameters can influence the result of soft tissue volume, particularly the extent of the peri-implant mucosa. The soft-tissue seal on the abutment portion of a dental implant (45) is composed of keratinocytes and gingival fibroblasts. This seal functions to protect the osseointegrated portion of the implant against bacterial infiltration.

Cell-to-cell contact, whether between cells of the same type or different types (such as keratinocytes and fibroblasts), triggers protein synthesis and aids in the development of the soft-tissue seal that surrounds dental implants.

When designing infection-resistant materials for dental implants, it is important to consider the complex three-dimensional structure of the soft-tissue seal, as shown in (Figure 2.5). However, this aspect is typically disregarded. As a result, the important physiological interactions between keratinocytes, fibroblasts, microbes, and implant surfaces are often ignored (24).

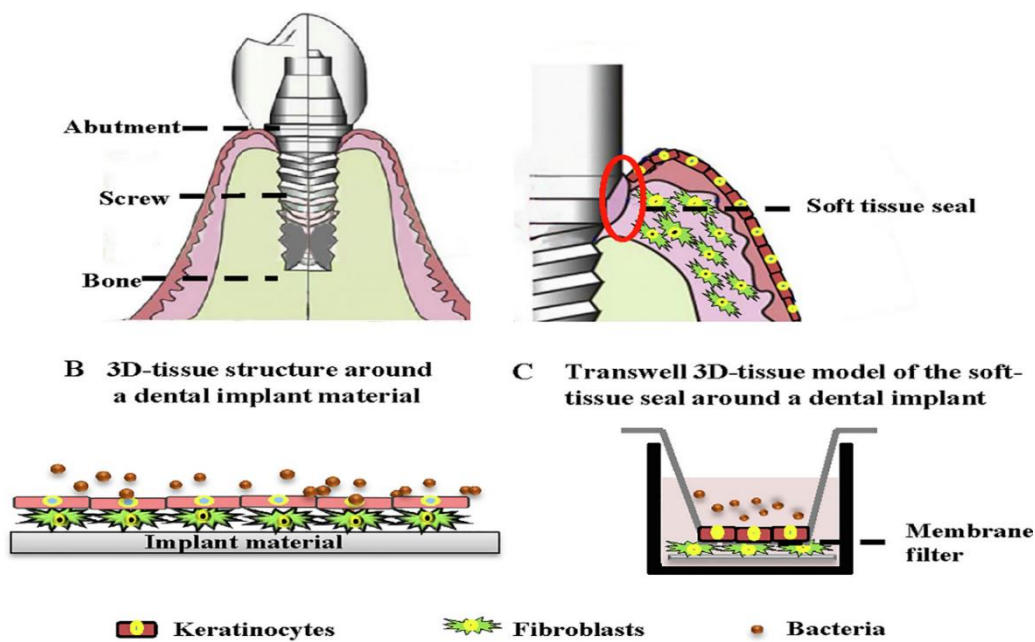


Figure.2. 5.The three-dimensional tissue architecture of the soft tissue seal surrounding dental implant (27).

The degree of adhesion between soft tissues and both natural dentition and titanium endosseous implant surfaces is influenced by the characteristics of the titanium surface (28). Two separate interfaces have been delineated in relation to endosseous dental implants:

- 1) The perimucosal interface refers to the area where soft tissue and the implant come into contact.
- 2) The endosteal interface refers to the area where the bone and the implant come into contact (29).

The crevicular epithelium, which is located at the perimucosal contact, is supported by connective tissue and creates the interface between the implant and the soft tissue. It is likely that a section of the epithelial cells around a titanium implant will create a hemidesmosome connection to the surface of the implant. Although the presence of attachment may not always be identifiable through clinical means, the presence of epithelium adhering to the implant should be visible when examined histologically (13).

There is controversial information about the similarities and differences in thickness, length, and adhesion strength between the epithelium around the tooth and the implant. Moreover, the morphology, configuration, placement technique (either one-stage or two-stage), surface characteristics, and material composition of implant fixings and abutments all influence the conditions of the epithelium that surrounds them (Figure 2.6) (18).

Although these disparities, the source of the epithelium surrounding the implants appears to be histologically analogous to the junctional epithelium encircling teeth (30). Hence, it is recommended to replace the term "junctional epithelium" with "epithelial attachment" in the context of peri implant epithelium (30).

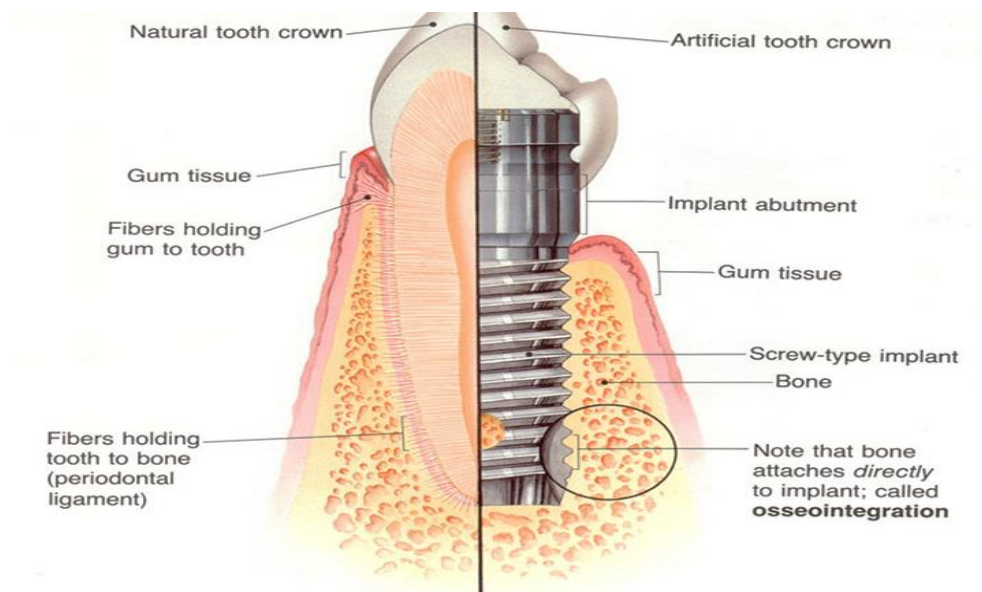


Figure.2. 6. A review of the differences between the dental system and implant system.

The potential connections between the connected section of epithelium and the implant surfaces can be categorized as (31):

A: The epithelial attachment can extend along the surface of the abutment without coming close to the junction between the abutment and the fixture.

B: The uppermost part of the connected layer of cells can be found at the point where the abutment and fixture meet.

C: The attached epithelium can either extend beyond the connection or be entirely positioned on the fixture above the junction.

D: The complete connected epithelium can be found on the fixture above the junction. The prevalence of these connections in vivo is uncertain (31).

The submucosal connective tissue surrounding titanium dental implants primarily consisted of fibroblasts, with macrophages and sporadic lymphocytes present. Collagen filaments and fibrils, which were frequently seen near the implant, consistently exhibited a proteoglycan layer measuring 20 nm in thickness, separating the fibrils and cells from the titanium metal oxide surface. No indications of hazardous or reactive reactions were seen (31).

2.2 Biomaterial

The definition of biomaterial has achieved a consensus among scientists globally and is currently described as "a substance intentionally shaped to guide the progress of therapeutic or diagnostic procedures through interactions with living systems" (32).

A biomaterial is a substance, either natural or synthetic, that is biocompatible and used to replace or support a part of an organ or tissue. It is designed to establish and maintain close contact with living biological tissue (33). Biomaterials are utilized in close proximity to the body, and it is crucial that the implanted material does not induce any harmful effects.

Biocompatibility is of the highest importance in this situation. Tissues have the tendency to undergo damage. by pathological or physiological reasons such as diseases and trauma, making the damaged tissue not functioning or partially functioning (25).

Biomaterials have been used to restore damaged tissues in every field of medicine. Dental implants dates back as far as 600 A.D, as scientists observed sea shells on Mayan remnants as replacing missing teeth and achieving the concept today we call "osteointegration", a seamless integration on bone (26).

A dental implant made of iron, dating back to 200 A.D in Europe, was discovered and described as "osteointegrated," indicating its successful integration with the bone (35).

Assessing the biocompatibility of a dental implant is extremely important since it is the part that comes into direct contact with the host organism, in addition to considering the material's bulk qualities.

Dental implants can be classified according to their interaction with the surrounding tissues, as described below (36).

- **Incompatible:** These implants emit compounds at amounts that are toxic, causing harmful consequences within the living organism and potentially leading to rejection of the implant.

- **Biotolerant:** Implants in this category emit chemicals, but not in concentrations that are harmful. This could result in the development of an encapsulation within the connective tissue.

- **Bioinert:** The term "bioinert" describes materials that exhibit minimal interaction with surrounding tissue once placed in the human body. Like biotolerant materials, they release substances, but not in toxic concentrations, often resulting in encapsulation within connective tissue.

- **Bioactive:** Bioactive implants foster positive interactions that facilitate tissue differentiation resulting in tight adherence at the implant's contact with the surrounding tissue.

Ceramic particle blasting/acid etching, titanium plasma spraying, electrochemical anodization, and calcium phosphate coatings can improve dental implant surface roughness (34). These techniques improve the implant's attachment to bone and live tissue.

2.2.1 Classification of implant biomaterials

Based on chemical composition Biomaterials can be classified into four groups (26).

- Composite implant biomaterials
- Ceramic implant biomaterials
- Polymer implant biomaterials
- Carbon Implants biomaterials
- Metallic implant biomaterial

2.2.1.1 Composite biomaterials

A composite material is formed by combining two or more materials to create a material with qualities that are superior to those of its individual components (37). A composite material consists of a base material or matrix combined with reinforcement, such as the addition of carbon fibers to a polymer.

The objective of utilizing this amalgamation or fusion of substances is to enhance the rigidity, mechanical robustness, and endurance of various composite materials derived from bioenergetic and bioactive ceramic materials, with the aim of attaining advancements in bioactivity and mechanical strength (37).

2.2.1.2 Ceramic biomaterials

Ceramics are a type of material that is composed of crystalline structures and lacks metallic properties. These materials form inter-atomic links through ionic, covalent, or metallic bonding. Originally, ceramics were predominantly found in a powdered state and utilized as fillers. However, it was subsequently determined that they had the capacity to induce bone regeneration. Ceramics exhibit lower density than metals and are distinguished by their hardness, brittleness, and restricted ability to undergo plastic deformation.

The importance of ceramics as biomaterials has significantly increased due to various crucial characteristics. These characteristics encompass their ability to interact well with living organisms, their strong ability to withstand damage from rust and pressure, their limited ability to conduct heat and electricity. Ceramics are particularly important in biomaterials because they are involved in the formation of bone, containing chemicals like hydroxyapatite (HA) or tricalcium phosphate (TCP) (38).

Zirconia dental implants are particularly beneficial for enhancing the appearance of front teeth due to their entirely white hue. Unlike traditional implants, zirconia implants do not have any visible metal components that could impair the natural color of the tooth (38). Despite their ability to facilitate bone ingrowth and osseointegration, these materials are insufficient in terms of strength and toughness for load bearing implant applications (39). Titanium ones are far less costly than them.

Zirconium dioxide (ZrO_2) is a novel ceramic substance employed by certain dental implant manufacturers. Zirconium is a metallic element that has comparable biocompatibility characteristics to titanium.

Numerous studies have been conducted on binary alloys consisting of titanium and zirconium. The composition of these has ranged significantly, with zirconium content ranging from 10% by mass (59), up to 50% by mass (60), and in one research, as high as 70% by mass (61). Zirconium has multiple advantages as an alloying metal for this particular purpose.

Zirconium instantly forms alloys with titanium and demonstrates an excellent resistance to corrosion, resulting in a little release of metal ions into the body (59). It is important to mention that Ti-Zr alloys have demonstrated a lower level of osseointegration with living bone in comparison to other materials (40).

In contrast, studies specifically targeting dental uses have shown that Ti-Zr alloys exhibit mechanical characteristics that are like those of commercially pure titanium (CpTi) (62). Furthermore, when the surface is properly prepared, they exhibit favorable biocompatibility and improved attachment of osteoblasts in comparison to CpTi (63).

These findings indicate that Ti-Zr alloys may provide specific benefits when used for dental implant applications. However, it is essential to acknowledge that most clinical trials conducted on zirconia implants have been of limited duration. Before zirconia-based implant systems may completely replace titanium in prosthetic rehabilitation, it is necessary to have extensive evidence supported by long-term clinical trials (41).

Alumina, on the other hand, is a bioinert material known for its superior biocompatibility, immuno-compatibility, and exceptional resistance to corrosion. Due to its polish ability and high mechanical wear resistance, alumina is used in the wear surfaces of joint prosthesis, specifically in the acetabulum and femoral heads for hip arthroplasty. However, it does have certain disadvantages, including low impact strength, challenges in reproducing properties, and difficulties in manufacturing and processing (27).

2.2.1.3 Polymer biomaterials

Polymers can be described as long chains of molecules made of repeating monomers. They can be obtained from natural or synthetic organic sources. Silicones used in finger joints; polylactic and polyglycolic acids used in sutures, polymethylmethacrylates used in bone cements are some examples to this type of biomaterials (28).

Polymer biomaterials have the advantages of easy fabrication and changeable surface properties, on the other hand polymer biomaterials have the disadvantages of weak mechanical properties, release of monomers to tissue (29).

Polyether ether ketone (PEEK) is a new polymer that is now being used to make dental implants. PEEK is utilized not in isolation but as a means to facilitate stress distribution (30). One of the primary advantages of PEEK, when compared to materials like titanium and zirconium, lies in its elastic modulus, which measures approximately 3.6 GPa. This modulus is closer in resemblance to that of natural bone. In addition, carbon fiber is being used to strengthen PEEK, resulting in a significantly increased modulus of elasticity of around 17.4 GPa. This value closely matches the modulus of elasticity observed in cortical bone (68).

2.2.1.4 Carbon implants biomaterials

Carbon, an elemental material, exists in various allotropes or forms. In the realm of implant dentistry, a significant milestone was reached in 1970 when the use of carbon and carbon-silicon compounds was initially reported.

These materials have garnered attention due to their commendable biocompatibility and mechanical properties that closely resemble those of bone tissue. Consequently, they are being considered as promising alternatives for orthopedic implant applications (31).

Carbon implant materials provide superior resistance to fatigue compared to other materials. Nevertheless, the material's low tensile strength and brittle characteristics have restricted its application to just minor load-bearing regions (69).

2.2.1.5 Metallic biomaterials

Metallic biomaterials have high metal content, used in load bearing areas such as implants and internal fixation devices (32). The intrinsic properties of metal materials encompass great tensile strength, mechanical dependability, elasticity, exceptional resistance to corrosion, wear, and fatigue, as well as toughness, and thermal and electrical conductivity., have established their dominance in the realm of medical devices. As a result, according to estimates, a substantial majority, ranging from 70% to 80% of implants used globally are made of metallic materials (47).

Metallic biomaterials are well-known for their corrosion resistance, it's important to note that when they do corrode due to the metabolic activity of surrounding tissues, they release ions and electrons into the adjacent tissues. This corrosion process can disrupt the physiological balance and, when these ions and electrons meet body fluids, they can potentially have adverse effects on various organs and tissues.

In the early twentieth century, stainless steel and chromium-cobalt alloys emerged as the pioneering metals for successful implant applications. The introduction of titanium and its alloys occurred in the 1940s, but it was not until the 1960s that titanium-nickel alloys, commonly referred to as nitinol (NiTi), began to gain recognition and be utilized in implant applications (71).

a) Stainless steel

316L stainless steel alloys have found extensive applications across various medical fields owing to their advantageous properties. These alloys are favored for their low carbon content, which contributes to high corrosion resistance, excellent cleanability, and biocompatibility.

Remarkably, stainless steel alloys made their debut in orthopedic surgeries as early as 1926, marking the beginning of their significant role in the realm of medical implants and devices (33). A standard composition of 316L stainless steel typically consists of 18% chromium, 12% nickel, 2% molybdenum, and 0.03% carbon.

These alloys contain at least 10% chromium as alloying contents for preventing rust formation. 316L stainless steel alloys also contain low amount of iron and nickel, nickel content increasing the fracture resistance (34).

316L Stainless steels alloys have higher density than bone. Its elasticity is less than bone (29). These disadvantages can negatively affect bone healing in the area where they are implanted. Despite its superior strength, affordability, and machinability, this alloy has inferior corrosion qualities compared to titanium. Due to the properties mentioned, stainless steel alloys have not received approval as a dental implant material (35).

These properties, including the potential for breakage during the initial phases of bone healing and the possibility of corrosion might lead to the liberation of metal ions from stainless steel implants into the body (49). These concerns highlight the need for alternative materials with better biocompatibility and performance characteristics for dental implant applications (76,77).

While chromium and nickel are believed to enhance the medicinal utility of stainless-steel implants, it is important to note that these chemicals can have harmful effects on the body, both locally and systemically (51).

Multiple animal investigations have demonstrated that the inhalation, intravenous, and intramuscular injection of ground nickel complexes can cause cancer. The toxicity of Cr appears to be strongly associated with its valence state (76).

b) Chrome cobalt

Chrome-cobalt alloys are high strength, temperature-tolerant, and wear-resistant. They resist corrosion longer than stainless steel alloys. In the Medical field alloys with chromium, cobalt, and molybdenum are widely utilized. These alloys are biocompatible and resistant to physical and chemical stresses, making them useful medical materials (82).

Specifically, chrome-cobalt alloys play a crucial role in the manufacture of total hip prostheses, knee implants, and dental implants (37). Their combination of biocompatibility and robustness makes them well-suited for these orthopedic and dental implant applications, where durability and compatibility with the human body are paramount.

Co-Cr dental alloys consist mostly of cobalt (Co) and chromium (Cr), but they may also include other metals including nickel (Ni), molybdenum (Mo), and manganese (Mn). The presence of these extra metals might lead to unwanted outcomes (61).

A systematic review was recently conducted to comprehensively examine the literature from the past 25 years regarding the toxicity of dental alloys containing cobalt and chromium (Co-Cr) (38). It's important to recognize that exposure to metals, particularly cobalt and chromium, primarily occurs through three routes: inhalation, ingestion, and skin contact.

When used in dental alloys, these metals affect the oral mucosa. A 2010 case report linked a cobalt allergy to dental restorations releasing cobalt, causing oral hypersensitivity. This example emphasizes the need of dental material allergy and hypersensitivity testing (77). Table (2.1) lists some of the materials utilized in dental implants.

Table.2. 1.Components utilized in dental implants (39)

Material used for Implants	Generic Name
Metals	
Titanium	CpTi
Titanium alloy	Ti6Al4V
Stainless steel	SS,316 L SS
Co-Cr Alloy	Vitallium, Co-Cr-Mo
Cold Alloys	
Tantalum	Ta
Ceramics	
Alumina	Al ₂ O ₃ , amorphous or single crystal sapphire Kyocera
Hydroxyapatites	HA, Ca ₁₀ (PO ₄) ₁₀ (OH) ₂
Beta-Tricalcium Phosphate	B-TCP Ca ₃ (PO ₄) ₂
Carbon	Vitreous, low temperature isotopic (LTI), ultra-low temperature isotropic (ULTI)
Carbon -silicon	C-Si
Bio -glass	SiO ₂ -CaO-Na ₂ O-P ₂ O ₅
Polymers	
Polymethylmethacrylates	PMMA
Polytetrafluoroethylenes	PTFE
polyethylene	PE
Polysulfides	PSF
Polyurethan	PU

c) Titanium and its alloys

Titanium is a chemical element represented by the symbol Ti. It is classified as a fourth-row transition metal on the periodic table, with an atomic number of 22 and an atomic weight of 47.88 U (universal atomic mass unit). Titanium, a highly reactive metal, develops a strong oxide layer that enhances its electrochemical passivity.

Titanium is the ninth most prevalent element and is abundant in the Earth's crust (40) (Table.2.2). It belongs to the category of light transition metals, characterized by its strength, metallic white color, lustrous appearance, corrosion resistance, and solid state at room temperature (41).

200 years ago, titanium was isolated. William Gregor discovered this element in England in 1790 while examining magnetic sand. In 1795, German chemist Heinrich Klaproth discovered it in rutile, which is mostly Titanium Oxides (TiO₂). A metallurgical process developed by Dr. William Kroll allowed metallic titanium to be commercially produced in 1946 (42).

Rutile and ilmenite are plentiful in US, Canadian, and Australian titanium reserves. The pigment industry uses most titanium ore, but 5% to 10% is used to make cp titanium and titanium alloys (57).

Table.2. 2. Displays the weight percentage of specific elements present in igneous rock (38)

Element	Percent (wt %)
Iron	5
Calcium	4
sodium	3
Potassium	3
magnesium	2
Titanium	0.6
manganese	0.1
Zirconium	0.3
Nickle	0.02
Copper	0.01
Lead	0.002

The ASTM Standard F1295 (58) is a set of guidelines established by the American Society of Testing and Materials, delineates various grades of titanium based on its purity, which is evaluated according to the oxygen content. All these grades have been considered medically acceptable and demonstrate effective osseointegration (59).

The first four grades, which are categorized as grade I to grade IV, are classified as pure titanium. On the other hand, grade V and grade 23 refer to titanium-aluminum-vanadium alloys. These two alloys are notably similar in properties, particularly when considering another designation.

Adding more fragments increases hardness, resulting in titanium grades II, III, and IV. The grades I through IV relate to commercially pure titanium (cp-Ti), which contains 99.5% titanium and 0.5% interstitial elements such as carbon, oxygen, nitrogen, hydrogen, and iron, with varying purity levels. The latter two alloys, Grade V (Ti6Al4V) and Grade 23 (Ti6Al4V ELI) (Extra Low Interstitial – highly clean), share comparable characteristics.

The purest form of titanium, known as grade I, is produced by melting titanium sponge. Grade I is considered the most pristine grade, as it is derived solely from titanium sponge. Combining titanium sponge with titanium fragments results in an increase in the levels of oxygen (O₂) and iron (Fe), hence making the titanium material tougher.

Beyond grades I through, four titanium alloys are created by introducing a variety of elements into the titanium matrix, giving rise to titanium grade V, according to ASTM specifications (43). Grade V encompasses two combinations of aluminum (Al) and vanadium (V). For instance, an alloy containing aluminum and vanadium enhances material hardness. Furthermore, vanadium, aluminum, and Ti-6Al-4V particles are toxic and mutagenic, causing Alzheimer's disease, osteomalacia, and neurological issues (44).

Hydrogen, nitrogen, carbon, and oxygen have a notable impact on the mechanical properties of commercial pure (CP) titanium. These elements dissolve interstitially in titanium and exert a strong influence on its mechanical behavior (43).

The impacts of these phenomena are indeed variably moved to titanium alloys. What makes interstitial elements unique is that they are much smaller than titanium atoms and can dissolve solidly into the titanium crystal lattice without taking over the titanium atom sites. Oxygen, carbon, and nitrogen tend to make alloys less tough as their quantity rises, eventually making the alloys very brittle (43). The chemical constituents of titanium and titanium alloys are presented in Table (2.3).

Table.2. 3. Composition of titanium and titanium alloys (wt%) (45)

ELEMENT	GRAE I	GRADE II	GRADE III	GRADE IV	Ti-6Al-4V	Ti-6Al-4V ELI
Titanium	99%	99%	99%	99%	90%	90%
Nitrogen	003 %	0i03 %	0.05%	0.05%	0.05 %	≤ 0.05%
Carbon	0.1%	0.1%	0.1%	0.1%	0i08%	≤ 0.08%
Hydrogen	0.15%	0.15%	0.15%	0.15%	0.015%	≤ 0.012%
Iron	0.2%	0.2%	0.2%	0.3%	0.25%	≤ 0.25%
Oxygen	0.18%	0.25%	0.35%	0.40%	0.13%	≤ 0.13%
Aluminum	-	-	-	-	5.50-6.75%	5.50-6.50%
Vanadium	-	-	-	-	3.50-4.50%	3.50-4.50%
Titanium	Balance	Balance	Balance	Balance	Balance	Balance

Commercially pure titanium (CpTi) is known for being one of the most challenging metals to work with due to its high melting point of 1670°C and its tendency to react chemically with investment materials at such temperatures (46).

As a result, the casting technique is limited in its routine use for dental prosthesis applications due to inherent defects such as porosities and insufficient mold filling, once the metallic infrastructures for the prosthesis have been obtained by casting (47).

The fundamental characteristics of titanium, such as its lack of toxicity, capacity to resist corrosion, controlled ability to degrade, modulus of elasticity, and strength against fatigue, have always been considered crucial elements in choosing suitable biomaterials for dental purposes.

Titanium grade III is exclusively produced from titanium sponge and undergoes a refining process to achieve a minimal oxygen concentration of approximately 0.18%. It possesses the highest level of purity among the four unalloyed titanium grades defined by ASTM. Nonetheless, it demonstrates the least amount of strength and the highest level of ductility at normal room temperature. Its exceptional formability is attributed to its low levels of oxygen and iron. Titanium grade I has exceptional corrosion resistance and formability. However, the overall mechanical strength is at its lowest point.

Like annealed austenitic stainless steels, grade II titanium has a minimum yield strength of 275 MPa. This material is characterized by a higher Young's modulus, signifying its stiffness and resistance to deformation under load. However, it exhibits relatively low mechanical strength, indicating that it may not withstand applied forces as effectively. Furthermore, it tends to have poor wear resistance, suggesting that it may be susceptible to wear and degradation when subjected to friction and mechanical stress (32) and a difficulty to improve the mechanical properties without reducing biocompatibility.

Titanium grade III has a maximum iron content of 0.30%, which is lower than the maximum iron content of grade IV (0.50%). It possesses a greater concentration of nitrogen and oxygen. The mechanical qualities are superior as compared to grade II. Grade III titanium exhibits inferior strength, diminished fatigue resistance, restricted flexibility, and a greater susceptibility to deformation in comparison to grade V titanium alloy.

Grade IV, with an oxygen concentration of around 0.4%, exhibits the highest level of strength and poorer formability when compared to other unalloyed ASTM categories(48). Most titanium implants are produced using Grade IV Cp-Ti because of its better Young's modulus of elasticity. The material exhibits a very low degree of mechanical strength, insufficient wear resistance (32), and presents difficulties in improving mechanical properties while maintaining biocompatibility.

Titanium grade V alloy, also known as Ti6Al4V, is often used as a replacement for dental implants because of its improved physical and mechanical properties. It consists of 6% Aluminum and 4% Vanadium (47). The titanium grade V alloy is an alpha-beta alloy, while the CpTi grades I to IV are alpha structure.

The addition of Aluminum and Vanadium showed an increased resistance to fatigue with an ultimate strength of 860 MPa of the Ti-6Al-4V. The presence of vanadium serves as an aluminum scavenger, likely inhibiting the creation of Ti-Al compounds that promote corrosion (49). In addition to having a higher hardness rating of 5, the Ti-6Al-4V alloy also demonstrated greater fatigue resistance compared to cp-Ti (50).

Grade 23 titanium alloy is characterized by its reduced levels of oxygen, iron, and nitrogen, as well as its exceptional ductility and fracture toughness. These qualities are especially advantageous for producing thin-walled implants and screws for abutments, whether for cement fixation or screw fixation of prosthetic components such as Multi Unit abutments.

The mechanical properties of titanium and its alloys not only meet but exceed the requirements for an implant material. Both orthopedic and dental implants necessitate strength levels that surpass those of bone, along with an elastic modulus that closely approximates that of bone.

The majority titanium alloy utilized for these specific applications is Ti-6Al-4V, due to its advantageous composition and reliable manufacturability (95). A comparison of the mechanical properties between CpTi and Ti-6Al-4V is provided in table (2.4).

Compared to bone, the yield strength of CP-Ti and Ti6Al4V is significantly higher, ranging between 550 and 900 MPa (49). In contrast, bone has an ultimate strength of 83 MPa (spongy bone) to 117 MPa (cortical bone).

The fatigue properties of biomaterials are closely connected to their microstructures, which can be altered by the processing and heat treatment techniques used. Significantly, the fatigue threshold of any metallic biomaterial typically surpasses that of bovine bone (50).

Generally, the fatigue strength is usually below 50% of the ultimate tensile strength (50,102). When comparing cp titanium with titanium alloys, there is a steady trend of strength increasing while the modulus remains relatively constant.

Occasionally, the fracture of structural biomaterials, particularly fatigue fracture, might become a significant concern among other forms of fractures. Fretting fatigue, also known as fatigue with fretting, is a specific type of tiredness that can arise from the relative movement between two objects, such as a bone plate and a screw (88).

Out of all the titanium alloys that are being studied as metallic biomaterials, pure titanium (Ti) has the lowest fatigue limit. By comparison, the fatigue thresholds of Ti-6Al-4V and Ti-6Al-7Nb, which are classified as alloys of the ($\alpha + \beta$ type), are among the most elevated in this category (6). Titanium and its alloys are the ideal materials in implant dentistry due to their high strength and low weight, with a density of 4.5 g/cm³ (62).

Table.2. 4. Titanium dental implant alloys: physical and mechanical characteristics (51)

Properties of titanium for dental implants and bone							
	Cp Ti grade I	Cp Ti grade II	Cp Ti grade III	Cp Ti grade IV	Ti 6Al4V	Ti -6Al- 4VELI	Bone
Strength/MPa	240	345	450	550	930	860	-
Elongation at failure/%	25	20	18	15	10	13-15	-
Density(glc)	4.5	4.5	4.5	4.5	4.4	4.42	-
E (GPa)	102	102	102	104	113	117	5-30

Strength is advantageous because it allows materials to withstand occlusal stresses without fracturing or failing. Therefore, researchers and manufacturers have produced alternative titanium alloys that have enhanced mechanical strength (50).

The occlusal forces exerted on each tooth are conveyed sequentially from the enamel, through the dentine, to the root. From there, they are communicated through the cementum, the periodontal ligament, and finally to the cancellous bone, reaching the end at the ramus of the jaw.

The mastication force exerted by natural teeth is usually within the range of 75 to 135 N, but the maximal biting force in the molar region can reach 230-450 N. The stresses applied to dental implants have a crucial role in maintaining the integrity of the implant and its integration with the adjacent bone (51).

For greater bone force transmission, implant biomaterials with lower modulus are better. (6,111). Ti-6Al-4V exhibits greater strength than bone and possesses a modulus of elasticity that closely resembles bone, surpassing all other implant materials except for pure Ti (18). As the bone and implant become more flexible, the stress at the critical bone-implant interface is distributed more uniformly (107).

Ensuring effective transmission of stress from the implant to the bone relies on obtaining optimal alignment between the implant's modulus of elasticity and that of the adjacent bone. The purpose of this realignment is to reduce stress shielding and subsequent bone resorption that may occur (31).

While Grade V titanium does have a relatively high Young's modulus (113 MPa), it does not significantly differ from Grade IV titanium, which has a Young's modulus of 104 MPa. Consequently, there should be no significant issues in this regard. It is worth mentioning that the Young's modulus of Grade V titanium is significantly higher than that of bone, specifically 17-20 GPa, and it may be approximately 3-6 times stronger than the Young's modulus of cortical bone.

Titanium alloy exhibits almost double the strength and half the elastic modulus when compared to Co-Cr-Mo alloys. When comparing the Ti-6Al-4V alloy to 316L stainless steel, it has almost the same strength but barely half the modulus (Table 2.5).

Zirconia-ceramic is stiff (200 GPa) and may have more relative motion or disuse atrophy due to stress shielding at the bone-implant interface. After osseointegration, loading zirconia implants may minimize crestal bone loss (110).

Table.2. 5 Presents the elastic modulus (GPa) of some important implant materials and bone (46)

Materials	Modulus of elasticity
Stainless steel	190
Ti-6Al-4V	109
Ti-6Al-4V ELI	114
Pure titanium	105
Chrome-Cobalt	180-210
Zirconia-ceramic	200
Cortical	5-23
Cancellous bone	0.01-1.57

Clinical usage of CpTi in dental implants began in 1965 (111). It has low tissue toxicity, good mechanical characteristics, and easy processing. With an ultimate strength of 550 MPa (111,115), CpTi has a good strength-to-weight ratio. However, Ti grade s I–IV (CpTi) dental implants have drawbacks such as increased young modulus, lower mechanical strength, poor wear resistance, and difficulties in improving mechanical qualities without compromising biocompatibility.

The response of the biological environment to artificial medical devices is contingent upon the surface characteristics of the implant material. Conventional manufacturing methods for titanium implants usually result in a surface layer that is oxidized, polluted, stretched, plastically deformed, uneven, and poorly defined.

These naturally occurring surfaces are not ideal for biomedical applications and need to undergo surface treatment to enhance their resistance to wear and corrosion, particularly for dental applications. Titanium and its alloys have favorable bulk properties such as low modulus, fatigue strength, formability, and machinability, which these treatments aim to preserve. Titanium and its alloys are categorized based on their microstructure into four types: α , almost α , α - β , and β (113) (Table.2.6).

Table.2. 6.Microstructure of titanium and titanium alloys (based on alloy elements) (62)

Alloy	Micro-Structure	Effect on structure
CpTi Grade 1	α	α stabilizer
CpTi Grade 2	α	α stabilizer
CpTi Grade 3	α	α stabilizer
CpTi Grade 4	α	α stabilizer
Ti-6Al-4V	$\alpha + \beta$	α and β strengthener
Ti-6Al-4V ELI	$\alpha + \beta$	α and β strengthener

Essentially, at normal temperature, pure titanium has a hexagonal close-packed (hcp) structure known as the α phase (52). α -type titanium alloys, which exclusively contain the α phase, are composed of different grades of commercially pure titanium (CP-Ti) and titanium alloys (53).

The elements (Aluminum, Oxygen, Nitrogen, Carbon) that possess a propensity to enhance the stability of the α phase are commonly known as α -stabilizers. The presence of these elements elevates the β transus temperature. Conversely, the addition of stabilizing elements such as (V, Mo, Nb, Ta, Fe, Cr, Fe, W, Si, Co, Mn, H) decreases the β transus temperature.

The β alloys are composed of an adequate amount of β stabilizers to ensure the retention of the β phase upon quick cooling to ambient temperature. Having a enough amount of β stabilizers avoids the formation of martensite. Beta alloys generally have remarkable strength and outstanding malleability. However, they have a relatively high density and restricted ductility (54).

The $\alpha+\beta$ alloys display a mixture of α and β phases at low temperatures and usually contain stabilizers for both α and β phases. The strength of these alloys can be increased with heat treatment and/or thermomechanical processing.

The alloys provide a moderate to high degree of strength and display satisfactory formability. Nevertheless, the α alloys have superior resistance to deformation at high temperatures compared to them (55). Out of the $(\alpha+\beta)$ -type titanium alloys, Ti-6Al-4V (sometimes referred to as Ti64) is the most used biomedical alloy. It makes up 50% of the overall titanium production, coming second only to commercially pure titanium (CpTi) which makes up 53%.

Near-alpha titanium alloys consist primarily of the alpha phase with a small amount of the beta phase (less than 5% by volume). This is achieved by incorporating a very low concentration of beta-stabilizers (1-2% by weight), which is significantly less than what is used in alpha-type titanium alloys. Both α -type titanium alloys and near- α titanium alloys possess similar attributes, such as outstanding corrosion resistance, good weldability, and elevated resistance to creep. Thus, they are suitable for utilization in high-temperature applications (71).

Aluminum, gallium, and germanium are some of the substitutional elements found in stabilizers. Aluminum is a crucial alloying element that serves to stabilize the alloy by simultaneously enhancing its strength and reducing its density through solid solution strengthening (52).

The use of α -type titanium alloys in load-bearing applications is restricted due to their poor mechanical strength and low fatigue strength (57). Furthermore, the use of near- α Ti alloys in biological applications has been limited up to this point. Commercially pure titanium (CP-Ti) has demonstrated superior performance in non-load-bearing applications that require corrosion resistance, when compared to near- α titanium alloys (53).

d) Binary alloys of titanium

To enhance the material qualities for dental implant applications, titanium has been investigated as an alloy material. Niobium (125,126), silver (56), gold (57), manganese (58), and zirconium are some of them (59). The primary aim of alloying is to enhance the efficiency of the material. But it is imperative to carefully evaluate any potential ramifications on biocompatibility.

The biocompatibility of some alloying components, such silver or chromium, may be negatively impacted. There is a chance that these elements might leak out of the alloy and into the surrounding biological environment if they are present (60). When present at dangerous amounts, both silver and chromium are known to cause adverse biological consequences (61).

As a result, even though alloying might improve material qualities, it's important to carefully assess any potential effects on biocompatibility and determine if the alloy can withstand the strict standards for dental implant materials. Any alloyed material's biocompatibility and safety must be extensively investigated and verified before it can be considered for use in dental implants.

Zirconium provides numerous advantages as an alloying metal in this application. It readily forms alloys with titanium and exhibits a high level of corrosion resistance (67), hence limiting the infiltration of metal ions into the body. However, Ti-Zr alloys demonstrate inferior osseointegration with living bone (62).

The TiZr alloy was synthesized through the amalgamation of a titanium alloy with a zirconium content ranging from 13 to 15 %. In comparison to titanium alloys, this alloy exhibits superior mechanical strength and possesses comparable biocompatibility with bone tissue (69). The use of TiZr alloy is recommended for the fabrication of small diameter implants, particularly in regions exposed to significant occlusal forces (63).

For dental applications, Ti-Zr alloy lacks the necessary strength and elastic recovery (also known as spring back) characteristics. Consequently, processing the Ti-Zr alloy might be difficult (64).

In comparison to titanium and titanium alloys, tantalum alloy (Ta) and niobium alloys (Nb) are better electrochemically and biocompatibly suited for the development of novel implant materials. In comparison to Ti and Ti alloys, natural Nb and Ta have weakened mechanical properties. According to reports, pure Nb is almost at the lowest safety limit for implant materials (65).

Titanium-Niobium (Ti-Nb) alloys have demonstrated high corrosion resistance. They often outperform CpTi regarding of hardness, yield strength, and tensile strength, but their tensile strength significantly lower (55). Experimental investigations have revealed that despite this advancement, human fibroblasts grow less rapidly and broadly on Ti-Nb alloys than on CpTi (66).

Titanium-containing binary biomedical alloys have been made from palladium (132), silver (130), and gold (131). Due to their better corrosion resistance over CpTi, the alloys' biocompatibility with bone and soft tissues is expected (136, 137). Experiments have only confirmed this in the Ti-Ag system (132). Due to their high cost, density, and weakness, noble metals are limited in biomaterials

There has been study conducted on manganese in binary alloys because it is an element that is typically acceptable physiologically (67). The effects have been positive despite the relatively modest levels, that is, 8 to 12 % by weight. When manganese is present, both hardness and density rise (58). While cell survival near these alloys was somewhat decreased compared to a CpTi implant (56), cell adherence seemed to be enhanced in the alloy (68).

Because it can stabilize the beta-phase of this phase with a low solute concentration, molybdenum (Mo), a strong β -stabilizer, is considered as the most suited β -stabilizer element. because it is a metal that doesn't melt easily and has a high melting point. therefore, the addition of this element to Ti raises the melting point, making the material extremely difficult to manufacture and offering a poor elasticity and flexural strength modulus (68).

There are several potential combinations with titanium that exhibit higher biocompatibility with cells and are less prone to corrosion. However, Ti-Zr is the only binary alloy that has so far produced truly significant advancements, and research on this substance as a potential dental implant alloy is still underway.

e) Titanium alloys with multiple components

Several alloys of at least three metals, including dental alloys like Ti-15Zr-10Cr, Ti-16Nb-13Ta-4Mo, and Ti-6Al-7Nb, have been investigated as potential implant materials.

Overall, it has been demonstrated that Ti-6Al-7Nb has very advantageous physical and biological characteristics for application in dentistry. However, judging on the literature, it does not appear to be especially frequently utilized for this purpose (45).

Three distinct pairings of vanadium (V) with aluminum (Al) are referred to as grade V (Ti-6Al-4V). For instance, an aluminum-vanadium alloy will enhance the material's hardness. Adding palladium alloy (Pd) will merely make the substance more corrosion-resistant (43). In addition to being harder than cp-Ti, Ti-6Al-4V alloy also demonstrated greater fatigue resistance (6,99).

Most of the research focuses on CpTi, a small number expressly mention Ti-6Al-7Nb, while some consider Ti-6Al-4V. This may alter in the future considering the empirical results achieved with implants made of this particular alloy (45).

2.3. Dental implant

Various studies in the medical field lead to developments that increase life expectancy and the quality of life in our world today. Although the life span of people can be extended within the framework of the joint studies of pharmacology, medicine, and psychology sciences.

The use of various biomaterials is an imperative to fully restore the functions of organs and limbs that lose their function as a result of various pathological and traumatic effects (69). The biomaterials that are used for restoring the functions of the damaged body parts are called implants (1).

The mechanical and physical functioning of lost tissue caused by diseased and traumatic effects can be restored using a variety of implants. Hip joint implants for replacing lost bone tissue, pacemakers for heart arrhythmia, dental implants for lost teeth tissue, can be used in order to maintain good life quality (70).

These implants may be required to remain in the body for a certain period of time or for the rest of one's life. Implant materials must be compatible with the human body since a foreign substance might trigger tissue reactions and allergic reactions .

2.3.1 Essential properties of implants

2.3.1.1 Elastic modulus:

In order to provide a more even distribution of load at the implant and to minimize movement between the implant and bone, it is important to choose an implant material with a modulus of elasticity that closely matches that of bone (18 GPa) (71).

2.3.1.2 Yield strength, fatigue strength

High yield and fatigue strengths are necessary in implant materials to prevent brittle fracture when subjected to cyclic stress (71) .

2.3.1.3 Ductility (Compliance)

Ductility is necessary for an implant to be molded and formed. The American Dental Association states that dental implants ought to be at least 8% ductile.

2.3.1.4 Hardness and toughness

Enhanced toughness prevents the occurrence of implant fracture, while heightened hardness decreases the probability of wear on the implant material.

2.3.1.5 Surface energy and tension

Wettability promotes stronger attachment of osteoblasts to the surface of the implant .The wettability of the implant may be assessed by the interaction between the wetting fluid (blood) and the cleanliness of the implant's surface (72).

2.3.1.6 Surface roughness:

Modifications in the surface roughness of implants have an impact on the reaction of cells and tissue. Various parameters, including roughness, texture, and the orientation of irregularities, have been used to classify implant surfaces (71).

Although titanium has many benefits, it cannot osseointegrate with the bone cells of humans since it is physiologically inert. Since these qualities cannot be incorporated into the metal during the production process, titanium must have its surface modified. Therefore, surface modification methods are needed to accelerate the development of osteogenesis and bonding on titanium and titanium alloys (73).

Acid etching, grinding, and other mechanical techniques; coatings such as hydrogen peroxide treatment using fluoride (F) and chloride (Cl) ions (74), hydroxyapatite (157,158), nitride (159,160), metal oxides (161,162), and silver (163,164). Empirical evidence supports the notion that these techniques may accelerate the pace of osseointegration, hence augmenting the implants' antimicrobial qualities, biocompatibility, and durability.

The primary contributing factors required to satisfy the criteria of a particular implant part are a combination of tenacity, elasticity, hardness, and ductility. Additionally, the device must be simple to manufacture and adhere to all clinical application specifications. Another issue associated with implant fabrication is the cost of production.

2.3.2 Selecting an implant

There is increasing clinical data that shows all six dental implant biomaterials now available on the market have shown outstanding biocompatibility, tissue response (74).

No titanium-based materials have shown superior biocompatibility compared to other groups. Without long-term comparison research, the choice of implant for a specific patient should be based on characteristics such as implant design, size, and material strength.

Implant materials vary in strength; thus, clinicians must choose them carefully. If a patient has parafunctional behaviors and implant breakage, the clinician should use titanium alloy rather than cp grade titanium. Also, small implants or implants with narrow walls require stronger materials.

Clinicians must use caution while selecting implant materials due to their varying levels of strength. When a patient exhibits occlusal parafunction and has implant breakage, it is advisable for the doctor to choose titanium alloy instead of pure titanium. In addition, smaller implants or implants with narrower walls need the use of harder materials.

If all other factors in implant selection are comparable, some doctors may choose to consistently use a stronger material, such as grade IV titanium pure titanium or a titanium alloy. These variables may include implant design, abutment availability, surface finish, and biomechanical concerns.

The manufacturers of the chosen implants and the materials they use are provided in Table 2.7. It is worth mentioning that 3i utilizes grade I cp titanium for its hex-top implants. This titanium is cold-worked throughout the production process to create a material that has the same mechanical properties as cp grade III titanium. While this approach enhances the material's yield strength without increasing the elastic modulus, it presumably reduces toughness. It is necessary to undertake independent investigations to assess these impacts.

Table.2. 7.Dental implant manufactures and implant material choices

	CpTi Grade I	CpTi Grade II	Cp Ti Grade III	Cp Ti Grade IV	Ti-6Al-4V-ELI Alloy	Ti-6Al-4V-Alloy
3i	=	=	/	*	*	*
Biohorizons	*	*	*	*	X	*
Lifecare	*	*	/	/	/	*
Nobel biocare	/	*	*	*	*	-
Paragon	*	*	*	/	/	*
Steri-Oss	*	*	*	/	/	*
Sulzer Calcitec	*	*	*	*	*	/
Straumann	*	*	*	X	*	*

3i company representatives use materials that meet cp grade I and grade I1 specifications, but that are cold worked to improve yield strength.

Today, materials used as implants are produced using metallic biomaterials. Wires, pins, screws, and plates used for internal or external fixation of damaged hard tissue are fabricated with stainless steel in terms of advantages such as low cost, biocompatibility, and high resistance to external forces. However, stainless steel metals can release the nickel and chromium elements in their structures by being corroded, which are considered having toxic effects on living tissue (1).

2.3.2.1 Guidelines for selecting implants biomaterial (71)

The ADA has provided specific criteria for the choosing of biomaterials for implants:

- Assessment of the physical features that guarantee adequate durability.
- The fabrication process should be simple, and the material should be able to undergo sterilization without experiencing degradation.
- Assessment of safety and compatibility, including cytotoxicity tests and evaluation of tissue interference properties.
- Absence of defects.
- Its efficacy is supported by at least two separate longitudinal clinical investigations.

There is few research on implant materials, that can remain in the region where they are applied without long-term toxic effects. To meet this need, implants are fabricated with Ti and Titanium alloys.

2.4 Biomaterials and biocompatibility

The term " Biocompatibility refers to the capacity of a substance to be used without endangering the species it is intended for (74). Implants and other biomedical devices are governed by several organizations worldwide, including organizations, such as the FDA (Food and Drug Administration) and the ISO (International Standards Organization) to assure their safety and to achieve the desired effects (ISO) (75).

The term "biocompatibility" refers to a variety of interactions between the body and the utilized substance (76). The relationship between a material and the tissue in the area where it is utilized should be taken into consideration when defining a substance's biocompatibility (76).

The utilization of Incompatible materials may significantly impair the body's immune system, making it more susceptible to diseases caused by bacteria, fungi, and viruses. How well a biomedical implant is biocompatible relies on various factors such as its physical, mechanical, degradation properties, sterilization to remove microorganisms, examination and treatment of toxins, the surgical method, the location of implantation and its ability to bear weight as illustrated in (Figure 2.7) (148).

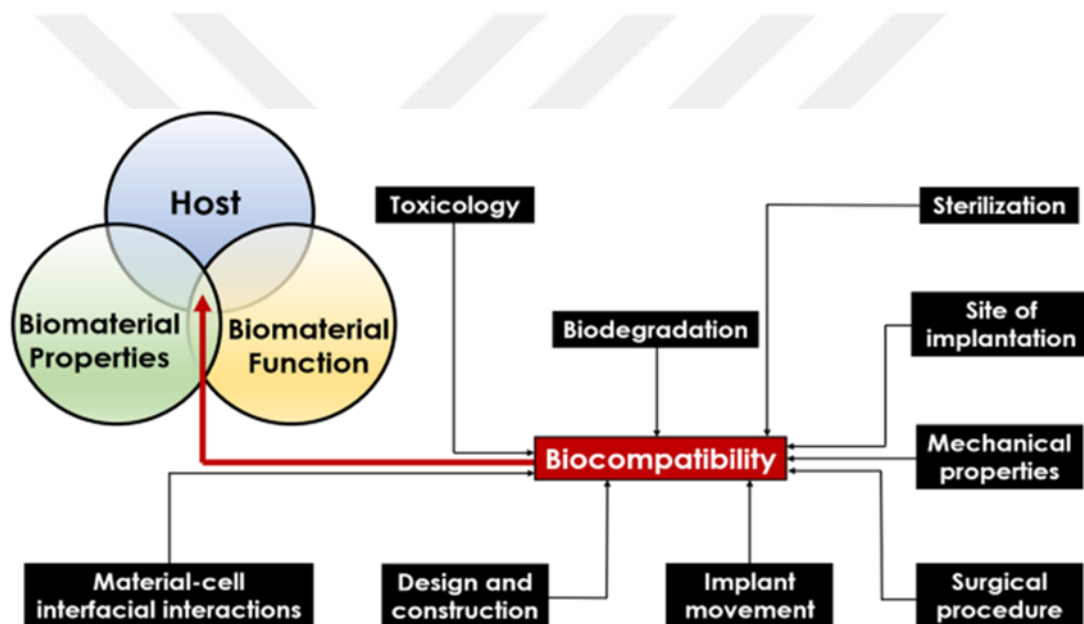


Figure.2. 7. Primary determinants of biomedical implants' functional biocompatibility implants (77)

2.4.1 Interaction of biomaterials with the living-organism

According to (Figure.2.8), protein adsorption, cell adhesion and dissemination, cell proliferation, and water-surface interaction are some of the main processes taking place at the surface. The water molecules react with the biomaterial surface in the early phase throughout the first few nanoseconds (ns), and depending on the surface's wetting characteristics, the surface can be classified as "hydrophilic" (contact angle 90°) or "hydrophobic" (contact angle $> 90^\circ$).

Because of its great attraction for water molecules, a hydrophilic surface encourages their spread and increases their interaction with the surface. A hydrophobic surface, on the other hand, repels water molecules and reduces their interaction with the surface, which results in the production of droplets.

"Contact angle" refers to the angle at which the surface of the biomaterial contacts the interface of the water molecules. The second phase also includes the quick adsorption of many proteins on the surface, followed by the fast migration of tiny proteins, then of large ones, generating a noticeably thick protein layer (78).

In the third phase, cells interact with the protein-soaked layer and then the surface topography for a few hours. This stage involves many molecular interactions on the biomaterial's surface. The biophysical environment and biological molecules (cell membrane, cytoskeleton, and extracellular matrix proteins) might affect it. The following physicochemical features evolved, including topography (surface protrusions and depressions), stiffness, wettability or wetting qualities, functional groups (such as trimethylammonium (hydrophilic) and n-butyl groups (hydrophobic)), and ion enrichment of the surface.

Proliferation, the fourth phase, continues the development of the other phases. Proliferation can take days for a biodegradable screw or years for a comprehensive hip replacement. However, surface topography and wetness greatly affect the third and fourth stages (165).

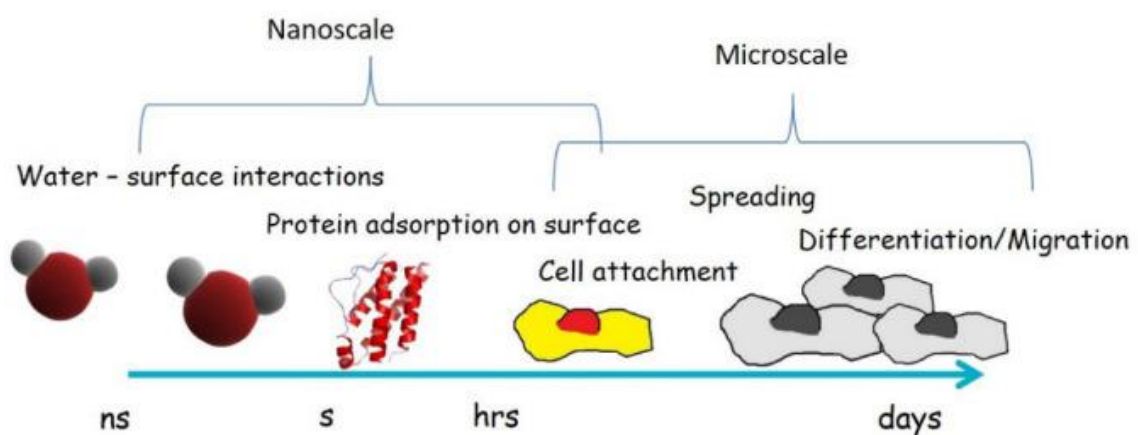


Figure.2. 8.Biomaterial's interaction with the living organism.

Ti used as dental implants is biocompatible for this application. Chromium-cobalt dental implants have not been shown to have the same capacity to osseointegrate with bone as titanium implants. Chromium-cobalt materials are reported to be more corrosion-resistant than titanium for hip prosthesis (79).

For this reason, it has been stated that hip prostheses made of Ti are more unsuccessful than hip prostheses made of chromium-cobalt. Based on these data, it is not possible to say that biocompatibility of Ti is better than chromium-cobalt or vice versa.

The phrase "biocompatibility" refers to both a material's and live tissue's physiological, chemical, and biological impacts on one another and on the material itself. The status of biocompatibility is dynamic rather than static.

The body's functions may have negative effects on the applied material, resulting in deterioration of the material's biocompatibility. Over time, as the body ages and various local or systemic diseases emerge, the body's response to the used material may change, resulting in material biocompatibility being compromised. The physiological reaction of the body to the substance and the corresponding reaction of the material to the living tissue might change over time since the interaction between the two is dynamic (74).

Biocompatibility cannot be defined with toxic effects, systemic or local factors alone. The production of the materials used in accordance with their desired functions affects the biocompatibility of the material (80).

High quantities of titanium, chromium, cobalt, and aluminum in body fluids from implants may cause acute and long-term injury. The long-term effects of implanted Titanium (Ti) are unknown. However, Ti implants have been associated with hypersensitivity and allergic responses (170,171).

Out of 1500 clinical trial participants, 0.6% (153 patients) had Ti allergic reactions. Research also shows that implant metal debris can cause marrow fibrosis, necrosis, and granulomatosis (175,173).

2.4.2 Oxide coating

The precise structure of the surface is pivotal in promoting the adhesion of fibroblast and osteoblasts, and the oxide layer typically exhibits favorable biological properties. However, the body still recognizes it as a foreign object, and under certain circumstances it could result in the creation of fibrosis around the implant (81).

Commercially pure titanium (CpTi) and Grade V, the two main alloys used in implanted products, have surfaces mostly composed of the oxide TiO_2 (158). This oxide layer contains hydroxyl groups in addition to oxide and is typically 4–6 nm thick.

Whenever subjected to an aqueous environment, or any electrolyte, most metals develop oxide coatings. The metal and the circumstances of its oxidation determine the composition of this oxide. The implant surface might be altered by anything that comes in contact with it. The way a metal behaves inside the body is determined by the characteristics of the oxide layer if the physiologic circumstances of the body don't change (82).

Theoretically, several oxides might arise from pure titanium. TiO , TiO_2 , and Ti_2O_3 are a few of them. TiO_2 is the most used of these under physiological settings since it is the most stable (83). When Ti is exposed to the environment, these oxides develop on their own. The sliced surface of pure titanium will develop a 0.1 nm oxide coating within moments after being in contact with air. That coating may grow to a thickness of 0.1 nm in under a minute (84).

The passive titanium oxide coating shields surfaces against corrosion. It can induce osseointegration (124) because the passive film of TiO_2 on the surface of CP-Ti contains many OH ions that can react with the mineral constituents on the surface of bones (e.g., Ca^{2+} and PO_4^{3-}) (69,122).

Passivated implant metals go through oxidation without deteriorating physiologically (167). Experts dispute how to define "passivity". Titanium can be passivated with this procedure, but the FDA requires Titanium implant suppliers to use nitric acid to cleanse their products prior to delivering them. The oxide layer should not degrade under physiological circumstances (97).

Titanium may be passivated readily, both in its pure form and as an alloy to preserve its passivity (85), producing a stable TiO_2 layer that shields the implant from undergoing corrosion. Ti is found in alloys in quantities of 85% to 95%. This oxide will immediately repair any damage it sustains, such as during the implantation of an implant (82).

2.4.3 Corrosion and corrosion resistance of titanium and titanium alloys

Corrosion is the reaction of the material with surrounding environment that leads to surface degradation and subsequent damage of the material properties (86). Several factors are needed for corrosion to occur, anode, cathode, medium for metal dissolution, ions, and electrons. The reaction can be electrochemical, chemical, physical, or a combination.

Tissue compatibility requires more than just a low rate of corrosion; one must also consider what will happen to the corrosion's undesired reaction product. The reaction products are available for the cells to absorb. This may or may not be harmful to the cells (87).

Ions/particles accumulate in peripheral areas due to titanium surface corrosion. Titanium diffusion from implant components to adjacent tissues (9) may enhance inflammation and lower alloy esthetic effects (170). Ti^{4+} , Co^{2+} , and Al^{3+} impair DNA synthesis, mitochondrial dehydrogenase activity, mineralization, and alkaline phosphatase mRNA expression.

Due to the development of TiAl_3 compounds, alloys with aluminum concentrations more than 6 % are especially vulnerable to this issue. Due to the reduction of the production of TiAl_3 compounds, vanadium lessens the susceptibility for corrosion cracking (88).

It is generally known that corrosion wear causes aluminum and, in particular, vanadium ions, to slowly leak into the environment (181,111). The ions that are produced may prevent cells from differentiating.

It is more likely that a response to a foreign substance is what causes the marginal bone loss surrounding dental implants rather than bacteria (76). In vitro and in vivo research by Pettersson et al., that showed how leaking titanium ions from dental implants caused inflammatory mediators validated the notion (91).

Dental implant fracture is an uncommon occurrence with serious clinical repercussions. Corrosion can significantly reduce the material's ultimate strength and fatigue life, which can result in mechanical failure of the implant. Metal deterioration has been shown to cause implant breakage.

Since titanium is a very corrosion-resistant implant material, the superstructures are what primarily cause metal ions to be released. As corrosion develops, ions begin to escape into the surrounding tissues (92) .

Titanium and its alloys exhibit high corrosion resistance because a thick and persistent layer of titanium oxide forms on their surface. Titanium oxide is responsible for chemical stability in the human body (93). This layer develops quickly due to titanium's reactivity with oxygen, which results in a range of oxides, the most prevalent being TiO₂.

Nano Al₂O₃-TiO₂ coating significantly enhanced the corrosion resistance and fretting wear of CpTi (93). In addition to the forementioned points, nanoceramic hydroxyapatite (HAP) coatings are utilized to enhance the process of osseointegration. To improve the adhesion between the metal and ceramic coatings, nanostructured graded metallochromic coatings have also been tested, and as a result, nanoceramic coatings are rapidly gaining more attention.

When exposed to the majority of mineral acids, even those that might be rather dangerous, such as hydrochloric acid or sulfuric acid, the corrosion resistance level is high, leading to very little corrosion in these circumstances (94). Because titanium alloys are so resistant to corrosion and fatigue, they are the material of choice when a high corrosion fatigue strength is needed (95).

Another family of materials with great biocompatibility and improved corrosion resistance are ceramics. Despite their frequent use in complete hip replacement, heart valves, dental implants, bone fillers, and scaffolds for tissue engineering, ceramics are brittle, have a high elastic modulus, and may break due to their limited flexibility.

There is some scientific plausibility to the idea that there is a relationship between corrosion, the presence of titanium particles, and biological difficulties. However, there is no evidence to support a single direction of the causing events. A complicated host reaction to foreign substances involves several feedback loops as well as participation from wear, corrosion, titanium particles, inflammation, and microorganisms. Material deterioration is caused by tribocorrosion, a process that results in the release of titanium particles as a result of wear, corrosion, and environmental conditions (96).

A process known as "biocorrosion" may trigger the release of titanium particles due to the acidic oral environment caused by bacterial biofilm and/or inflammatory processes. When titanium is broken down, it directly activates osteoclasts and osteoblasts and triggers macrophages and lymphocytes to release inflammatory cytokines. This upsets the balance between bone production and resorption (97).

2.4.4 Intracellular uptake and toxicity

The word "cytotoxicity" refers to the series of molecular interactions that disrupt macromolecular synthesis, resulting in undeniable cellular, functional, and structural damage (97). Therapeutic uses for cytotoxic-mediated responses include cancer treatment (98). However, it is important to preserve the greatest amount of tissue vitality during dental therapy, and cytotoxic responses must be avoided; for this reason, all dental chemicals must be screened before being applied clinically.

Since there are almost many methods to cause cellular disturbance, cytotoxicity is a challenging process to define. The ability of cellular proteins to promote or prolong cell survival (99) and the genetic propensity of cells to trigger processes of cell death add to the complexity of comprehending this intricacy.

Since no reports of titanium toxicity have been made in a long time, titanium has been regarded as an innocuous substance with great biocompatibility. However, in a few instances, allergic responses, such as lichenoid reactions, erythema, irritation, and inflammation, may be brought on by titanium (alloy) implants (191,192).

Despite their perceived biocompatibility, titanium-based implants have been associated with negative outcomes like hypersensitivity and allergic reactions. (170,171). Tissues and implants may have interacted to produce these adverse effects (89, 193). Alternatively, the body may circulate and transport potentially cytotoxic and genotoxic titanium ions and particles.

Dental implants often use titanium alloys (Grade V), which include four to eight metals like vanadium, chromium, nickel, aluminum, and iron, to overcome the lower fatigue strength of CpTi (Grade IV) ((101, 102).

Conversely, titanium alloys have a propensity to corrode in bodily fluids (103) and release metallic ions. As a result, adverse effects such osteolysis, allergic responses, hypersensitivity, carcinogenesis, and toxicity (104) may occur.

Because dental implants include a variety of intricate structures that are always changing, it is difficult to determine which one has fewer negative effects (105).

Ti ions have been shown in the past to cause cytotoxicity and DNA damage in human cells (198,199). After Ti-based implants were applied, peri-implant animal tissues included Ti-particles/debris (0.5-250 μm) (114). Ti-particle phagocytosis has the potential to produce cytotoxicity in MG63 and rat calvarial osteoblasts (116,117); Moreover, Ti-particles have the potential to cause genotoxicity, which in turn causes apoptosis in mesenchyme stem cells (119).

It is well known that fibroblasts are toxic to high particle contents, which also affects fibroblast viability and proliferation. High particle concentration alters the viability and proliferation of fibroblasts and is harmful to them. According to in vitro research, fibroblast growth may be boosted by 0.001% titanium particles. Nevertheless, those cells' ability to proliferate is inhibited by greater amounts (114).

The genotoxic and cytotoxic potential of TiO₂ nanometric particles (100-100 nm) generated during the bio-corrosion of dental implants was also shown by recent investigations (115). Other investigations have shown that cells cultivated in the presence of titanium nanoparticles had lower cell viability and a higher frequency of mutations, resulting in binucleated and micronucleated cells (115).

Rat lung alveolar macrophages subjected to titanium-based nanoparticles underwent oxidative stress studies, which demonstrated the particles' cytotoxic and genotoxic effects (116).

Despite prior concerns about the cytotoxic effects of V and Al ions in Ti-6Al-4V alloy, which Woodman confirmed in a work with baboons (117), he was unable to identify the toxicity of this alloy in the investigation.

We can assess the biocompatibility of metal alloys using the cations generated by corrosion and wear. For instance, short-term in vitro tests have shown that the Ti-6Al-4V alloy exhibits Ti⁴⁺, Al³⁺, and V⁵⁺ cations in solution, all of which are harmless. Long-term tests have, however, shown that the same V⁵⁺ cation is significantly cytotoxic (118).

As a result, it is impossible to predict implantable materials' long-term behavior based solely on short-term in vitro studies. Europe and the US continue to use the Ti-6Al-4V alloy in hip prostheses due to the absence of clear toxicity issues. Researchers have reported similar findings regarding the acute cytotoxicity of Cu, Ni, and Be elements (119).

2.4.5 Evaluation of biocompatibility and cytotoxicity

The number of substances and materials being studied for biocompatibility is rapidly increasing. To obtain the most accurate results on the studied material, these tests must adhere to international standards (16,94).

We regularly revise or adjust the criteria used to assess the biocompatibility, cytotoxicity, and clinical efficacy of restorative materials in response to new scientific discoveries or to prevent the recurrence of previously encountered issues (120).

The International Organization for Standardization (ISO) is a worldwide federation that comprises national standards organizations, referred to as ISO member entities. At present, there are well-defined national standards for evaluating the methods used in biocompatibility testing. International standards, such as ISO 7405 for dental materials and ISO 10993 for medical devices, cover both dental materials and medical equipment (104).

ISO 10993 standards are a set of guidelines designed to assess the biological safety of materials intended for use on living creatures. The primary objective is to safeguard both humans and other organisms. These constantly updated standards serve as a guide for evaluating biological response by performing various tests prior to using new materials intended for use on humans (7).

An evaluation of the dental industry's medical equipment compatibility before clinical trials is the main objective of the international standard ISO 7405 (26). (The World Dental Federation collaborated in the development of this ISO standard. It refers to the preclinical assessment of dental materials and serves as an adjunct to ISO 10993 (127).

Biological evaluation of new materials which are considered to be used for various dental procedure, is determined in three different test stages (85).

- In-vitro assays
- In-vivo experiments
- Clinical trials

2.4.5.1 In vitro tests

In-vitro assay are tests done with cell cultures, outside of living organisms. In-vitro tests are performed with the cells in the region intended for material to be used, or cells like those cells (104).

The effects of the material in contact with these cells are studied, investigating the effects on the number of cells able to live, effects on cell growth rate and the effects on the metabolic activity speed on cells. With in-vitro tests, the cellular and systemic toxicity of the investigated material is studied, revealing the materials toxic profile (106).

For in-vitro studies, mouse fibroblasts (L929) or cells that show similar characteristics to cells in the region where the material is intended to be used are chosen(107).The cells selected are primary or secondary cells with high proliferation rate and have similar feature of tissue cells (108).

In-vitro tests are fast, cost-effective, and easy to standardize; but in-vitro tests cannot fully mimic the in-vivo environment .This disadvantage may cause the response of the complex biological tissue's response to differ from the in vitro tests when studying the material in vivo (109).

The general goal of in-vitro cytotoxicity testing is to compare the outcomes with the control to determine the impact on the cells exposed to the extract of the researched substance on cell viability and proliferation (109).

2.4.5.2 In-vivo tests

In-vivo tests are secondary experiments carried out to examine the systemic and local effects of the material to be tested on certain experimental animals such as mice, rats, cats, dogs or pigs (110). Although more comprehensive results, regarding living creatures reaction, are obtained compared to in-vitro tests, but standardization on in-vivo tests are difficult (111).The in vivo test is expensive, subject to ethical regulations, and may have adverse consequences for experimental animals (112).

2.4.5.3 Clinical studies

These are studies in which the biocompatibility of the material is best evaluated. The materials investigated in clinical studies are tested on human-like mammals or on volunteered humans (7).It should be demonstrated that the material used in clinical studies is not cytotoxic in tests both in vitro and in vivo (112).

Disadvantages of clinical studies are the long time requirement for data acquisition and evaluation of the data obtained; the cost of these clinical studies being high and these studies needs to be ethically correct and must be done within the framework of the law (74).

2.4.5.4 Cell culture

Quantitative evaluations of many biological endpoints in diverse cell types were included in the cytotoxicity investigations of various dental materials (133). The first stage in determining biocompatibility is often cell culture research.

They conduct a toxicity investigation using a simplified approach that lessens the influence of complicated factors. (128) Cell culture techniques for testing dental materials are somewhat simple to use, repeatable, economical, and allow for meticulous control of the results (113) .

Cell culture is formed by separating the cells from the mechanically taken parts from living tissue with the help of proteolytic enzymes and reproducing them in the correct medium in the laboratory.

Cell cultures are applications that can be repeated easily, giving minimal to no harm to living creatures and having low cost. Because of these advantages, they have widespread use .In cell culture studies, three different cell types are used; primary, secondary and continuous cell cultures (114) .

Primary cell cultures are cells that are isolated from living tissue and carry the genotype and phenotype characteristics of the original tissue cells until the first passage of cells (105). Primary cells may better suit the in vivo environment since they possess a distinct metabolic capacity that is similar to that of target cells (115).

Secondary cell cultures are cells obtained when primary cell cultures are subcultured. They contain up to 90% of the primary cell properties up to 20 to 50 passages depending on the cell type obtained (116).

Continuous cell cultures are also being used, as primary cells are not easy to obtain. These cell lines are obtained from malignant cell sources (108). Human cervical carcinoma (HeLa) cells (117), human colon carcinoma (HCT115) cells (118),bone osteosarcoma (SaOs-2) are used for in-vitro tests (119).

Because their growth rates and biological responses are always the same, continuous cell lines as 3T3 or L929 murine fibroblasts are often used to test how cytotoxic dental materials are.

Furthermore, worldwide standards recommend using these cells for dental equipment testing because it is easy to manage the cell development environment. (120).

Continuous cell cultures are products based on embryonic and cancerous cell types (126, 35). Although cells in continuous cell cultures may be genetically different from primary cells, their effects on the test results are minimal since they are metabolically more stable. Continuous cell cultures are easier to standardize for in-vitro studies (121).

2.4.5.4.1 MTT cytotoxicity assay

An alloy needs to pass a few tests before being deemed biocompatible and capable of osseointegration. For starters, cytotoxicity testing is crucial. Like other metals, titanium alloys are generally non-toxic, although they may become so when they corrode and produce ions or when they wear and produce particles (122).

The 3 (4,5 dimethyl 2 thiazolyl) 2,5 diphenyl 2H tetrazolium bromide test, also known as the MTT test, evaluates the activity of mitochondrial dehydrogenases. The applicable International Standard for the biological evaluation of medical equipment (133) uses this test to determine the cytotoxicity of metal ions or wear debris.

According to the applicable International Standard for the biological evaluation of medical equipment (120) ,it determines whether metal ions or wear debris are cytotoxic.

The MTT assay, based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) by the cells that remain viable after exposure and incubation with a test chemical or device, provides a rapid assessment of cell proliferation and cytotoxicity, while a colorimetric assay measures cell metabolism or function.

96-well plates are frequently used for this experiment, allowing for high throughput. The main idea behind it is that mitochondrial dehydrogenase breaks the tetrazole ring at the cytochrome b and c sites of living cells, converting the yellow, water-soluble MTT into a purple crystalline formazan (123,124).

Physiologically balanced solutions dissolve the MTT reagent, typically administered to cells in culture at doses of 0.2 to 0.5 mg/cm². After a brief incubation period of one to four hours, a calibrated colorimeter measures the absorbance at 570 nm to determine the quantity of purple formazan produced. Cells in optimal health that have high mitochondrial activity create the deepest purple color (125).

The MTT test does, however, have several disadvantages that are dependent on the cell's ability to withstand cell death. The possibility that injured mitochondria might still convert MTT to formazan crystals is an amazing disadvantage (126).

2.4.5.4.2 ATP assay

The primary chemical energy source in cells, adenosine triphosphate, or ATP, is required for a variety of biological functions, including synthesis, signaling, transport, and movement. Therefore, one of the most sensitive endpoints, cellular ATP, can determine cell viability (140). As a result, cells that suffer deadly damage and lose their capacity to synthesize ATP see a sharp decline in ATP levels (141).

The ATP test's foundation is the transformation of luciferin into oxyluciferin. The ATP assay is the fastest, most accurate, and least prone to artifacts when compared to other viability assays. However, rather than being a byproduct of the assay chemistry, the repeatability of pipetting several samples usually limits the sensitivity of the ATP test (127).

2.4.6 Evaluation of cell adhesion, morphology, and spreading

The interaction between the material and its surface cells determines how well a novel biomaterial performs (171). Shape, adherence, and cell spreading are indicators of growth behavior and a suitable cell response to the chemical under investigation (172).

We can study cell-material interaction using electron, confocal, and fluorescence microscopy. Because of their high opacity, cells adhering to materials are challenging to see using optical microscopy (173). Confocal laser scanning microscopy and fluorescence can overcome this barrier (174). With great resolution, SEM measures biomaterial surface coverage and cell dispersion. Seed, disseminate, and track cell orientations. Transmission electron microscopy (TEM) has the best magnification and resolution, but its small sample size and long processing time limit cell-material interactions

Detection of metallic particles within the soft tissue around the implant, combined with M1 macrophages and an elevated level of titanium concentration detected in lymphocytes (9). The study also assessed the damage to the implant surface caused by the release of metal particles during the implantation operation (128).

The application of synchrotron X-ray microfocus spectroscopy revealed a diffused and varied distribution of titanium (Ti) in inflammatory tissues surrounding Ti implants that had penetrated the skin.

Implant implantation debris is not anticipated to be the main contributor, based on the position and distribution features of Ti particles. Evidence of Ti in tissues can be acquired from surface crack corrosion and micro-motion.

2.5 Hypothesis study:

Null hypotheses evaluated as followed:

1st hypothesis: The cytotoxicity of Ti6Al4V (grade V) alloy was compared to grade IV Commercially Pure Titanium to see whether it was equivalent.

2nd hypothesis: We hypothesized that Ti6Al4V (grade V) structures would be as biocompatible as commercial pure titanium grade IV.

2.6 Specific aims

The specific aims of this study were:

1. To analyze the cytotoxic response of grade IV pure titanium and its alloy Ti-6Al-4V (Grade V) on gingival fibroblasts using MTT assay.
2. Another value of this thesis is to evaluate the fibroblast cells response to Ti6Al4V (Grade V) relative to (Grade IV) commercially pure titanium.
3. To supply valuable data and recommendations on the biocompatibility of grade IV titanium and grade V titanium alloy for dental implant applications based on the findings of the MTT assay.

3.MATERIALS AND METHODS

3.1 Sample's description

The study examines the differences in fibroblast cell reactivity between commercially available titanium IV and grade V titanium alloy using MTT tests. A total of 32 commercially pure titanium grade IV (ASTM F67) and titanium grade V (Ti6Al4V) specimens, 16 per group, were tested in a lab according to ISO 7405 guidelines (243) (Figure.3.1). Grade IV specimen disks were 6 mm diameter and 2 mm thick, whereas grade V disks were 8 mm and 2 mm thick (Figure 3.2) depicts implant manufacturing sample preparation.

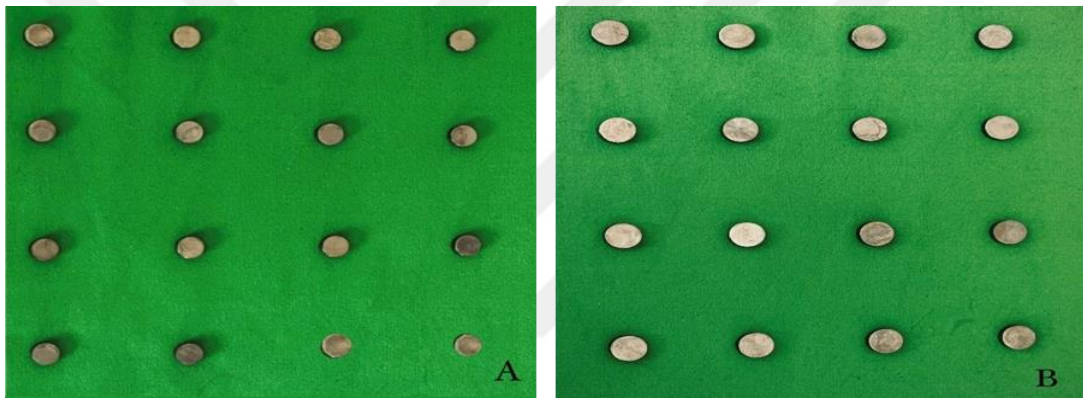


Figure.3. 1.Two groups' specimens (A) grade IV titanium (B) grade V titanium alloy

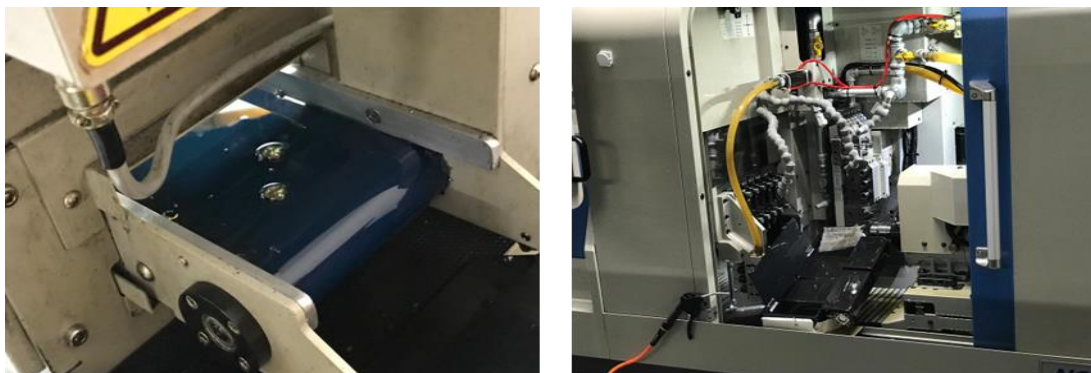


Figure.3. 2.The figure illustrates the process of sample preparation in an implant manufacturing company.

3.2 Study intervals:

The specimens were examined at three distinct time intervals: 24 hours, 48 hours, and 72 hours. These intervals represent the specific time points at which the specimens were observed and analyzed during the study.

3.3 Observations and analysis:

At each time interval (24 hours, 48 hours, and 72 hours), samples were examined, and data collected.

The purpose of studying the specimens at multiple time points was likely to observe any changes or developments over time and gather information about the behavior or characteristics of grade IV and grade V Titanium.

To summarize, (Table.3.1) and (Figure.3.2), demonstrates that there is an equal distribution of specimens between grade IV and grade V groups, with each group comprising 50% of the total. The total number of specimens in the study is 32, as indicated in the "Total" row, which represents 100% of the sample.

Table.3. 1 .Specimens' distribution among study groups

		N	%
Group	Grade IV	16	50%
	Grade V	16	50%
	Total	32	100%

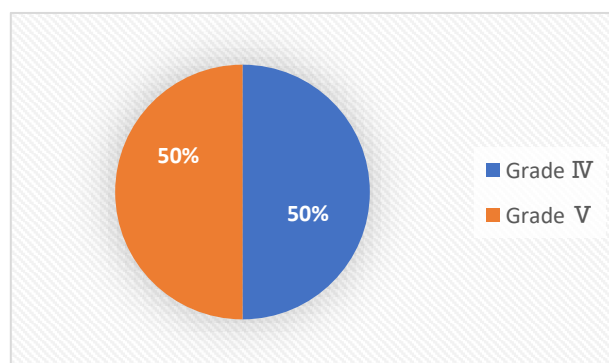


Figure.3. 3.Specimens distributed among study courses.

3.4 Specimens' preparation:

Before conducting the tests, all specimens were metallographically polished with white coarse grit wheel 22×3.7 (Reddish Stone. Italia) (Figure.3.4), to remove the main dirt, followed by white knife edge rubber aluminum oxide wheel 7.8×22mm (Richemplus. USA) for extra finishing. The white wheel is fine and non-contaminating and extremely flexible (Figure.3.5).



Figure.3. 4.White coarse grit wheel 22×3.7 (Reddish Stone. Italia for polishing)



Figure.3. 5.White knife edge rubber aluminum oxide wheel 7.8×22 mm (Richemplus.USA)

Using a digital ultrasonic cleaner CD-4820 (China, certified by ISO CE.), all polished specimens were ultrasonically cleaned to remove any dirt (Figure.3.5). Deionized water was used for 10 minutes (Figure.3.6).

After the initial cleaning with deionized water, the specimens were subjected to a second cleaning step using 70% propanol. Propanol is a common alcohol-based cleaning agent. The specimens were immersed in the ultrasonic cleaner, which now contained the 70% propanol solution. This step further ensured the removal of any remaining impurities from the specimens' surfaces. The specimens were cleaned in propanol for a duration of 10 minutes.

The purpose of this cleaning process was to ensure that the specimens were free from any dirt, contaminants, or residues that could potentially interfere with the subsequent analysis or testing procedures. The use of deionized water and propanol as cleaning agents helps to achieve a high level of cleanliness and prepares the specimens for further examination or experimentation.



Figure.3. 6.Digital ultrasonic cleaner CD-4820) for specimens cleaning

3.5 Specimens study grouping

The specimens have been categorized into various groups according to the treatment they received and the purpose they were used for. The following is a list of the study groups, along with the treatments and samples that correlate to them (Table 3.2):

Table.3. 2.Experimental groups and treatments

Group	Treatment	Sample (n)	Purpose
Negative Control Group	Cells treated with RPMI + 10% FBS medium	N/A	Serve as a control to compare the effects of other treatments. Represents the baseline or normal condition
Positive Control Group	Cells treated with 10% DMSO and 1% Phenol	N/A	Used to validate the effectiveness of the assay. Expected to produce a known response or effect.
Test Group (Cp Grade V)	N/A	16	Includes conducting tests that specifically examine the impact of grade V discs on cells.
Test Group (CpTi Grade IV)	N/A	16	Involves testing the effects of the CpTi grade disks on the cells

N/A = Not Applicable

Following that, discs were autoclaved for 20 minutes at 134 °C and 2 bar of gauge pressure by (Systec vertical autoclave GmbH & Co. Germany), which is ISO 9001 and ISO 14001 certified (Figure.3.7).

The autoclaving process effectively destroys or eliminates microorganisms, including bacteria, viruses, and fungi, that may be present on the discs' surfaces. This ensures a clean and sterile environment for subsequent procedures, reducing the risk of contamination.



Figure.3. 7.Systec vertical autoclave (GmbH & Co. Germany)

3.6 Cell culture

Cell culture was conducted using L929 cells (Yıldız Technical University / central laboratory). A total of 10^4 cells were cultured on 24-well plates using RPMI1640 media with an acidity indicator, 10% FBS, 1% penicillin/streptomycin, 1% l-Glutamine. This culture medium provided the necessary nutrients and growth factors to support the growth and viability of the L929 cells. Cultures were incubated for 24 hours. The experiment was conducted in a controlled environment with a humidified atmosphere consisting of 5% CO₂ in air, at a temperature of 37°C and a relative humidity of 95%. This configuration is specifically engineered to minimize the evaporation and condensation of medium.

The following day, when the cell population had reached at least 80% confluency (indicating sufficient cell proliferation) as shown in (Figure 3.8), the cells were detached using a solution containing 0.10% trypsin and 1 mM EDTA-4Na. A volume of 100 mL of the cell suspension, containing 10^4 cells/mL, was placed on the test samples and kept in an incubator at a temperature of 37°C with 5% CO₂ for a duration of 24 hours.

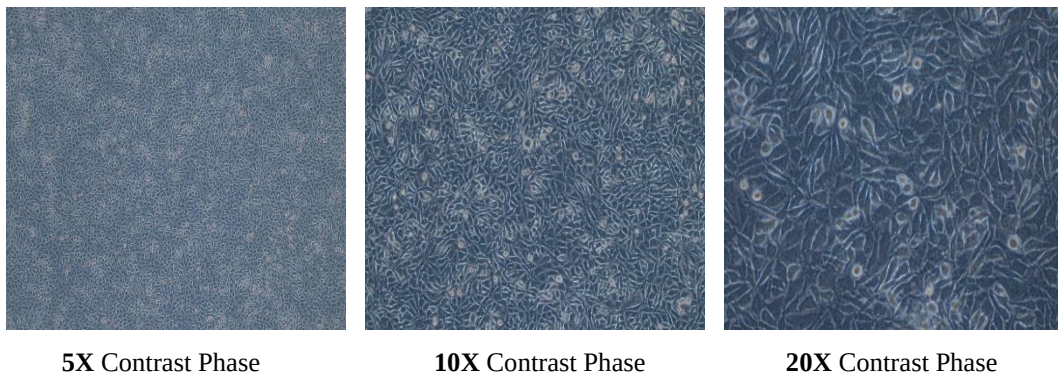


Figure.3. 8.L929 Cells before seeding (Invert Microscope: Zeiss Axio Observer USA)

3.7 Cell viability assay (MTT Assay)

At the 24-hour time point, the next day, the cell culture media was collected, and the samples were introduced to the cells and cultured for another 24 hours.

After incubation, the container was emptied, and each small area was treated with 5 mg MTT/mL in liquid medium (Figure 3.9). The cells were then incubated for 4 hours at 37°C with 5% CO₂. They were then agitated for 1 minute. To verify reliability and reproducibility, the MTT assay was replicated twice. It accounts for experimental heterogeneity and gives data statistical significance.

Repeating the studies twice helps identify outliers and calculate the average or mean value. Subsequently, the MTT solution was substituted with 500µL of dimethyl sulfoxide (DMSO) per well to facilitate the dissolution of the formazan crystals, the resulting solution typically has a deep purple or dark blue color (Figure.3.10).

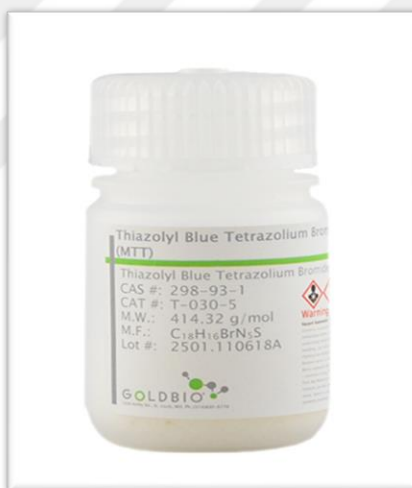


Figure.3. 9.Thiazolyl blue tetrazolium bromide (MTT).



Figure.3. 10.24-well cell plate after fixation and formazan crystals staining.

Upon dissolving the formazan crystals with 500g/L/well of DMSO, the resultant solution generally exhibits a deep purple or dark blue hue. The optical density (OD) of the solution is subsequently determined at a specified wavelength of 570 nm using a spectrophotometer (Thermo Scientific Multiskan ELISA Reader GO, U.S.A) (Figure.3.11).

The OD measurement at 570 nm correlates with the amount of formazan produced proportional to the number of viable cells in the sample. The greater the OD reading, the more the number of viable cells, and vice versa. By comparing the OD readings of different samples, cell viability was assessed.

Similarly, at the 48 and 72-hour time points, following the first 24-hour incubation, the cell culture media was collected, and the samples were introduced to the cells and cultured for an additional 48 and 72 hours. After the incubation period, the substance in the container was removed, and each well was treated with the MTT solution and left to incubate for an extra 4 hours at a temperature of 37°C with a 5% concentration of carbon dioxide. The following procedures, which involved replacing with DMSO and measuring optical density, were carried out in the manner previously described.



Figure.3. 11.Microplate photometer (Thermo Scientific Multiskan Go, USA)

3.8 Statistical Assessment:

The statistical analysis was carried out using version.26 of the statistical package for the social sciences (SPSS) (IBM.SPSS, New York, New York). It was determined that the level of statistical significance was set at $p < 0.05$.

Samples taken independently T-tests were carried out to investigate the impact that the grade of titanium had on the proportion of cells that were viable at each time point.

A one-way analysis of variance (ANOVA) was conducted using SPSS 14.0 for Windows software to examine the impact of time on cell viability in different types of titanium grades. This was followed by a post hoc Tukey's test.

4. RESULT

4.1 Sample description

The data were found to follow a normal distribution in all time intervals, as shown by the Shapiro-Wilk's test ($P > 0.05$) (Table 4.1) and (Figure 4.1).

Table.4. 1.Shapiro-Wilk's test for normal distribution.

Time Interval	Group	statistics	Df	P-Value
24 Hours	Grade IV	0.842	16	0.066
	Grade V	0.927	16	0.220
48 Hours	Grade IV	0.917	16	0.152
	Grade V	0.933	16	0.276
72 Hours	Grade IV	0.940	16	0.346
	Grade V	0.807	16	0.052

Statistics: mean viability of the cells

Df: The number of independent observations available for analysis

P: $< 0,05$

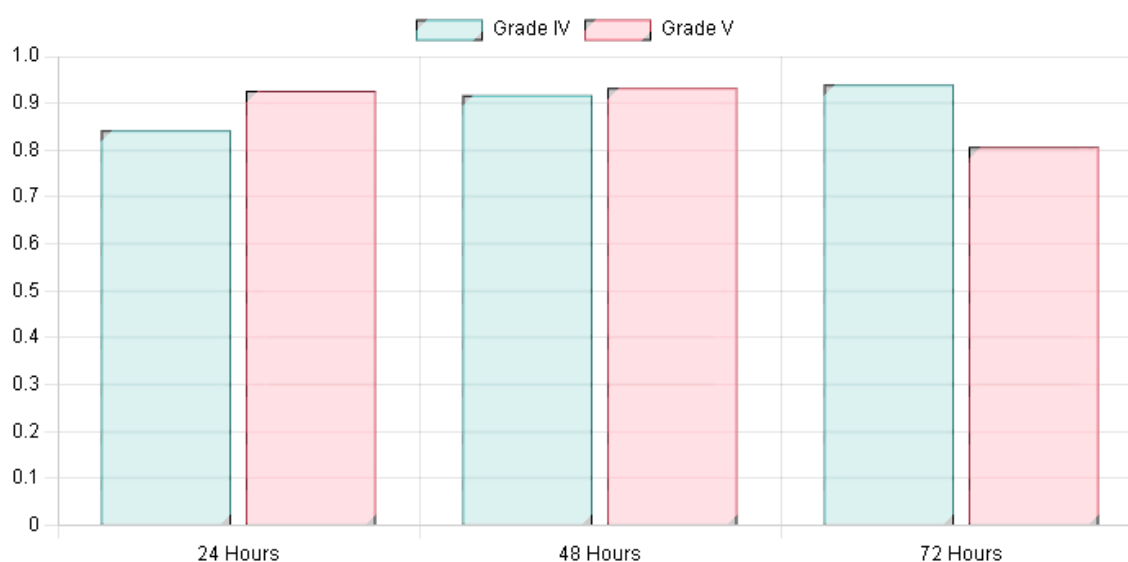


Figure.4. 1.Shapiro-Wilk's test findings for Grade IV titanium and Grade V alloy cell viability data normalcy at three time points: 48, 72, 24 hours.

In the context of the provided Shapiro-Wilk's test results:

Grade IV at 24 hours: $W = 0.842$.

The p-value of 0.066 is above the generally employed significance level of 0.05, suggesting that the Grade IV data at 24 hours conforms to a normal distribution.

Grade V at 24 hours: $W = 0.927$.

The p-value of 0.220 is also greater than 0.05, so the Grade V data at 24 hours also follows a normal distribution.

Grade IV at 48 hours: $W = 0.917$.

The p-value of 0.152 is greater than 0.05, so the Grade IV data at 48 hours follows a normal distribution.

Grade V at 48 hours: $W = 0.933$, $df = 16$, $p\text{-value} = 0.276$

The p-value of 0.276 is also greater than 0.05, so the Grade V data at 48 hours follows a normal distribution.

Grade IV at 72 hours: $W = 0.940$.

The p-value of 0.346 is greater than 0.05, so the Grade IV data at 72 hours follows a normal distribution.

Grade V at 72 hours: $W = 0.807$.

The p-value of 0.052 is greater than 0.05, so the Grade V data at 72 hours also follows a normal distribution.

Based on these results, none of the p-values were found to be below the significance level of 0.05 between the grade IV and grade V groups, indicating that there is insufficient evidence to reject the null hypothesis of normality for any of the groups within the different time intervals. Therefore, it can be reasonably assumed that the data in each group and time interval follows a normal distribution. This means that parametric statistical tests can be used to properly look at how the two groups are different.

4.2 Cell viability

The cell viability of the Ti-6Al-4V (grade V) group specimens were compared with that of CpTi (grade IV), among time intervals and they were expressed as the cell viability ratio (%) relative to CP Ti (grade IV).

Grade IV titanium has higher cell viability than Grade V titanium, according to graph analysis. Variable cell viability is significant. Grade IV titanium cell viability rises with time, but Grade V titanium is stable (Figure.4.2).

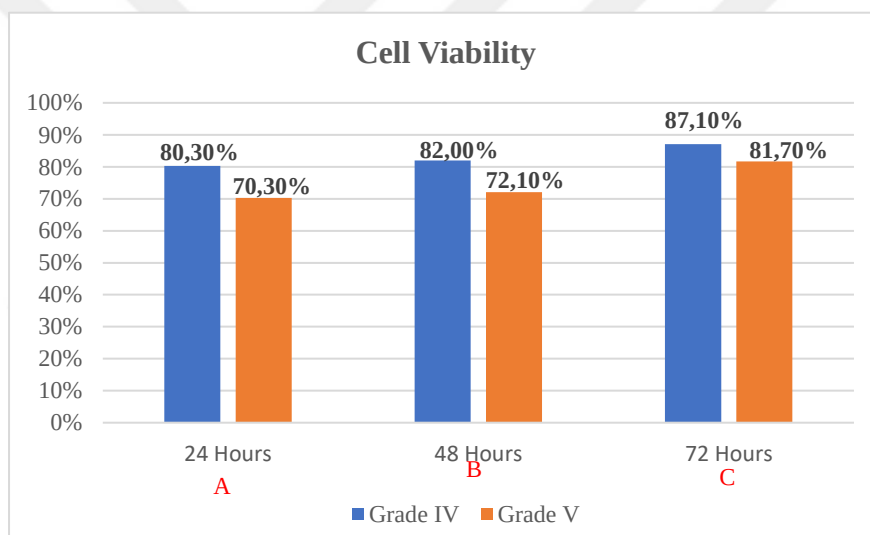


Figure.4. 2.Cell viability among the (A) 24 hr, (B) 48 hr and (C) 72 hr of medium extracts of titanium alloys obtained from MTT assay.

As shown in (table.4.2):

after 24 hours, cell viability in grade IV titanium (80.3 ± 5.1) % was statistically significantly higher than Grade V Titanium (70.3 ± 4.1) % by 10% ($p < 0.001$).

After 48 hours, cell viability in grade IV Titanium (82 ± 6.7) % was statistically significantly higher than grade 5 Titanium (72.1 ± 3.2) % by 9.9% ($p < 0.001$).

Also, after 72 hours, cell viability in grade IV Titanium (87.1 ± 2.5) % was statistically significantly higher than grade V Titanium (81.7 ± 4.5) % by 5.4% ($p = < 0.001$).

In grade IV Titanium, no statistical difference was observed in cell viability between 24 and 48 hours ($p = 0.338$), however, cell viability % after 72 hours was statistically higher than both after 24 hours by 6.9% ($p = 0.001$) and after 48 hours by 5.2% ($p = 0.003$).

Also in grade V Titanium, no statistical difference was observed in cell viability between 24 and 48 hours ($p = 0.272$), however, cell viability % after 72 hours was statistically higher than both after 24 hours by 11.4% ($p < 0.001$) and after 48 hours by 9.6% ($p < 0.001$).

Table.4. 2. Descriptive statistics of cell viability in study groups among time intervals

Time Interval	Group	Mean	Sample standard deviation	Minimum	Maximum	95% CL for Mean	
						Lower CL	Upper CL
24 Hours	Grade IV	80.3	5.1	69.7	85	77.6	83
	Grade V	70.3	4.1	62.5	77.1	68.1	72.5
48 Hours	Grade IV	82	6.7	71.9	90.7	78.4	85.5
	Grade V	72.1	3.2	65.9	76.9	70.4	73.8
72 Hours	Grade IV	87.1	2.5	82.7	91	85.8	88.5
	Grade V	81.7	4.4	76.9	87.2	79.4	84

Certainly, based on the pairwise comparisons of cell viability % between titanium grades, here is a summary (Table.4.3):

- **Titanium grade IV:**

Comparing 24 hours and 48 hours: The mean cell viability at 48 hours is, on average, **1.7** lower than at 24 hours. Nevertheless, this discrepancy does not have a significant statistical impact ($p = 0.388$).

When comparing the viability of cells after 24 hours and 72 hours is, on average, **6.9** lower than at 24 hours. This difference is statistically significant ($p = 0.001$).

Comparing 48 hours and 72 hours: The mean cell viability at 72 hours is, on average, **5.2** lower than at 48 hours. This difference is statistically significant ($p = 0.003$).

- **Titanium grade V:**

Comparing 24 hours and 48 hours: The mean cell viability % at 48 hours is, on average, **1.8** lower than at 24 hours. Still, this distinction lacks statistical significance ($p = 0.272$).

Comparing 24 hours and 72 hours: The mean cell viability % at 72 hours % is, on average, **11.4** lower than at 24 hours. This difference is statistically significant ($p = 0.000$).

Comparing 48 hours and 72 hours: The mean cell viability % at 72 hours is, on average, **9.6** lower than at 48 hours. This difference is statistically significant ($p = 0.000$).

In summary, for titanium grade IV, there is a statistically significant decrease in cell viability within the time frame of 24 to 72 hours, as well as from 48 to 72 hours. However, the difference between 24 hours and 48 hours is not statistically significant.

For titanium grade V, there is a statistically significant decrease in cell viability percentage between 24 hours and 72 hours, as well as between 48 hours and 72 hours. The difference between 24 hours and 48 hours is not statistically significant.

These data indicate that the cell viability of both titanium grade IV and titanium grade V diminishes over time, although the rate of reduction may change across different time intervals.

These additional findings provide more specific comparisons within each titanium grade and between different time intervals. They indicate that, regardless of the titanium grade, there were significant decreases in cell viability over time, with the largest decrease observed at the 72-hour.

However, for some specific comparisons, such as 24 hours vs. 48 hours, the differences were not statistically significant, suggesting that there may not be a substantial change in cell viability within that time frame.

Table.4. 3.Pairwise comparisons of cell viability percentage between titanium grades.

Titanium Grade	Time intervals		Mean Difference	p	95% CL for Difference	
					Lower	Upper
Grade IV	24 Hours	48 Hours	1.7	0.388	5.7	2.4
		72 Hours	6.9	0.001	10.5	3.3
	48 Hours	72 Hours	5.2	0.003	8.3	2
Grade V	24 Hours	48 Hours	1.8	0.272	5.1	1.5
		72 Hours	11.4	0.000	15.3	7.5
	48 Hours	72 Hours	9.6	0.000	11.7	7.6

The study conducted a comparison between grade IV titanium and grade V titanium at various time intervals, including 24 hours, 48 hours, and 72 hours. Below is an analysis of the discoveries (Table 4.4):

1. Grade IV Titanium:

- After 24 hours, the mean cell viability was 80.3% (± 5.1 sd).
- The mean cell viability after 48 hours was 82% (± 6.7 sd)
- Following a period of 72 hours, the average cell viability was 87.1% (± 2.5 sd).

2. Grade V Titanium:

- **After 24 hours**, the mean cell survival rate was 70.3% (4.1 sd).
- **Within 48 hours**, the mean of living cells was 72.1% (3.2 sd).
- **During the 72-hour period**, the mean proportion of viable cells was 81.7% (4.4 sd).

The mean difference between grade IV and grade V titanium was calculated for each time interval:

- **At 24 hours**, the mean difference was 10% (grade IV - grade V).
- **At 48 hours**, the mean difference was 9.9 %.
- **At 72 hours**, the mean difference was 5.4%.

The statistical analysis showed that the mean differences across the titanium grades exhibited statistically significant results ($P < 0.001$) across all time intervals. Indicating that the choice of titanium grade had a significant effect on cell viability.

The confidence intervals of 95% (CI) for the mean differences provide a range within which the true difference between the two titanium grades is likely to fall. The lower and upper bounds of the 95% CI were also provided:

- At 24 hours, the 95% CI for the mean difference was 6.6 to 13.3 %.
- At 48 hours, the 95% CI for the mean difference was 6.1 to 13.7 %.
- At 72 hours, the 95% CI for the mean difference was 2.8 to 8%.

Overall, the data suggests that grade IV titanium consistently exhibited higher cell viability percentages compared to grade V titanium across all time intervals (24, 48, and 72 hours). These disparities were determined to have statistical significance. Furthermore, within each titanium grade, there were significant differences in cell viability percentage between the different time points.

Table.4. 4.Effects of titanium grades and time on cell viability percentage.

	Grade IV Mean $\pm$ sd	Grade V Mean $\pm$ sd	Mean difference	P	95% CL for Difference	
					Lower	Upper
24 Hours	80.3 $\pm$ 5.1	70.3 $\pm$ 4.1	10	0.000	6.6	13.3
48 Hours	82 $\pm$ 6.7	72.1 $\pm$ 3.2	9.9	0.000	6.1	13.7
72 Hours	87.1 $\pm$ 2.5	81.7 $\pm$ 4.4	5.4	0.000	2.8	8
P (Between time intervals)	0.001	0.000				

5. DISCUSSION

The study aimed to determine if grade V titanium alloy Ti-6Al-4V was equal to grade IV commercially pure titanium in terms of its toxicity to fibroblast cells in the peri-implant environment.

Titanium has been the most effective and popular dental implant material to date (129). The utilization of dental implants in the treatment of individuals with partial or complete tooth loss, it has been possible to restore function to the stomatognathic system, while also maintaining dental tissues and ensuring the treatments durability. Because of its excellent success rate, dental implant has become a widely favored treatment choice for many patients (130).

Dental implants are commonly constructed from unalloyed titanium CpTi (Grade IV). However, in dentistry, titanium alloys (Grade V) consisting of four to eight metals, such as vanadium, chromium, nickel, aluminum, and iron, are used to enhance their resistance to fatigue (126).

Titanium of Grade IV often includes trace quantities of iron, carbon, oxygen, and nitrogen. These trace elements are more abundant and improve the mechanical characteristics of the material (50).

While titanium-based implants are generally regarded as biocompatible, it is important to note that the particles and ions they produce may not be. The implants are deemed biocompatible due to the little occurrence of cell death (necrosis) in the vicinity of the implants within a short period of time. Additionally, they successfully pass in vitro cytotoxicity tests, such as ISO-10993 and ISO-7405, since they do not emit any harmful substances when immersed in cell culture experiments.

Earlier studies have shown a total success rate of 95.3% for implants implanted in partially and fully edentulous areas after 3-7 years of loading (131). Nonetheless, and unsurprisingly, many difficulties associated with dental implants continue to occur, and dental surgeons must be cognizant of this condition to prevent further biomechanical issues and implant failures (132) .

When implants that are essentially inactive are too large to be engulfed by phagocytes, they undergo fibrous encapsulation. If particles are generated, they may be sufficiently minuscule for macrophages to absorb them. This would lead to impaired phagocytosis and localized chronic inflammation, both of which might contribute to bone resorption in the adjacent region (133).

Conversely, titanium alloys tend to corrode when exposed to bodily fluids (179), resulting in the release of metallic ions. This may lead to many negative consequences including clinical implant failure, osteolysis, allergic responses, cytotoxicity, hypersensitivity, and carcinogenesis (128).

While grade V titanium (Ti-6Al-4) is an alloy of titanium that also contains aluminum and vanadium in addition to iron, grade IV titanium is a commercially pure titanium alloy that only contains iron. The chemical and physical characteristics of the material, such as its resistance to corrosion, mechanical strength, and biocompatibility, can be affected by these alloying components.

However, grade V (Ti-6Al-4) alloy is rarely used as an uncontested material for biomedical implants. A new study suggests that dental implants have the tendency to degrade and release metallic particles (180). It may be inferred that fibroblasts are not negatively impacted by Ti-6Al-4 V alloy when they are not exposed to challenging conditions, such as acidic environments (134).

Grade V titanium alloy is often used for medical and dental implants due to its superior physical, mechanical (156, 157), and biological properties compared to other easily available biomedical metals and alloys. Specifically, this substance surpasses other metals in terms of corrosion resistance, outstanding biocompatibility, lightweight design, and exceptional tribological qualities, making it very beneficial for dental applications (158,59).

Studies have shown that titanium alloys may be harmful to skeletal muscle when they come into close contact (139). Nevertheless, the current knowledge on human biopsies is scarce.

Cytotoxicity is the term used to describe a cascade of molecular events that interfere with the synthesis of complex molecules, leading to evident harm to the functionality and organization of cells (140).

Fibroblasts, being the primary cell type of mesenchymal origin, act as a perfect prototype for examining for investigating the characteristics and actions of other cells from the same origin. While it is not possible to accurately anticipate the activity of cells resembling osteoblasts based on the activity of fibroblasts, their common launching fundamental qualities for growth and differentiation can serve as a foundation for future study and development (160,161).

The cytotoxicity test is a biological evaluation and screening process that investigates the impact of medical and dental devices on cell growth, reproduction, and morphology. This test utilizes tissue cells that are cultivated in vitro (132).

This study was an in-vitro study, which is relatively effective in terms of cost and time compared to in-vivo studies. There are numerous elements that need to be considered because in vitro environments cannot accurately reproduce in vivo situations. Nonetheless, it is feasible to determine the fundamental cellular response for a single-type cell, which may serve as a model for cells of similar origin (143).

The study investigated the cell viability of two different titanium grades, Grade IV and Grade V, over three-time intervals: 24, 48, and 72 hours. Cell viability refers to the percentage of cells that remain alive and functional after a specific period. The study aimed to compare the cell viability between the two titanium grades and observe any changes over time.

When examining the in vitro study methodology, an effort was made to obtain samples from all groups that exhibited identical characteristics. Our attempt to obtain discs with uniform width was unsuccessful due to technical constraints. To make sure that comparisons between groups were as accurate as possible, the cells were carefully put on the disc using a droplet that did not touch the disc's edges. Thus, the quantity of cells utilized, and the extent of expansion were uniform across all groups (144).

The cell viability was assessed using the MTT test, as indicated by the International standard ISO 7405 standards. This test relies on the capacity of the mitochondria's Succinate dehydrogenase (SDH) to transform the yellow MTT dye, which is a solution of tetrazolium salt, into a purple insoluble substance called formazan. The quantity of formazan is closely correlated with the number of viable cells. The optical density of the resulting solutions was quantified at a wavelength of 570 nm using a microplate reader after the addition of the solution. The optical density was converted to a percentage relative to the controls for each cell culture plate. (160,161).

Based on the results we provided, it shown that at all time points, the cell viability of the grade IV and grade V titanium alloys differs statistically significantly. Grade IV titanium's cell survival after 24 hours was 10% greater than in grade V titanium alloy, and after 48 hours, it was 9.9% higher. After 72 hours, the difference was 5.4% higher in grade IV titanium compared to grade V titanium alloy.

Across all time intervals, there was notable variability in cell viability within each group, as indicated by the standard deviations. This variability may arise from various factors, such as inherent biological differences, experimental procedures, or other unidentified variables influencing the cell viability outcomes.

In both grade IV and grade V titanium alloy, there was no statistical difference in cell viability percentage between 24 and 48 hours. However, cell viability after 72 hours was statistically higher than after 24 and 48 hours in both groups. This suggests that the cytotoxicity of both grades' titanium decreases over time.

The causes of the comparatively reduced survival rate of cells and the possibility of increased cell mortality on grade V titanium are not explicitly discussed in the available information. The surface properties of grade V Titanium alloy, including its composition (which includes aluminum and vanadium), roughness, and potential interactions with cells, can influence its performance (160,161). These parameters can influence cell adhesion, proliferation, and overall cell health, which may result in elevated cell mortality.

Nevertheless, the impact of toxicity was seen to reach a stable state and diminish over time. The presence of a TiO₂ passivating layer over time reduced the toxicity of the test materials by preventing Ti, Al, and V ions from entering tissue cells when they came into contact with them (164).

The time-dependent variations in cell viability observed within each titanium grade are also noteworthy. Across all time intervals, the cell viability percentages generally increased, indicating that the cells may adapt and proliferate over time in response to the titanium substrates. This finding suggests that longer exposure to the titanium surfaces promotes cell growth and survival.

The statistical analysis conducted in this study strengthens the validity of the results. The significant p-values obtained for both the comparisons between titanium grades and the comparisons between time intervals indicate that the observed differences in cell viability are unlikely to have occurred by chance. The inclusion of 95% confidence intervals provides additional information about the range of possible differences, enhancing the precision of the estimated effects.

Overall, these results suggest that grade IV titanium has higher cell viability compared to grade V titanium at all time intervals studied. However, it's significant to remember that these results are based on study done in vitro, and further research is needed to validate them in vivo.. One possible explanation for the difference in cell viability between grade IV and grade V titanium alloy could be related to differences in the chemical composition of these materials.

Multiple studies, both in vitro and in vivo, have supported this finding. Cultured fibroblasts and osteoblasts were used in the tests to examine how cells react to different surfaces, as well as human and animal participants.

Chander et al (143) , examined the cytotoxicity and corrosion behavior of grade I pure titanium and grade V titanium alloy on human gingival fibroblasts using the MTT assay in, 0.1 N Sodium Chloride (0.1 N KCL) and artificial saliva with and without Sodium Fluoride (NaF). The disks made of Grade I titanium and Grade V titanium alloy had a width of 8 mm and a thickness of 5 mm. Cell metabolic activity decreased with Ti-6Al-4 V dose after 48 hours. In both samples, TiO₂ synthesis decreased cytotoxicity over time, validating the present results.

In their study, Galeotti et al (146), placed three different types of grade V Mini-screws, specifically Mini screw (Micerium Avegno, Italy), Spider screw (HDC Sarcedo, Italy), and Ortho screw (Novaxa, Cinisello Balsamo, Italy), into a solution of 0.9% (w/v) NaCl with pH levels of 7 and 4. The Mini-screws were submerged for durations of 1, 7, 14, 21, and 28 days. The findings indicated that HGF cell lines exhibited higher viability compared to untreated cells in eluates conditioned at pH 7. Nevertheless, eluates conditioned at pH 4 significantly reduced the viability of HGF.

Malkoc et al (147), used Orthodontic (grade v) Mini Implants (Leone, Florence, Italy), MTN (Istanbul, Turkey), AbsoAnchor (Dentos, Daegu, South Korea), IMTEC Ortho (3M Unitek, IMTEC, Ardmore, Oklahoma), and VectorTAS to study the cytotoxic effects of orthodontic mini-implants on gingival fibroblasts and osteoblasts. According to ISO 10993-5, the specimens grow for 72 hours in Dulbecco's modified Eagle's culture medium. To assess cell survival, a real-time cell analyzer (xCELLigence, Roche Applied Science, Mannheim, Germany; ACEA Biosciences, San Diego, California) was used. Implantation did not cause any damage to gingival fibroblasts.

Velasco-Ortega et al (135), examined the cytotoxic and genotoxicity effects on human and mouse fibroblasts of a commercial titanium (Cp) and Titanium alloy grade V. In accordance with the protocols outlined in ISO 7405:2008, the cytotoxicity of the titanium alloy was investigated using the agar-diffusion method and its variation using a filter-diffusion model. It was confirmed that both titanium and titanium alloy were compatible with living tissues and had no cytotoxic effects on human and mouse fibroblasts. The filter-method resulted in a cell survival of 90% on Ti-6Al-4V discs, which was comparable to the negative control. Nevertheless, the positive control decreased cell viability to 66% of the initial levels.

In an in vitro investigation, villa and colleagues (136), studied the biocompatibility of NIH 3T3 mouse fibroblast cells with titanium and Ti-6Al-4 V alloy. For duplicate cell culture tests, metal samples were cut into 2cm² (200mm) coupons. The samples underwent polishing using cold mount plastic and were then evaluated for cell proliferation using MTT assay. To measure cell proliferation, flow cytometry was also used. Cell viability is marginally higher on titanium samples than Ti-6Al-4V samples, supporting the current findings.

Ti-6Al-4V, Ti-6Al-2Nb-1Ta, CP Ti (not specified), and other titanium alloys were examined for cytocompatibility by Okazaki and collaborators (148). Growth ratios of L929 and MC3T3-E1 cells were evaluated in the research. Compared to CP Ti (0.96) and Ti-6Al-4V (0.97), the growth ratio of cells on Ti-6Al-2Nb-1Ta (about 0.9) was lower, which bolsters the findings of this research.

Kim and colleagues (149), cultivated 3 T3 mouse fibroblast cells directly on mirror polished grade II cpTi and grade V titanium alloy disks with a diameter of 5 mm and a thickness of 3 mm. The biocompatibility test samples were polished and washed ultrasonically in ethanol for 30 min, and incubated at 37 °C. The samples were tested for cell proliferation toxicity and using MTT procedures. After 5 days of incubation, grade II cpTi, Ti-6Al-4V, and polystyrene tissue culture plastic showed no significant differences.

Grade V titanium alloy and four distinct commercially available grades of II titanium (Super-TiX1 800, 51AF, 21SRx, Super-TiX1 800, 51AF, 21SRx) were investigated in 2007. The experiment was conducted in a controlled laboratory setting, utilizing in vitro techniques. The positive controls consisted of Teflon 1 and cp Ti (ASTM grade II). The specimens were meticulously cleaned and disinfected. Balb/c 3T3 fibroblasts were promptly exposed to all sterilized samples for a period of 72 hours. The cytotoxicity of the extract was assessed by quantifying the activity of succinic dehydrogenase (SDH) using the MTT assay. The metal's cytotoxicity was comparable to that of grade V, CpTi, and Teflon (138).

Experimental Titanium-based binary alloys and commercially pure titanium were tested for cytotoxicity in Balb/Cell 3T3 murine fibroblasts. The study included two commercial alloys, Grade V and Ti-6Al-7Nb (T-alloy Tough), and five custom binary alloys, Ti-20 wt% Cr, Ti-10 wt % Mn, Ti-25 wt % Pd, Ti-5 wt % Cu, and Ti-10. There were no significant differences in cell toxicity between commercially available titanium alloys and binary titanium alloys as assessed by succinate dehydrogenase (SDH) (54).

These findings matched earlier research looked at cytocompatibility of numerous titanium alloys (167,168,167)) is being examined. L929 and MC3T3-E1 cells were utilized to examine the relative growth ratio of these cells. While the cells and parameters of their study differed from those of the present study, their results were comparable. Specifically, the growth ratio of these cells for Ti-6Al-2Nb-1Ta was approximately 0.9, which was lower than that for cp (0.96) and Ti-6Al-4V (0.97).

Evans (153), studied the effects of CpTi (grade unknown, ground particle diameter 14 μm , unground particle diameter 49 μm) and Ti-6Al-4 V alloy particles (diameter 8.9 μm) on primary rat skin fibroblasts at a dose of 0.5 mg per 104 cells. After two days of culture, cells that were directly exposed to ground cpTi or Ti-6Al-4 V alloy particles had a much lower cell count than the control group that received no particles. The unground cpTi particles were nontoxic.

Indirect exposure to metal particles, such as using 0.4 μm pore size cell culture inserts, did not alter cell count. While metallic ion release by phagocytosis may result in a drop in cell number. It is currently unclear how direct contact with particles affects cells, however it has been postulated that it may be due to membrane damage (153)

The results of the osseointegration process associated with grade V Mini Transitional Implants (MTIs) placed in the anterior region of the mandible and immediately loaded by over dentures in humans have provided unusual evidence of the successful osseointegration of grade V titanium. There were no notable variations in the toxicity to cells between the commercially available titanium alloys and the binary titanium alloys (154).

In a recent systematic review conducted by Willis et al (155), the cytotoxicity of titanium alloy grade V on gingival fibroblasts was evaluated. The study indicated that the medium may experience the release of ions, specifically vanadium, which can subsequently lead to an increase in cytotoxicity.

Mostardi along with colleagues (141), collected a human synovial fibroblast from four patients who had undergone complete knee arthroplasty. The average donor age was 64, ranging from 48 to 73. The donors were three women and one man. These fibroblasts were grown with cpTi (grade undetermined) or grade V titanium alloy particles. Both cpTi and grade V titanium alloy particles reduced dose-dependent cell count, however this decrease was not statistically significant. No particle size was mentioned, however fibroblasts consumed particles by lysosomal engorgement and activity.

Rae and coworkers (142), conducted tests in which human synovial fibroblasts were subjected to different metal and alloy preparations, including pure titanium and the presence of particulate matter resulting from the degradation of V grade titanium alloy. Under the experimental conditions, fibroblasts that were exposed to pure titanium and titanium alloy did not exhibit any notable elevation in lactate dehydrogenase levels, nor did they undergo any observable morphological alterations.

Rocher et al (156), examined cytotoxicity of nickel titanium, commercially pure titanium and grade titanium alloy using electrochemical assays and cell culture tests involving three cell lines: human embryonic cells, human embryonic palatal mesenchymal cells human embryonic palatal mesenchymal cells and NIH3T3 mouse connective tissue fibroblasts. All experiments showed high biological and electrochemical performance. NIH3T3 cells exhibit superior proliferation on cp-Ti compared to Ti6Al4V and NiTi, with no statistically significant differences seen between the groups, which support the current study's conclusions

Faria along with colleagues (157), examined 12 x 5 mm entirely porous CpTi, Ti-6Al-4V, and Ti-35Nb alloys in vitro and in vivo. To evaluate cell viability, osteoblast cells (derived from rat calvarium) were grown at 2×10^4 /disc/well for 3 and 10 days. Cell survival on all materials grew from days 3-10 ($p < 0.05$) and declined at 14 days, except for Ti-6Al-4V and Ti-35Nb alloys. From 10 to 14 days, nothing changed. ANOVA demonstrated significant alloy type and cell culture duration differences on alkaline phosphatase test, but no factor interaction differences. Across all samples, ALP activity reduced between 3-10 days ($p < 0.05$), with no significant difference between groups.

In 2020, Rosa et al (144), compared commercially pure titanium to titanium grade V in vitro and in vivo. Dental implants (Oxtein Implant, Madrid Spain) are used to make titanium discs. In the in vitro experiment, the titanium discs of grade IV measured 1 cm in width and 0.2 cm in thickness, while the grade V discs measured 1.5 cm in width and 0.2 cm in thickness. Cell cultures were conducted to evaluate the adherence and proliferation of osteoblasts on titanium discs. The experiments used MG-63 human osteoblasts on 48-h osteoblast cell growth cultures, all in vitro groups responded similarly.

Three 5-month-old, 4.0-kg New Zealand rabbits were used for in vivo experiments. Oxtein L6 grade V implants and L35 grade IV implants were implanted in the right and left tibias of rabbits. 3D microcomputed tomography was performed on samples using SkyScan 1172 (Kontich, Belgium) to analyze bone density. The result showed cell culture, adhesion and proliferation were low in the first 6 and 24 hours between groups. After a period of 48 hours, the observed response in terms of cell proliferation was identical. micro-CT and histology study revealed no notable variations across groups (144). The result suggested that both titanium alloys have good results regarding osseointegration.

María Rizo-Gorrita et al (144), examined 18 implants on the tibias of three groups of New Zealand rabbits in 2020. The implants were Zimmer grade V, Biohorizons grade V, and Ziacom t grade IV titanium implants. The implants were placed 8 mm apart and 1.5 mm supracrestally. Cell Viability assessed using MG-63 osteoblastic cells. Titanium grades IV and V showed no statistically significant changes in cell viability

Ribeiro da Silva et al (158), tested the in vivo reactions of dog bone cells to grade IV and V titanium. In order to compare bone area fraction occupancy and bone implant contact, 36 implants were implanted inside the radius of 18 dogs. They used various materials and procedures to analyze groups, but they found no statistically significant difference.

In their *in vivo* experiments, Piozzi et al (159), found that titanium had neither cytotoxic nor genotoxic effects. The rats' tibias were surgically implanted with internal fixture of little titanium alloy miniplates. The lung, liver, and kidney were excised 30 days, 90 days, and 180 days after implantation for histological and genotoxic testing. The samples were stained with hematoxylin-eosin and tested for genotoxicity using single-cell gel electrophoresis. No statistically significant differences in DNA damage were seen in any of the experimental groups when compared to the negative control (animals that not received any implant) across all tested tissues. Furthermore, histological examination revealed no major morphological alterations.

Alessandra Cremasco and co-authors (160), studied the cytotoxicity Ti-35Nb, Ti-35Nb-7.5Ta, Ti-35Nb-4Sn, Ti-25Nb-15Zr, Ti-25Nb-8Sn, Ti-6Mo, Ti-7.1Cu, commercially pure titanium, and Ti-6Al-4V alloys on Vero cells, (a kind of African green monkey kidney fibroblast cells) from the Adolfo Lutz Institute in São Paulo, Brazil. All MTT-based investigations were statistically analyzed using ANOVA and Tukey's test. The rates at which cells grow on nickel titanium alloy and Ti-6Al-4V alloys are slightly higher than on commercially pure titanium.

In an *in vitro* biocompatibility testing of three newly developed titanium alloys for oral implantology: Ti-31.7Nb-6.21Zr-1.4Fe-0.16O, Ti-36.5Nb-4.5Zr3Ta-0.16O and Ti-20Nb-5Ta.comparison. whether the use of these alloys can have any toxic or injurious effects on biological systems and to evaluate mouse pre-osteoblast MC3T3-E1 cell line, subclone 4 (CRL-2593™, American type Culture Collection) response. In terms of comparison, a commercial Ti6Al-4V alloy was used as a reference material. The study found that, Cell survival, adhesion, morphology, and proliferative potential were good in the newly developed alloys, and cell differentiation tests showed that they support MC3T3-E1 cell osteoblastic differentiation to a similar level as the reference material. Therefore, these alloys may be ideal dental implant candidates (161).

Milan Stoilov and his colleagues (162), conducted an in vitro study to examine the effects of sandblasting procedures (SA in acid etching) of cpTi and Ti6Al4V on Immortalized human fetal osteoblasts (hFOB 1.19 cells; obtained from ATCC: LGC Standards GmbH, Wesel, Germany). Proliferation and differentiation of osteoblasts were assessed over a period of 12 days using crystal violet staining, MTT assay, ALP activity assay, and Alizarin Red staining assay. No noticeable cytotoxicity or differences were seen between the cpTi and Ti6Al4V SA surfaces.

In contrast Citeau et al (163), found that osteoblast viability was decreased in contact with titanium-alloy (Ti-6Al-4V) discs with different surfaces on MC3T3-E1 cells after 4, 8, and 15 days in culture. In culture, this reduction was almost completely restored after 8 days and eliminated after 15 days. Scientists say the chemical constitution causes this brief cell viability change..

Faria et al (164), studied the cytotoxic effects of discs obtained from wax patterns (13-mm diameter and 4-mm thickness) that were cast from different alloys and commercially pure (cp) Ti on human oral squamous carcinoma SCC 9 cells and found that cell viability was not affected by CpTi and Ti-6Al-4V. The study determined that cp Ti and Ti-6Al-4V alloys were found to be non-cytotoxic, followed by Ni-Cr alloy, however other alloys were shown to be cytotoxic, particularly Ni-Cr-Be alloy.

In 2021, Teodora et al (165), conducted an in vitro study to examine grade IV titanium and Ti-4Al-6V in powder form. The analysis examined mammalian model (lymphocyte cell cultures) proliferation by micronucleus test. Each type of sample was examined using two concentrations: 1.5 mg/ml and 3 mg/ml. Micronuclei were identified in both materials evaluated at the two concentrations; however, the levels were within the normal range. While the variations are within acceptable ranges, it is important to acknowledge that the occurrence of the mentioned phenomenon was more frequent in Ti-6Al-4V at both concentrations compared to grade IV titanium. This result is consistent with our own studies.

A comparative study examined the short-term inflammatory potential of Ti-6Al-4V, Ti-6Al-7Nb, and commercially pure titanium (cp-Ti) on striated muscle nutritive perfusion and leukocytic response. They measured primary and secondary leukocyte-endothelial cell interaction, extravasation, microvascular diameter change, and capillary perfusion in skeletal muscle collecting and postcapillary venules in 28 hamsters using dorsal skinfold chamber preparation and intravital microscopy. Compared to the other metals, animals treated with Ti-6Al-4V showed a modest increase in leukocyte recruitment and slower recovery of inflammatory parameters, suggesting a little biocompatibility difference, which is compatible with our study (139).

However, direct, and indirect induction of apoptosis in human mesenchymal stem cells (hMSCs) in response to titanium particles has been reported by Wang et al (166). Direct contact with CpTi particles reduced cell viability by causing apoptosis, which elevated levels of the tumor suppressor proteins p53 and p73 in a way that depended on the kind of material, the dose of the particles, and the duration of the exposure. Furthermore, conditioned media obtained from hMSCs exposed to CpTi particles was hazardous to hMSCs and caused apoptosis in the absence of particles.

The cytotoxicity of titanium alloys comprising aluminum and vanadium on fibroblast cells was shown to be greater than that of commercially pure titanium, according to different research by Poma et al (167). Aluminum containing titanium alloys such as Ti-6Al-4V, exhibit an oxidized layer on their surfaces containing aluminium oxides that may exert some cytotoxic effects.

The deposition of the bone matrix around the implant determines the success of the matrix, which depends directly on the process that induces the osteoblast proliferation, differentiation, secretion of extra cellular matrix proteins, and tissue mineralization (168). Along with the above, osseointegration is an adynamic process which alternates between bone formation and bone resorption. Consequently, the magnitude, direction, and period of any forces applied over the implant bone zone will determine whether maintenance or failure of osseointegration equilibrium results (169).

In comparison to bone implants for orthopedics, oral implants not only need to integrate into the surrounding bone but also into the adjacent soft tissues to achieve a long-term clinical implant survival. This is due to the fact that two components are necessary for an implant-supported replacement of teeth: (1) an endosseous implant part placed into the jawbone and (2) a transgingival part piercing the oral mucosa and allowing for the anchorage of the prosthetic restoration, such as a crown (164, 165)

Dental implants typically consist of an abutment inserted into the implant body and an implant body fixed in the bone. The implant body is often composed of CP titanium grade IV, while the abutment is typically made of titanium grade V alloy or zirconia. Due to the recognized micromovement of this screwed joint, wear and fretting develop. It has been shown that metal wear particles are formed at the implant–abutment interface (172).

Titanium oxide (TiO₂) coating forms on titanium implants exposed to air. This 1.5–10 nm layer forms because Ti has a high oxygen affinity(173). This may make titanium implants corrosion resistant.

Successful incorporation of dental implant strongly depends on affirming long standing adhesion of the tissues surrounding the implant. It's important to guard against bacterial invasion and subsequent infection in deeper periodontal tissues. The chemical composition and topography of the surface, as well as the bulk material's qualities, have an impact on the cellular response(174).

Peri-implantitis was analyzed for the incidence of different elements. In one pilot study (175), biopsies of patients with peri-implantitis were analyzed for the incidence of different elements by means of Synchrotron radiation X-ray fluorescence spectroscopy (SXRF). In 75% of the samples, a higher amount of titanium was detected. In some biopsies, this was accompanied with an increased iron concentration (up to $2.4 \cdot 10^5$ counts); this might possibly be due to the composition of the (unalloyed) commercially pure titanium (Grade IV) based on the American Society for Testing and Materials (ASTM)International Standard F67, respectively, DIN EN ISO 5832-2 with the following elements: O₂, N, H, C, and Fe (175).

Surface characteristics have an impact on cellular responses. The chemistry, topography, and roughness of titanium surfaces affect cell adhesion, multiplication, and differentiation. Titanium implant surface roughness affects gingival fibroblast attachment and proliferation (174). After a decade of study, the effects of implant roughness and other factors on biological response are still controversial (163).

A good wound repair, with the formation of adequate gingival seals around titanium implants, is mandatory for the success of this kind of surgery (150,151). This raises the question of whether titanium surface treatments may interfere with gingival tissue repair after implant surgery.

It is crucial to remember that the outcomes of in vitro research could not accurately reflect how these materials would behave biologically in real life. The biocompatibility of dental implants can also be impacted by elements including the host immunological response, implant design, and surgical technique. To corroborate the results of this study and assess the long-term biocompatibility of grade IV and grade V titanium in vivo, more research is thus required.

There isn't yet a consensus on the danger of particles released from Ti, but doctors would be wise to carefully assess the materials utilized and to take into account any possible concerns associated with the individual components of any alloy, as this study suggests (152).

Grade V has a high Young's modulus (115GPa) yet does not significantly differ from that of CP grades. This shouldn't raise concerns about this alloy. Ti-6Al-4V alloys use different alloying elements to replace Al and V. The alloy Ti-13Nb-13Zr uses niobium (Nb) and Zr. Ti-13Nb-13Zr is ideal for orthopedic implants due to its excellent strength-to-weight ratio and low Young's modulus (77 GPa). Dental use of 8 Ti-13Nb-13Zr is being investigated (51).

As an in vitro study, the present study cannot simulate the complex oral microenvironment and validate our conclusions in vivo. Conducting in vivo or ex vivo studies would provide a more realistic assessment of the biocompatibility of different titanium grades.

The present study primarily concentrated on evaluating cell viability inside a controlled laboratory environment, with less consideration for its clinical applicability. Although these discoveries enhance our comprehension of cellular function, their immediate applicability to therapeutic outcomes may be limited.

The investigation only concentrated on cell viability as the primary assessment criteria. Although cell viability is a crucial aspect, it may not provide a comprehensive understanding of the cellular response to titanium. The incorporation of further characteristics, such as markers of inflammation, cell adhesion, or gene expression studies, would contribute to a more comprehensive comprehension of the biocompatibility of various grades of titanium.

Despite the obvious strength advantages of grade V, no material is perfect, or else the development and testing of new combinations would have stopped long ago. At the very least, superhard alloys are more difficult to machine. Grade IV is quite good in basic terms. The only exceptions are screws and other thin parts operating under heavy loads.

Grade V titanium alloy is often used in the manufacturing of medical and dental implants because it has better physical, mechanical, and biological qualities compared to other commonly used biomedical metals and alloys. This material demonstrates exceptional features in comparison to other metals and alloys, particularly in terms of its corrosion resistance, superb biocompatibility, lightweight structure, and remarkable tribological characteristics. These attributes render it highly advantageous for various dental applications.

The only drawback of the present study is lack of comparative examination with other materials. Research focused on comparing grade IV and grade V titanium. There are other titanium grades and biomedical materials. Examining the cellular response to different grades of titanium compared to other materials would help explain titanium's biocompatibility and appropriateness for many uses.

In conclusion, titanium grades in vivo biocompatibility and comparison to other biomedical materials need more study. Grade V titanium alloy is recommended for dental implants due to its outstanding qualities, but machining and application matter. Despite this, more comparative studies are needed.

6. CONCLUSION

Based on the constraints of this in vitro investigation, it is possible to draw the following conclusions:

1. Ti dental implants are the gold standard in implant dentistry due to their high biocompatibility and physicochemical characteristics. Grade IV titanium delivers superior physical qualities as well as remarkable biocompatibility and survival.
2. There are significant differences in cell viability between Grade IV and Grade V titanium at 24, 48, and 72 hours ($p < 0.001$). Grade IV consistently exhibited higher mean cell viability compared to Grade V, indicating that Grade IV titanium may be more biocompatible and cell-viable than Grade V titanium.
3. Grade IV titanium exhibits greater cell survival than grade V titanium alloy at all time intervals tested, even after considering the limitations of the current investigation. Aluminum and vanadium are included in grade V titanium alloy, which reduces cell viability. For dental and medical uses, grade IV titanium and grade V titanium alloy are equally appropriate.
4. CP-Ti vs Ti-6Al-4V for dental implants has been debated in the literature. CP-Ti is marginally preferred, however in vitro experiments have shown that Ti-6Al-4V is better for these purposes. Many dental metallurgy researchers propose using titanium grade V alloy (Ti6Al4V) instead of pure titanium, which is commonly used in screw-type dental implants. Its higher tensile strength and superior oral behavior make Ti-6Al-4V the favored alloy. However, studies show aluminum and vanadium ions in peri-implant tissue following dental implant osseointegration.
5. Further research is required to thoroughly assess the biological reaction of these materials in vivo since these findings may have consequences for their biocompatibility in dental implant applications

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8. BIOGRAPHY

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EDUCATIONAL STATUS

Degree	Field	Name of institution of graduation	Year of graduation
PHD	Oral and Maxillofacial Surgery	Yeditepe University	2024
Master's degree	Oral Surgery	University of Malaya / Malaysia	2007
BDS	Bachelor of Dentistry	University of Tripoli / Libya	1989-1995
High school	Science	Qasr Bin Ghasheer High School /Tripoli/ Libya	1985-1988