T.C.
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CYTOTOXICITY OF TWO DIFFERENT PHYSICAL FORMS OF TITANIUM DIBORIDE IN AN IN-VITRO STUDY

DOCTOR OF PHILOSOPHY THESIS

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 28.01.2021 and numbered 2021/01-63.

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text

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LIST OF SYMBOLS and ABBREVIATIONS

Ar-TiB_2	Titanium diboride sintered under 70 mPa pressure in Argon atmosphere at 1800°C
B	Boron
B_2O_3	Boride trioxide
cpTi	Commercially pure Titanium
FBS	Fetal Bovine Serum
h	Hour
ISO	International Organization for Standardization
L929	Mouse fibroblast
SPS	Spark plasma sintering
Ti	Titanium
TiB_2	Titanium diboride
TiB	Titanium boride
Ti6Al4V	Grade 5 titanium
V70-TiB_2	Titanium diboride sintered under 70 mPa pressure in vacuum atmosphere 1750°C
V50-TiB_2	Titanium diboride sintered under 50 mPa pressure in vacuum atmosphere at 1600°C

ABSTRACT

Tekin H. (2021). Cytotoxicity of two different physical forms of titanium diboride in a in-vitro study. Yeditepe University, Institute of Health Science, Department of Oral and Maxillofacial Surgery, PhD Thesis, Istanbul.

The aim of this study is to investigate the cytotoxicity of titanium diboride (TiB_2) ceramic in two physical forms. The cytotoxicity of the chitosan scaffolds, containing TiB_2 powder was determined by direct contact test method on periodontal ligament stem cells. Alkaline phosphatase (ALP) enzyme levels in medium were examined on 7th day. TiB_2 bulk ceramics produced with spark plasma sintering method (SPS) by using Argon atmosphere under 50mPa pressure at 1800°C (Ar-TiB_2), vacuum atmosphere under 70mPa 1780°C temperature (V70-TiB_2) and under 50mPa at 1600°C (V50-TiB_2) were used. Osteosarcoma cell line (SaOs-2) with normal cell medium was used as control, and cell mediums with extracts were used for cytotoxicity of TiB_2 plates. After being kept in cell media for 24 hours, four different dilations of 100%, 50%, 25% and 12,5% extracts were taken from each plates. The cytotoxicity in both forms of TiB_2 on 24 h and 48 h were evaluated according to ISO guideline. The scaffolds containing TiB_2 powder and chitosan showed no cell viability on measuring by ALP levels. The extracts taken at dilations of 50%, 25% and 12,5% in all plates did not have cytotoxic effect on SaOs-2 in 24 h. V70-TiB_2 plate was found to be more cytotoxic on 24 h ($p<0.001$). Statistical significant differences were found between 12,5% extracts of Ar-TiB_2 and V50-TiB_2 ($p=0.025$), and between V70-TiB_2 and Ar-TiB_2 ($p=0.034$). After 48 h cell viability was observed to decrease below 70% for all the plates and extracts. Statistical significant differences were found between %12,5 extracts of V70-TiB_2 and V50-TiB_2 ($p=0.015$). Negative correlation between the cell viability and material content were found on 24 and 48 h ($p<0.001$). The cell viability of 100% extracts of all plates on 24 h and 48 h was below 70% and statistically significant difference were found between all plates and their extracts rates ($p<0.05$). Both powder and bulk plate forms of TiB_2 showed cytotoxic effect on cell viability according to ISO guideline. Further studies are needed exposed to temperatures after produced bulk material for evaluating the cytotoxicity.

Key Words: titanium diborde, TiB_2, titanium, cytotoxicity, ALP.

ÖZET

Tekin H. (2021). Titanyum diborür'ün iki farklı fiziksel formunun sitotoksik etkisinin in-vitro incelenmesi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü Ağız Diş Çene Cerrahisi Anabilim Dalı Doktora Tezi. İstanbul.

Bu çalışmanın amacı, titanyum diborür (TiB_2) seramiğinin sitotoksisitesini iki farklı fiziksel formda araştırmaktır. TiB_2 tozu içeren kitosan iskelelerinin sitotoksik etkisi periodontal ligament kök hücreler üzerinde direkt temas yöntemi ile belirlendi. 7. Günde besiyerindeki alkalin fosfataz (ALP) enzim düzeyleri incelendi. 1800°C sıcaklıkta 50mPa basınç ve argon atmosferi altında (Ar-TiB_2), 1780°C sıcaklıkta 70mPa basınç ve vakum atmosferi altında (V70-TiB_2), 1600°C sıcaklıkta 50mPa basınç ve vakum atmosferi altında (V50-TiB_2) kıvılcım plazma sinterleme yöntemi (SPS) ile üretilen TiB_2 plakalar kullanıldı. Normal hücre ortamına sahip osteosarkom hücreleri (SaOs-2) kontrol olarak kullanıldı ve TiB_2 plakalarının sitotoksisitelerini incelemek için ekstraklı hücre ortamları kullanıldı. Hücre ortamında 24 saat bekletildikten sonra her plakadan %100, %50, %25 ve %12,5 oranında dört farklı dilatasyon alındı. 24 ve 48 saatteki her iki TiB_2 formun sitotoksisitesi, hücre canlılığının %70'in üzerinde gerektiği ISO klavuzuna gore değerlendirilmiştir. Tüm plakalarda %50, %25 ve %12,5 dilatasyonlarda alınan ekstrakların 24 saatte SaOs-2 üzerinde sitotoksik olmadığı görüldü. V70-TiB_2 plağı 24 saatte daha sitotoksik bulundu ($p<0.001$). %12,5 Ar-TiB_2 ile V50-TiB_2 ekstraktları arasında ($p=0.025$), V70-TiB_2 ile Ar-TiB_2 ($p=0.034$) arasında istatistiksel olarak anlamlı farklılık görüldü. %12,5 V70-TiB_2 ve V50-TiB_2 ekstrakları arasında 48 saatte istatistiksel fark gözlendi 48 saat sonra hücre canlılığının tüm plakalar ve ekstreler için %70'in altında düştüğü belirlendi. Hücre canlılığı ile material içeriği arasında negative korelasyon bulundu ($p<0.001$). Tüm plakaların %100 ekstraktlarının 24 saat ve 48 saatteki hücre canlılığı %70'in altında olduğu görüldü. Tüm plakalar ile ekstrakt oranlarının 24 ve 48 saatlik karşılaştırmalarında istatistiksel olarak anlamlı farklılık bulundu ($p<0.05$). TiB_2'nin hem toz hem de plaka formlarının, ISO klavuzuna gore hücre canlılığı üzerinde sitotoksik bir etkiye sahip olduğunu gösterdi. TiB_2'nin üzerinde oluşan (B_2O_3) tabakasının yüksek sıcaklık ile uzaklaştırılarak, materyalin sitotoksisitesi üzerinde daha fazla çalışma yapılması gerekmektedir.

Anahtar Kelime: titanyum diborür, TiB_2, titanyum, sitotoksisite, ALP

1. INTRODUCTION and PURPOSE

Biomaterials are obtained by using natural materials or by using various physical and chemical reactions, producing metals and metallic alloys, composites and ceramics. It can be used for diagnostic or treatment purpose, in order to increase the lost function of organs or tissues and cosmetic appearance or to assist in healing process (1).

One of the most common uses of biomaterials in current medical field is as medical implants, which can restore, support or enhance lost tissue function. Implants can remain in the body for a certain period of time, such as fixation screws and plates used to fix broken bones, as well as long-term retention may be required in the body such as hip or hearth prostheses and dental implants (2).

Titanium (Ti) and Ti alloys, which are frequently used in orthopedics and dentistry as implants, are durable, biocompatible and it possess high corrosion resistance. The elasticity of Ti is close to elasticity of trabecular bone, which makes Ti to be an efficient biomaterial (3). Ti alloys such as Ti-6Al-4V and commercially pure titanium (cpTi) are being used as medical implants for replacing lost bone tissue and for replacing lost dentition as dental implants (4). It has been stated that, Ti implants can wear over time, causing inflammatory response in surrounding tissue (5). In addition cpTi, such as plates has been reported the risk of fracture between 2.9-10.7% in dental field (6). To eliminate such risks, it is attempted to introduce Ti alloys that are more stable and withstand more strength.

Studies on surface modification are considered an alternative production method for Ti implants. Zirconium, niobium and B have been used for improving the surface strength of Ti implants, and producing Ti alloys that can withstand higher external forces as well (7,8)

Boron (B) has been the subject of many studies, considering its antibacterial, antifungal and antiseptic properties (9,10). It is found in nature as B minerals, having effect on different metabolic activities such as bone formation (11) and wound healing (12). It can form compounds with Ti, resulting in titanium boride (TiB) and titanium diboride (TiB_2). Studies on surface coating of Ti with TiB, reported improvement of hardness and wear resistance of produced implants (13). TiB does not have negative effects on cell development when used as Ti-TiB composite (5).

TiB_2 is the most stable of TiB compounds. TiB_2 is a hard material, able to withstand high forces and has high wear resistance (14). However TiB_2 is currently used in applications such as impact resistant armor, cutting tools, crucibles and wear resistant coatings.

ISO 10993 guideline is a standardization method aimed at maximizing the safety of the material as a result of the study in biomaterial research. This guideline includes test samples selection methods, suitable extraction methods, and illustrations of control groups. ISO 10993 standard is a guideline created to investigate the biocompatibility of the studied material on the relevant cells in a controllable in vitro environment before proceeding to the in vivo research phase in biocompatibility studies. The main purpose of this guideline is to ensure the safety of studied material at the highest point. The study conducted within these standards ensures the safety of the researched material by ensuring similar results in repeated studies (15). It recommends conducting an in vitro study of previously untested materials. The overall purpose of in-vitro cytotoxicity tests is to examine the effect on the cells exposed the extract of the studied material to cell viability and proliferation of the studied material, by comparing the results with the control ISO 10993 standards is a guideline created for biological evaluation of a material intended for use on living creatures. Its first goal is the protection of humans and living creatures. These standards, which are constantly updated, are a guide for the evaluation of biological response by performing various tests before using the new materials intended for use on human beings (16). It is mandatory to describe cell viability in medium above 70%.

Direct, indirect contact and extraction test methods can be used for evaluating the biocompatibility of a material in vitro. Method selected depends on physical and chemical properties of the material (17).

Best to our knowledge, there is no study on TiB_2 material regarding its cytotoxicity on cells. The aim of this study is to investigate the cytotoxicity of titanium diboride (TiB_2) ceramic in two physical forms on cell lines.

2. LITERATURE REVIEW

2.1 Bone Tissue Engineering

Bone tissue engineering has been a topic that researchers have been exploring since 1980. Bone tissue engineering aims to prevent problems such as creating a second surgical field, limited accessibility, rejection of the immune system or pathogen transfer (18).

In studies on bone tissue engineering, bone tissue scaffolds must have features such as biocompatibility, positive effects on bone tissue healing, such as increasing osteogenic growth factors, or not to have a negative effect while new bone is formed, while showing similar physical properties of bone tissue.

Bone tissue engineering requires a multidisciplinary approach. In order to determine the biocompatibility of tissue scaffolds to be produced for bone tissue engineering, scientists with the knowledge of biomaterials; for imitating the physiological and histological properties of bone tissue, scientists with the knowledge of bone biology and surgeons who can perform the discovered bone scaffold on patients are required to work in collaboration (19).

2.1.1 Bone Biology

Bone is a hard, impact resistant, mineralized connective tissue. Bone has many functional and protective purposes including; locomotion, support, protection of soft tissue and internal organs as well as storage of calcium and phosphate, harboring of bone marrow (20).

Bone is composed of organic and inorganic materials. Water, calcium phosphate, collagen and fibrotic proteins are the essential materials composing bone. Magnesium, fluoride and sodium are some of the inorganic materials seen in bone.

Bone structure can be classified into two general groups, one being compact (cortical) and the other being trabecular (cancellous) bone. Although cortical bone and trabecular bone layers show different properties, they consist of the same elements histologically. Cortical bone layer is more calcified than trabecular bone. Cortical bone

layer provides support and protection to the body; while metabolic functions are intense in the trabecular bone layer.

There are four different types of cells in bone tissue; osteoblasts, osteoclasts, osteocytes and bone lining cells. Osteoblasts and osteocytes are composed of osteoprogenitor cells that differentiate from mesenchymal stem cells; osteoclasts are of hematopoietic stem cell origin (21).

Although the bone structure seems simple, it is in a continuous cycle of resorption and formation. Bone is constantly resorbed by osteoclasts and formed by osteoblasts.

2.1.2 Bone Tissue Cells

2.1.2.1 Osteoprogenitor cells

Osteoprogenitor cells are found in the lower layers of periosteum and endosteum where there is no bone resorption or bone synthesis. Osteoprogenitor cells are derived from mesenchymal stem cells. These cells can multiply and differentiate into other cells. Osteoprogenitor cells can migrate and differentiate into osteoblasts on sites that are undergoing bone synthesis and can differentiate into osteoclasts on sites that are undergoing bone resorption (22).

2.1.2.2 Osteoblasts

Osteoblasts are cuboidal cells that are known for their bone forming function. Osteoblasts are located alongside the bone surface (23). These cells have abundant rough endoplasmic reticulum, prominent Golgi apparatus and secretory vesicles like protein synthesizing cells. Osteoblasts secrete osteoid on bone forming sites (24).

Osteoblasts are derived from mesenchymal stem cells by activation of different genes. Mesenchymal stem cells differentiate into osteoprogenitor cells with the activation of bone morphologic protein (BMP) and Wingless (Wnt) pathways (25). Runt-related transcription factor-2 (Runx2), osterix (Osx) and distal-less homebox 5 (Dlx5) are crucial for osteoblastic differentiation. Runx2 upregulates osteoblast-related genes, collagen type 1 alpha 1 (Col1A1), alkaline phosphatase (ALP) and osteocalcin (OCN) genes (26).

2.1.2.3 Bone lining cells

Bone lining cells are flat-shaped osteoblasts, covering the bone surface where there is no bone resorption or formation (27). Bone lining cells have cytoplasm that extends along the bone surface. These extended cytoplasm, displays cytoplasmic organelles such as profiles of rough endoplasmic reticulum and Golgi apparatus. Bone lining cells also have processes that extends towards canaliculi, adjacent bone lining cells and osteocytes (28).

Although the functions of bone lining cells are not fully known, they are thought to prevent direct contact of the osteoclasts and bone matrix, as they cover the bone surface, and are involved in osteoclast differentiation (29). It has also been proven that bone lining cells are involved in the synthesis of osteoprotegerin and the RANKL pathway (30).

2.1.2.4 Osteocytes

Osteocytes are derived from mesenchymal stem cell lineage through osteoblast differentiation. At the end of bone formation cycle, osteoblasts that get trapped in the non-mineralized bone matrix (osteoid) are called osteocytes. These cells go through some morphological changes and size of their organelles decreases, whereas their cytoplasm ratio increases, resulting in decreased protein synthesis and decreased secretion.

Osteocytes are the most abundant cell type that can be seen in the bone (31). Osteocytes form a network of canaliculi amongst themselves, permeating the entire bone matrix. This network is created within the lacunae surrounded by mineralized bone matrix with osteocytes showing dendritic morphology (30,31).

2.1.2.5 Osteoclasts

Osteoclasts are cells that can differentiate into macrophages and monocytes, derived from hematopoietic stem cells of the mononuclear lineage (34). Hematopoietic stem cell lineage differentiates into osteoclasts under the influence of macrophage colony-stimulating factor and RANK ligand (35).

Osteoclasts have developed lysosomes. Acid phosphatase, cathepsin K, collagenase and proteinase are actively synthesized by the osteoclasts. In bone resorption regulated by a number of factors, such as tumor necrotizing factor-6 (TSG-6) and calcitonin, osteoclasts take part in local destruction of bone matrix and mineral parts of the bone. Following bone matrix resorption, calcium rises and parafollicular cells of thyroid gland secrete calcitonin to inhibit the function of osteoclasts (36).

2.1.3 Bone Tissue Healing

Bone remodeling is a complex process of old bone being replaced by new bone that can be described in four different stages (37). (Figure 1)

Activation phase: First phase of bone remodeling is activation phase. This phase starts when the physical integrity or homeostasis between bone resorption and formation is disturbed. Mechanical stimulation, effects of estrogen or parathyroid hormone can also start bone remodeling. In this phase, the number of osteoclasts increases in the region that will undergo bone remodeling.

Resorption phase: Multinuclear osteoclastic cells start resorption in remodeling region. Osteoclastic function is done in two steps; first being acidification of bone matrix in order to dissolve inorganic components of bone, and second step being release of lysosomal enzymes, such as cathepins K, MMP9, dissolving the organic components of bone (38). After 4 to 12 days of multinuclear osteoclastic function, mononuclear osteoclasts appear in the region. Mononuclear osteoclasts have less resorption power then multinuclear osteoclasts and they are considered to smoothen the rough bone and prepare the site for osteoblasts.

Reversal phase: Within 7-10 days after mononuclear osteoclastic cells cease their activation, cement (rich in proteoglycans, glycoproteins and acid phosphatase but has very little collagen) can be found in the region. In this phase, osteoblasts are signaled

Formation phase: Formation phase starts after reversal phase with osteoblasts migrating to the site where resorption occurred and synthesizes extracellular matrix (osteoid). These osteoid have collagen like structure. Osteoblasts have vesicles that are rich with alkaline phosphatase and mineralization starts from these areas and continues towards osteoid thus completing bone remodeling.

Resting phase: After formation phase, in the region of bone remodeling, homeostasis is restored and cells on the region continues their basal metabolic activity. Osteoblasts on the region undergoes apoptosis or get trapped in osteoid and becomes osteocytes.

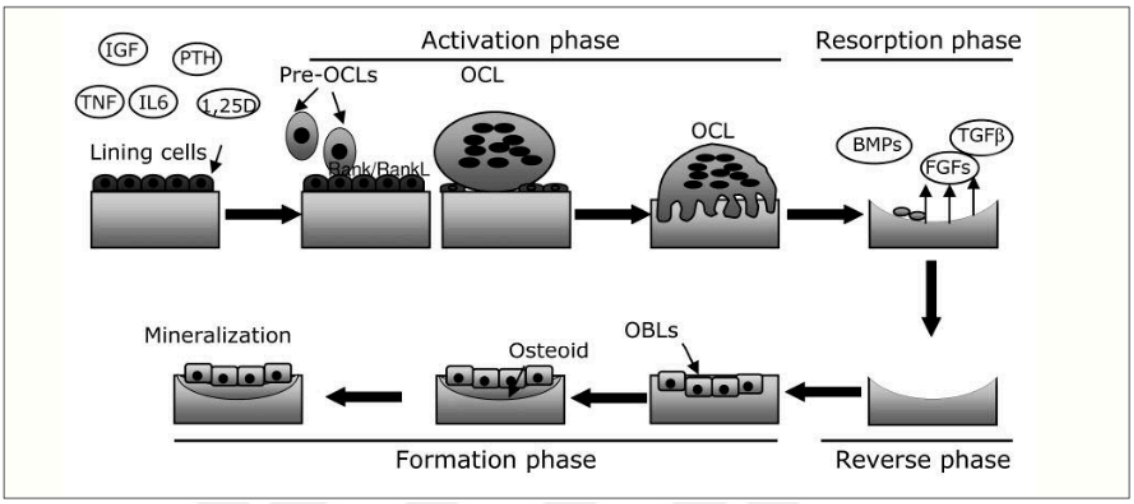

Figure 2.1 Schematic representation of the bone remodeling process

2.1.4 Bone Formation Markers

Bone formation markers are products of osteoblastic activity during bone remodeling. Different bone markers are considered reflecting different osteoblast activity and bone remodeling phase. Bone formation markers are measured in plasma and serum (39).

2.1.4.1 Osteonectin

Osteonectin, also called SPARC, is a type of protein that can bind to calcium and hydroxyapatite region of the bone (40). Osteonectin can be classified as an early bone formation marker as osteonectin expression was found to be high in the first phases of bone remodeling, and decrease overtime as osteoblasts undergo differentiation and convert to osteoid (41). Earlier it was thought to be expressed only in mineralized hard tissues; however, studies have shown that it is expressed in mineralized and non-mineralized tissues (42). It has been shown in studies that osteonectin plays a critical role

in bone remodeling, and it is involved in the formation of bone matrix, its mineralization, osteoblast and osteoclast differentiation (43). There are studies on non-mineralized tissues that osteonectin deficiency causes skin, heart, liver and kidney diseases (44).

2.1.4.2 Osteocalcin

Osteocalcin is a protein that is secreted only by osteoblasts. Although osteocalcin was initially thought to be involved in extracellular matrix mineralization, however studies have shown that it does not have a major effect on extracellular matrix mineralization in osteocalcin deficiency (43,44). Osteocalcin is thought to have a physiological role in the regulation of endocrine. In the development of the brain, it has been shown in studies that it has a role in insulin secretion (47).

Osteocalcin is a late marker of osteoblastic activity, but considered as unstable molecule as it has a short half-life (39).

2.1.4.3 Alkaline phosphatase

Alkaline phosphatase is an membrane-bound glycoprotein that catalyzes the hydrolysis of phosphate monoesters at neutral pH values (48). Alkaline phosphatase can be isolated many human tissues. Liver, intestines, kidney, placenta, bone tissue, tooth enamel are some examples.

Human alkaline phosphatase can be classified into four subgroup isoenzymes; placental alkaline phosphatase, intestinal alkaline phosphatase, tissue-nonspecific alkaline phosphatase and germ cell alkaline phosphatase (48).

More than 80 years ago, high level of alkaline phosphatase expression in bone was found, and in 1924, first hypothesis was put forward that alkaline phosphatase played an important role in bone formation (49). Since then, the role of alkaline phosphatase has been researched in hard tissue formation. More than 280 publications were published in the last 2 years (50). Most of these publications used alkaline phosphatase as a biomarker for hard tissue formation. Collective hypothesis of these publications, alkaline phosphatase became the marker of choice assessing the development of early mineralized hard tissue cells.

2.2 Implants

Various studies in the medical field lead to developments that increase the 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 in an imperative to fully restore the functions of organs and limbs that lose their function as a result of various pathological and traumatic effects (51). The biomaterials that are used for restoring the functions of the damaged body parts are called implants (2).

Various types of implants can be used for restoring the mechanical and physical functionality of lost tissue due to pathological and traumatic effects. Hip joint implants for replacing lost bone tissue, pacemakers for hearth arrythmia, dental implants for lost teeth tissue, can be used in order to maintain good life quality (52). These implants may need to stay in the body for a defined time period or for the remainder of life span. These implants need to fulfill their function and not cause any harmful effects on the human body (52). In this regard, a definition called biocompatibility have an important role identifying which material is biocompatible and which material is harmful to the human body (53). There is a good level of understanding of biocompatibility definition, mostly revolving around the concept of materials placed inside the body that doesn't cause irritation or don't have toxic effects on tissue or organs (52).

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 (2). 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 (2). These long term negative effects caused researchers to investigate nickel-free stainless steel implants or replacing nickel content with other elements that would not have toxic effect (54).

There are many researches on implant materials, that can remain in the region where they are applied without long-term toxic effects. To meet this need, implants fabricated with Ti and Ti alloys are used for total hip replacement, knee and dental surgeries (55). Ti implants have excellent mechanical properties with high resistance to corrosion and high biocompatibility (56).

2.3 Scaffold Fabrication Methods

The preparation techniques of tissue scaffolds constitute the mechanical and chemical properties of the scaffolds (57). Therefore, scaffolds are produced, using different scaffold fabrication techniques according to the tissue used. These fabrication techniques include; solvent casting, membrane lamination, peptide self-assembly, phase separation, 3-D micro plotting, micro patterning, freeze-drying.

Different tissue scaffold fabrication techniques have their own advantages and disadvantages. Fiber bonding technique is an easy technique for scaffold production but it lacks control over porosity control; on the other hand in solvent casting method, the porosity of the scaffold can be controlled as requested but the scaffold created lacks the required mechanical strength for load-bearing tissues. Micro patterning technique can only be used for 2-D scaffold production whereas macro plotting creates 3-D scaffolds but lack the structural integrity (58).

In this study, freeze-drying method and crosslinking are used to fabricate a 3-D chitosan/Ti and chitosan/TiB_2 composite structure, therefore freeze-drying method and crosslinking techniques will be discussed in the following sections.

2.3.1 Freeze Drying

Freeze-drying is a process used to form solid, porous scaffolds from liquid-containing mixtures. Freeze drying process is made by following two main phases. The first step is to freeze the liquid-containing mixture at low temperature. The temperature selected determines the freezing rate and the size of the ice crystal that will form inside the sample (59). Since the degree of freezing temperature determines the freezing rate, it also determines the size of the ice crystals within the scaffold. With this knowledge, it can be argued that, the size, frequency and distribution of porosity within the scaffold can be controlled (60). If the liquid content is frozen to a very low degree, the ice crystals formed in it take up large spaces and after sublimation reaction, the sample will have bigger porous structure. If the liquid content is frozen to a higher degree, the ice crystals will form slower and smaller, causing the scaffold to have a smaller pore structure after lyophilization reaction.

The second step of freeze-drying method is lyophilization. For this reaction to occur, samples are placed into a lyophilizer and subjected to low pressure and temperature conditions. As a result, ice crystals formed within the samples are sublimated and 3-dimensional scaffolds are formed (61).

2.3.2 Crosslinking Scaffolds

Studies have been carried out on the use of natural or synthetic polymer scaffolds in bone tissue regeneration. However, polymeric scaffolds have low integrity and low mechanical strength limiting the use of polymeric scaffolds on bone tissue engineering (62). Crosslinking is a method used to modify the physical and chemical properties of polymeric tissue scaffolds. Therefore, researchers have been studying on crosslinking agents on polymeric scaffolds for amplifying their mechanical and chemical strength, trying to create scaffolds that are more stable, more sturdy and more resistant to resorption (63). Crosslinking can be achieved by various agents such as glutaraldehyde, genipin, N-hydoxysuccinimide (NHS). Glutaraldehyde is a widely used cross-linking agent capable of crosslinking with a wide range of biomaterials (64). However, although glutaraldehyde cross-linking positively affects the mechanical properties of scaffolds in the desired direction for bone tissue regeneration, studies have shown that it can have toxic effects on tissues. Studies, using glutaraldehyde as crosslinking agent, on polymeric scaffolds showed that glutaraldehyde increases mechanical strength of polymeric scaffolds by microstructural fibril rotation changes (65).

2.4 Chitosan

Although chitin was found in early 1800s, chitosan was not found until 1859 by a French physiologist named Charles Rouget (66).

Chitin is a linear polysaccharide, found on the exoskeletons of insects, crustaceans and fungi. Chitosan, primary derivative of chitin, belongs to polysaccharide family, consisting of ß-(1-4)-2-acetamido-2-deoxy-D-glucopyronase repeating units. Chitosan is an amino-polysaccharide in crystalline formation obtained by deacetylation of chitin. Chitosan is insoluble in water pH 7 or above. However, chitosan goes into soluble form at pH 7 and below by the amino groups acting as positive charge towards acidity (67).

Since its solubility depends on pH, it has an easily processable structure. Besides the property of being easily processable, recent studies on chitosan showed that it also has antimicrobial (68), antioxidant (69), biocompatible, biodegradable and antitumoral properties. Due to the characteristic properties, chitosan has been a material studied in biomedical area for wound healing (70), scaffold and tissue engineering (71), scaffolding with Nano-particles for formation of tissue skeletons (72) and drug delivery systems (73).

2.5 Biomaterials

Tissues can be damaged by pathological or physiological reasons such as diseases and trauma, making the damaged tissue not functioning or partially functioning. Biomaterials are synthetic or natural materials used to replace the damaged tissue or used to improve the function of tissues with reduced functions. These biomaterials can be used individually or combined with other materials.

The use of biomaterials dates back to 32.000 years as scientist found fossils sutured with animal sinew. Early Egyptians used linen sutures. Catgut was used in the Middle ages in Europe. Synthetic ears, teeth, eyes have been found on Egyptian mummies.

Throughout the history, scientists studying human physiology and anatomy have aimed to replace organs that have lost their function or fail to function fully by replacing organs with other people's organs, or by using biomaterials to replace the organ that has lost its function. In mid-1950s, Dr. Paul Winchell patented an artificial heart design and in 1957, Dr. Willem Kolff and his team studied Dr. Paul Winchell's heart design on animals.

Biomaterials have been used in order 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 (74). Also, a dental implant was found in a corpse dated 200 A.D in Europe. This dental implant was made out of iron and also described as "osteointegrated" into the bone (75).

Biomaterials can be classified into four groups;

- Composite biomaterials

- Ceramic biomaterials
- Polymer biomaterials
- Metallic biomaterials

2.5.1 Composite Biomaterials

Composites are materials that consist of two or more distinct parts. Composites consist of at least two phases, one being continuous phase, called the matrix and one being discontinuous phase, called the reinforcement. Different continuous phase and discontinuous phase create different composite materials. Shape, size and distribution of reinforcement, reinforcement properties and volume percentage, bioactivity of the reinforcement, matrix properties (ex. Molecular weight, grain size etc..), and reinforcement-matrix interfacial state make up the structure of composites (76).

2.5.2 Ceramic Biomaterials

Ceramics are crystalline, inorganic, nonmetallic materials that have inter-atomic bonds with ionic, covalent or metallic bonds. Ceramics have good compressive strength and biological inertness. Ceramics, designed to repair and reconstruct damaged tissue or organs are called bio ceramics (76).

2.5.3 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 (77).

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 (78).

2.5.4 Metallic Biomaterials

Metallic biomaterials have high metal content, used in load bearing areas such as implants and internal fixation devices (79). They have high yield strength, resistance to corrosion, high tensile strength and resistance to fatigue. These biomaterials are mostly used in orthopedic and dental application, such as hip, knee prothesis and dental implants as well as making surgical handpieces (80). Although metallic biomaterials are resistant to corrosion, when they are corroded as a result of the metabolic activity of the tissue, ions and electrons emit into the surrounding tissues, disrupting the physiological balance and when mixed with body fluids, acting on various other organs and tissues.

Most used metals are stainless steels, chromium-cobalt metals and cpTi and its alloys (77).

2.5.4.1 Stainless steel

316L Stainless steel alloys are used in many medical fields because of their low carbon content, high corrosion resistance, high cleanability rate and biocompatibility. Stainless steel alloys were first used in orthopedic surgeries in 1926 (72). 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 (81).

316L Stainless steels alloys have higher density than bone. Its elasticity is less than bone (82). These disadvantages can cause them to negatively affect bone healing in the area where they are implanted. Although the chromium it contains increases the corrosion resistance against bodily fluids, they are exposed to corrosion due to their long stay in the area where they are implanted (83). These properties of stainless steel alloys may cause the implant to be broken in the early period of bone healing resulting in malunion (84). In cases of corrosion, stainless steel implants can release metal ions that they contain into the body (85,81). Although chromium and nickel are thought to have a positive effect on the medical use of stainless steel implants, these substances are known to have toxic effects when they affect the body locally or systemically (86).

2.5.4.2 Chrome Cobalt

Chrome-cobalt alloys have great strength, high temperature tolerance and have high wear resistance. They can withstand corrosion effects longer than stainless steel alloys. They are used in the medical field as chromium, cobalt and molybdenum alloys. Besides being biocompatible, they have high resistance against physical and chemical forces which opens up the option to use these alloys in the medical field (87). Chrome-cobalt alloys are used in the production of total hip prostheses, knee implants and dental implants (88).

Although chrome-cobalt alloys are said to be biocompatible in their use for orthopedic and dental surgeries, they are considered less biocompatible than Ti alloys (89).

2.5.4.3 Titanium and titanium alloys

Ti is a material that doesn't exist as a pure element in the nature. Pure Ti is obtained as a result of reduction process with sodium which is called the Kroll process (56).

Ti due to its high resistance to physical forces, its low density, its non-toxic structure on living cells, its high resistance to corrosion and its low elastic modulus, is used in medical implant production, in aircraft, space and chemical industries (90).

Ti is considered a biocompatible material as it rapidly gets oxidized when it is exposed to oxygen, creating a stable layer on the outside, allowing attachment to biological molecules. This surface oxide attaches serum proteins, producing a protein layer and allowing cells to attach and differentiate on the surface (91).

However recent clinical reports indicate, upon its use as prostheses, implants and plates, Ti allergies developed. Ti also has been reported possibly causing generalized health problems regarding human tissue response to this oxide layer (92).

2.5.4.4 Titanium diboride

Boron is a transition element that is not found free in nature but as boron minerals. It is known that there are nearly 230 boron mineral types in nature (93).

Boron is a trace mineral that is involved in many different metabolism activity in living organisms (94). There are various studies showing the effect of boron in bone formation and regulation (11), wound healing (12), inflammation formation, magnesium absorption (95), estrogen, testosterone and vitamin D metabolism (96). Boron also plays an important role in regulating brain nerve-impulse transition (97).

Boron mineral and its derivatives have antibacterial, antifungal and antiseptic properties (9,10). Due to these properties of boron derivatives, there are various studies for their use in various applications in the field of medicine. It has been shown that the biofilm layer produced from boron derivatives can show protective properties against bacteria and yeast by inhibiting biofilm formation (98,99).

Use of boron and boron compounds are very diverse. Boron and boron compounds are used in the production of materials in many industries such as glass, ceramics, nuclear industries; as well as an active, side or protective agent in the medical, pharmaceutical and cosmetic field (98).

Boron can form compounds with transition group metals such as zirconium diboride, chromium diboride and TiB_2; with zirconium, chromium and Ti metals respectively. This transition group metal boride compounds are categorized as ceramic materials (100).

TiB_2 is a ceramic material with high strength and durability. The fact that it has high melting temperature and high abrasion resistance has enabled TiB2 to be used in armor resistant armors in heavy arms industry and in the production of hand tools that are desired to be durable (14).

TiB_2 with a density of 4.52g/cm3 is the most stable material among boron and metal alloys. The resistance of TiB_2 to oxidation is high. At temperatures approaching 600 ℃ the amount of oxidation increases; however, the borate film layer formed around this temperature reduces the amount of oxidation, making TiB_2 highly resistant to oxidation (101).

TiB_2 is mostly used outside the medical field. Its high resistance against oxidation ensures that the TiB_2 's resistance to fracture, abrasion and corrosion is higher than most of the alloys made by Ti. Studies for its use in the medical field are limited.

Yavuz Kaplan et al obtained a TiB and TiB_2 coating on the grade 5 Ti (Ti6Al4V) material, which is used in implant production. Their study investigated the biocompatibility, resistance to corrosion and strength of the material. Researchers reported an increase to corrosion resistance and external forces compared to Ti6Al4V and that TiB_2 layer on the surface didn't have negative effect on biocompatibility (93).

In the study of Mitun Das et al, coated surfaces of Ti6Al4V material with TiB-TiN, they reported no negative effect of coated surfaces on cell viability compared to cpTi (102).

Study designed by F.M.Makau et al, have shown that TiB_2 does not have cytotoxicity, but the composite compounds made by TiB_2 with Ti have been studied, cell interactions of this material itself have not been examined (5).

2.6 Biocompatibility

Biocompatibility can be defined as the fact that a material does not have a negative effect on the species in which it is use (103). These negative effects include systemic or local negative effects; soft or hard tissue reactions, as well as any allergenic, mutagenic or carcinogenic effects (103). The damage of the material used to the DNA structure is called mutagenic effect, if this damaged DNA is transferred to the new, proliferated cell, is called carcinogenic effect (104,105).

The term biocompatibility includes the physiological, chemical and biological effects of a material on living tissue, as well as the effects of tissue on the material. Biocompatibility is a non-static but a dynamic state (103). It can cause various changes in the material that the body interacts with time. Corrosion, fatigue as a result of body functions may have negative effects on the applied material, causing deterioration of the biocompatibility of the material used initially as biocompatible, over time. With the aging of the body, the emergence of various local or systemic diseases, the response to the used material can change; causing biocompatibility of the material to be impaired. Since the

interaction between the material and the body is a dynamic relationship, body's response to the material and the materials response to the living tissue can vary over time (103).

The definition of biocompatibility includes various types of interaction between the body and the material used (103). In order to define the biocompatibility of a material, its relation with the tissue in the region where this material is used should be taken into account (103). For example Ti used as dental implants is biocompatible for this application. It has been observed that dental implants produced from chromium-cobalt material don't have the ability to osseo-integrate with bone like Ti. For hip prostheses, chromium-cobalt materials are known to be more resistant to corrosion than Ti (106) For this reason, it has been stated that hip prostheses made of Ti are more unsuccessful than hip prostheses made of chromium-cobalt (106). Based on these data, it is not possible to say that biocompatibility of Ti is better than chromium-cobalt or vice versa. 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.

2.6.1 Evaluation of biocompatibility

The number of substances and materials investigated for biocompatibility increases drastically. In order to get most accurate results on studied material, these tests needs to follow the directions of international standards (16, 103).

ISO 10993 standards is a guideline created for biological evaluation of a material intended for use on living creatures. Its first goal is the protection of humans and living creatures. These standards, which are constantly updated, are a guide for the evaluation of biological response by performing various tests before using the new materials intended for use on human beings (16).

Biological evaluation of new materials which are considered to be used for various dental procedure, is determined in three different test stages; (107)

- In-vitro tests
- In-vivo tests
- Clinical studies

2.6.1.1 In-vitro tests

In-vitro tests 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 similar to those cells (108) The effects of the material in contact with these cells are studied, investigating the effects on the amount 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 (109).

In-vitro tests are fast, cost-effective and easy to standardize; but in-vitro tests cannot fully mimic the in-vivo environment (107) 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 (110).

The overall purpose of in-vitro cytotoxicity tests is to examine the effect on the cells exposed the extract of the studied material to cell viability and proliferation of the studied material, by comparing the results with the control (111). 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 (112). The cells selected are primary or secondary cells with high proliferation rate and have similar feature of tissue cells (113). Continuous cell cultures are also being used, as primary cells are not easy to obtain. These cell lines are obtained from malignant cell sources (113). Human cervical carcinoma (HeLa) cells (114), human colon carcinoma (HCT116) cells (115), bone osteosarcoma (SaOs-2) are used for in-vitro tests (116).

In-vitro tests, in the form of direct cell culture, can be done by examining the direct effect of the material on the cells in question; or by examining the effect of the extract obtained from the material, on the cells (16). According to the ISO 10993 standard, it has been said that cytotoxicity evaluation can be made by using extracts obtained from the studied material, as well as the material itself (16).

2.6.1.2 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 (104). Although more comprehensive results, regarding living creatures reaction, are obtained compared to in-vitro tests, but standardization on in-vivo tests are difficult (17). Due to their high cost, ethical rules and possible negative effects on experimental animals, in-vivo tests are applied as secondary tests for research (108).

2.6.1.3 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 (16). It should be demonstrated that the material used in clinical studies is not cytotoxic in in-vitro and in-vivo tests (108).

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 (103).

2.7 Cell Culture

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 have low cost. Because of these advantages, they have widespread use (117).

In cell culture studies, three different cell types are used; primary, secondary and continuous cell cultures (118).

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 (119).

Secondary cell cultures are cells obtained when primary cell cultures are sub cultured. They contain up to 90% of the primary cell properties up to 20 to 50 passages depending on the cell type obtained (120).

Cells obtained by sub culturing secondary cells cultures for 70 or more times are called continuous cell cultures (120). These cell cultures can be passaged infinitely. However, with the increase in the amount of passage, the differences of the karyotype from the primary cell culture increases. Continuous cell cultures are produces based on embryonic and cancerous cell types (113, 120). 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).

2.8 Spark Plasma Sintering (SPS)

Spark plasma sintering (SPS), although not a very old technique, is currently being used for fabricating bulk metals using powdered metal derivatives. This method has many advantages over conventional sintering methods, including the ability of control over the microstructure of fabricated material, flexibility by controlling the heating range and the ability to control the porosity of the sintered material, reaching the desired density (122).

SPS technique can be used on conductive and non-conductive materials. This technique can be applied on many materials such as Ti and alloys (123), bioceramics (124), composite materials, even on nanostructured materials ([125]), which are difficult to sinter using conventional sintering methods. The ability of versatility of SPS technique has been proven on many materials, which contributed to the development of innovative materials using SPS technique (126).

Even though the process of changing the densities and fabricating powder-type metals and ceramics using electrical current discharge dates back to 1900s (127), in general, patents in this field does not appear until 1980s (128). With the development of commercial machines, which are able to pulse electric fields to powders, created the opportunity in the metallurgy field, made possible to achieve quick consolidation of powdered metals and ceramics while preserving the particle size and density of these materials (128).

SPS technique allows the production of powder materials in predetermined shapes and sizes. At the same time, since it is a method that works with electrical discharge, the nanostructures of the materials do no deteriorate and allows for the desired shape and sizes to be fabricated in a very short time (129).

The machine used for SPS technique consists of a graphite die system, pressing devices, upper and lower punches which are also used as electrodes, a water-cooling reaction chamber, a vacuum control mechanism, which can include desired gas with controlled atmosphere pressure, a DC generator which gives out pulsed electricity, temperature, position and pressure regulating systems, controlled by an external computer control unit (127) Figure 2 shows a basic setup of a SPS system (130).

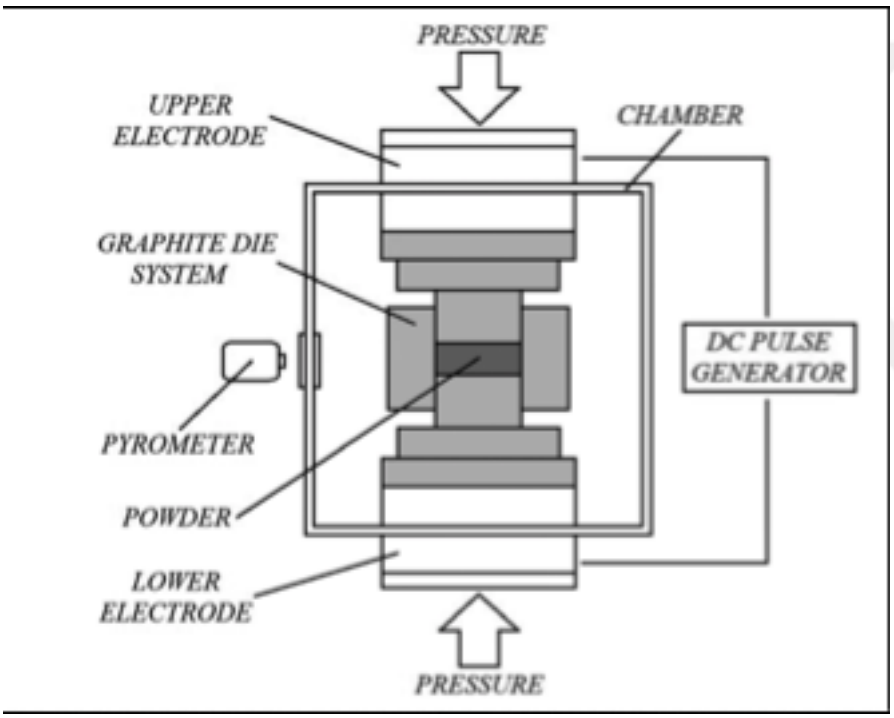

Figure 2.2 Basic Setup of SPS system (130)

Before using SPS system, desired powder is loaded into the graphite die. After loading the graphite die, sintering chamber is kept under vacuum or under used inert gas to avoid oxidation of powder particles (129). During sintering process, DC generator sends out pulsed electricity, making graphite die a heating source, causing the powder to be heated up from inside and outside (131). With SPS system, very fast heating and cooling can be achieved, making SPS system a fast occurring sintering technique (132).

SPS system is characterized by the spark plasma generated by the DC generators electrical current, heating the substance in the graphite die. Spark plasma is generated

between the gap of two electrodes in the electrical discharge (132). This generated plasma, creates over 2000 ℃, which is 200 ℃ to 500 ℃ lower than conventional sintering methods (133).

SPS system is a sintering system with various advantages over conventional hot pressing hot isostatic press sintering systems (129). In conventional sintering processes, the surfaces of the substances to be treated, must be free of the naturally occurring oxide layer and other contaminants (134). If not treated properly, materials sintered in conventional sintering process physical and chemical properties may not be at the desired level (134). SPS system, however, uses pulsed electrical currents during the sintering process, which with the pressure applied, breaks down naturally occurring oxide layer, resulting in diffusion and allowing bonding to occur between sintered substances enabling sintering in full density (135).

SPS can achieve full density with nearly every material used; metallic, ceramic or composites (129). Properties of SPS system, makes it a viable option in sintering conventional and unconventional materials.

3. MATERIALS AND METHODS

The materials used in this study were consisted of powder and plate forms of TiB_2. (Sigma Aldrich, Germany). The study on cell viability of TiB_2 powder form was conducted at Yeditepe University Faculty of Genetics and Bioengineering.

TiB_2 in powder form were used. Chitosan (Sigma-Aldrich, Germany) was chosen as the carrier material for TiB_2 powder and used as the scaffolding material. The scaffolds produced were produced contain 1mg and 2mg of TiB_2 at 0.005% and 0.0025% respectively. Chitosan scaffolds without TiB_2 powder were evaluated as the control.

Chitosan scaffolds were prepared as 4 samples each in each group. Study groups are as follows; (Fig 3)

1.Group; Control, chitosan scaffold without TiB_2 powder

2.Group; Chitosan scaffold containing 0.005%TiB_2 powder

3. Group; Chitosan scaffold containing 0.0025%TiB_2 powder

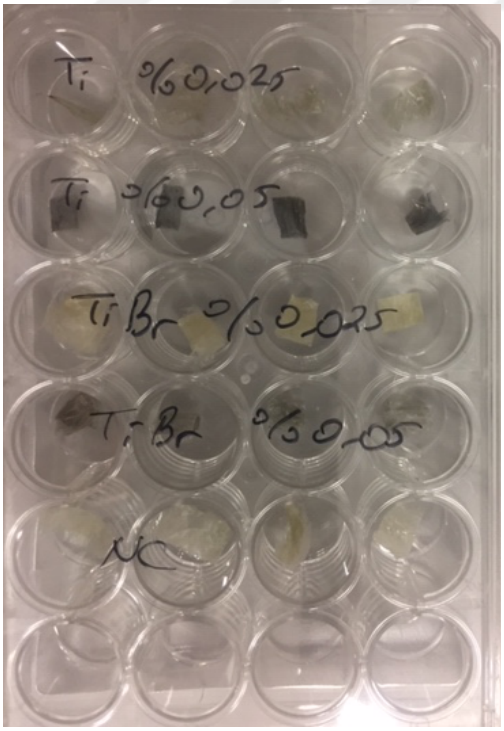

Figure 3.1 Study groups containing different rate of TiB2 powder

Glutaraldehyde was chosen as the chemical for crosslinking produced scaffolds (59). 0.025% Glutaraldehyde was prepared with double distilled water and mixed with dissolved chitosan achieving 20:1 volume. Periodontal ligament stem cells (PDLSC) were used for the powder form of TiB_2. ALP enzyme activity was studied for determining the cell viability in the produced scaffolds.

The study conducted on plate for of TiB_2, fabricated by using spark plasma sintering method was used. TiB_2 plates produced under three different atmospheric gases (argon and vacuum), atmospheric pressure (50 mPa and 70mPa) and temperatures (1600 °C, 1780 °C, 1800 °C) were used (100). Extracts of TiB_2 plates were used for determining the effects of TiB_2 on cell viability in SaOs-2 cell line.

TiB_2 plates were placed on phosphate buffered saline-free (PBS-Free) cell medium DMEM F12 (Thermo Fischer, England). Extracts were prepared in 4 different dilutions from TiB_2 (100%, 50%, 25%, 12,5%). 3 samples were prepared for each group for measurement. Study groups are as follows;

Control; SaOs-2 with normal medium.

Ar-TiB_2 ; Extracts of TiB_2 sintered under 50 mPa pressure in Argon atmosphere at 1800 °C was used at different percentages as cell culture medium.

V70-TiB_2; Extracts of TiB_2 sintered under 70 mPa pressure in vacuum atmosphere at 1780 °C was used at different percentages as cell culture medium.

V50-TiB_2; Extracts of TiB_2 sintered under 50 mPa pressure in vacuum atmosphere at 1600 °C was used at different percentages as cell culture medium.

3.1 Preparation of Scaffolds

Chitosan (SigmaAldrich, Germany) was dissolved in 1% acetic acid by using magnetic stirrer to make 2% chitosan solution. The chitosan solution was dispersed into 5 tubes, each tube containing 10 ml of dissolved chitosan. Control was consisted of no powder into pure dissolved chitosan. First study group consisted of dissolved chitosan containing 2mg of TiB_2 in order to achieve 0.005% Ti. Second study group consisted of dissolved chitosan containing 1mg of TiB_2 for 0.0025% TiB_2. 0.25% glutaraldehyde was prepared with double distilled water and added to each group, at 20:1 volume. Mixtures

were vortexed for 5 minutes and frozen at -80 degrees for 24h. Afterwards, frozen mixtures lyophilized at -105 degrees for 24 h in HyperCOOL (GZ-HC3110-CHR, Hanil Scientific Inc., Korea) lyophilizer (Fig 4,5). The lyophilized porous scaffolds were cut into smaller pieces and added to 24-well plates and neutralized with 2% NaOH for 2 h and all scaffolds were washed with ddH2O and frozen at -80 degrees for 24 h and lyophilized for 24 h at -105 degrees (Fig 6).

Figure 3.2 HyperCOOL (GZ-HC3110-CHR, Hanil Scientific Inc. Korea) lyophilizator

Figure 3.3 Lyophilization of chitosan scaffolds.

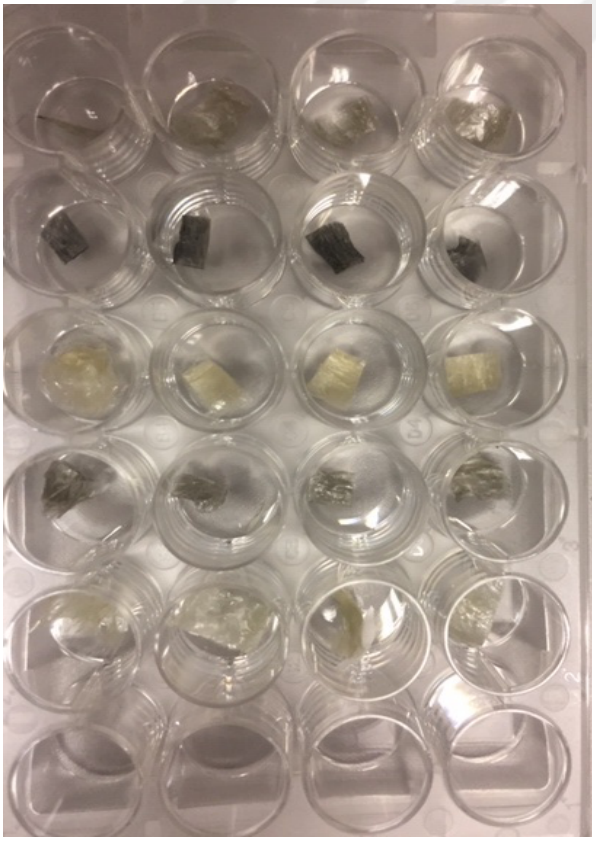

Figure 3.4 Placement of chitosan scaffolds containing TiB_2 powders into cell wells

3.2 Preparation of Cell line for cytotoxicity test on TiB_2 powder

Human periodontal stem cells (PDLSC) in the cell stock were removed from -196 °C and heated in a 37 °C water bath. Defrosted stem cells were incubated in t75 flasks with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F12 SD222-500, Serox, Germany) containing 20% Fetal Bovine Serum (FBS, A0503010, Cegrogen Biotech, Germany) and 1% penicillin/streptomycin inside a laminar flow cabinet. Stem cells were incubated for 7 days in a humid 5% CO2 incubator at 37 °C. The growth medium was refreshed every 3 days. After 80% confluence of cells was obtained, cells were passaged.

When the cells reached the 1x106 cells/mL for cytotoxicity analysis, the medium on the cells was withdrawn and the cells were washed with Ca2+, Mg2+free PBS. 0.05% Trypsin-EDTA (SM-2002-C, Sigma Aldrich, Germany) solution was added to the cells and kept in the incubator for 2-3 minutes.

Reverse phase light microscope was used for observing detachment of cells from the surface. The cells were collected in the centrifuge tube with their medium and centrifuged. After centrifugation, the cells were suspended in the medium. Cells were counted in Neubauer Hemocytometer after staining with trypan blue and it was added in 96 microplates at a concentration of 5x105 cells/mL in 150 µL volumes and incubated for 24 h at 37 °C with 5%CO2 and 95% humidity

3.3 Preparation of ALP test

Abcam (ab83369,Abcam,England) Alkaline Phosphatase Assay Kit (colorimetric) was used for ALP measurement. (Figure 7) Analyzes were carried out in line with the manufacturer's recommendation.

Figure 3.5 MTT test kit (Abcam, England)

Chitosan scaffolds containing TiB_2 powders were incubated for 7 days in 37°C, 5% CO2 conditions. After the incubation period, the cell culture media was aspirated and the wells were washed with PBS. 20 µL of stop solution was added to wells for stopping ALP activity. After aspirating the stop solution, 50 µL of 5 mM ₚNPP solution was added to each well containing chitosan scaffolds with TiB_2 powders. 10 µL of ALP enzyme solution was added to each well. The plate was incubated in 37 °C for 60 minutes, protected from light. After 60 minutes of incubation, 20 µL of stop solution was added and mixed well. ALP measurements were made in a microplate reader at a reference wave size of 405 nm.

3.4 Preparation of TiB_2 plates

TiB_2 powders were mixed, prior to sintering to ensure homogenization in ethanol medium. Then, ethanol content was removed by putting the mixed powder mixture into an oven at 105 °C. Ethanol free powders granulated until it can pass through a 160 µm sieve. When producing TiB_2 plates, electrical current was applied to TiB_2 powders with 12:2 on-off combinations, every pulse being 3.3 milliseconds, under 150 °C/min heating rate.

After creating three different sintered TiB_2 plate samples, they were reduced to the desired dimensions for present study, by using electrical discharge machining (EDM) system. Each sample was cut into 1mm thickness with 8x8mm dimensions. (Fig 8,9,10)

Figure 3.6 Sterilized Ar-TiB_2 plate

Figure 3.7 Sterilized V-70 TiB_2 plate

Figure 3.8 Sterilized V-50 TiB_2 plate

3.5 Preparation of Cell Line for cytotoxicity test on TiB_2 plates

SaOs-2 in the cell stock were removed from -196 °C and heated in a 37 °C water bath. The tube content was collected into a centrifuge tube with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F12 SD222-500, Serox, Germany) containing 10% Fetal Bovine Serum (FBS, A0503010, Cegrogen Biotech, Germany) inside a laminar flow cabinet. The collected cells were centrifuged at 1000 rpm for 5 minutes at 4 °C. After pouring the supernatant, the cell pellet was suspended with medium and was collected into a T75 flask (Cellstar, 658175, Greiner, Germany). Cells were incubated at 37 °C in an incubator containing 5% CO_2 and 95-98% humidity.

When the cells reached $1x10^6$ cells/mL for cytotoxicity analysis, the used medium on the cells was withdrawn and the cells were washed with Ca^{2+}, Mg^{2+}free PBS. 0.05% Trypsin-EDTA (SM-2002-C, Sigma Aldrich, Germany) solution was added to the cells and kept in the incubator for 2 to 3 minutes.

Reverse phase light microscope was used for observing detachment of cells from the surface. The cells were collected in the centrifuge tube with their medium and centrifuged. After centrifugation, the cells were suspended in the medium. Cells were counted in Neubauer Hemocytometer after staining with trypan blue and were added in 96 microplates at a concentration of $1x10^5$ cells/mL in 100 µL volumes and incubated for 24 h at 37 °C with 5% CO_2 and 95% humidity

3.6 Preparation Of Cytotoxicity Test

Sterile test materials were placed in FBS-free DMEM F12 media as 3 cm2/mL and were extracted for 24 h, at 37±0.1 °C. (Fig 11) Extraction was performed according to ISO 10993-12: 1998 (In vitro Cytotoxicity Test Sample Preparation and Reference Material Selection Standard (140). The material extracts were diluted with DMEM F12 at 50%, 25%, 12.5%rates. Together with 100% extract, all extracts prepared contained 10% FBS, 1% Sodium Pyruvate (L0473, Merck, Germany), 0.1% Penicillin-Streptomycin (A2213, Biochrom, Germany).

Cytotoxic effects of material extracts were analyzed at 24 h and 48 h after incubation in four different dilutions (100%, 50%, 25%, 12.5%) using MTT test (3-(4,5-

Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, (M5655, Sigma Aldrich, Germany). In control cells incubated with standard cell culture medium without test substance extracts. After 24 h and 48 h the used media was removed from cell cultures and DMEM 12 containing 10% MTT (5mg/mL) were added and cell cultures were incubated for 4 h (Fig 12). After the incubation period was completed and the medium MTT was removed and DMSO (802912, Merck, Germany) was added in order to dissolve formed formazan crystals. Absorbance was obtained by reading the microplate reader at a wavelength of 570 nm.

Cell viability were measured according to ISO 10993 guideline, considering its requirement of percentile method comparison between control and study groups.

Figure 3.9 Gibco Dulbecco's Modified Eagle Medium F12 (Thermo Fischer Sci. England)

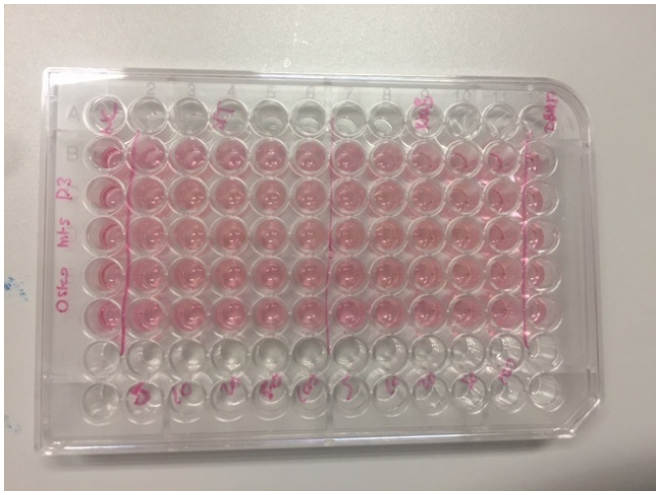

Figure 3.10 Placement of TiB_2 extracts and control medium into wells with SaOs-2 cell line

3.7 Statistical Analyses

All parameters were analyzed by using IBM SPSS Statistics (version 24). One-Way ANOVA test were used for comparing of cell viability between groups ($p<0.05$). In the homogeneity of variances, Tukey test was chosen for post-hoc comparison when Levene's test result was $p>0.005$ and Tamhane test was used for post-hoc comparison when Levene's test result was $p<0.005$. Paired t-test was used for comparing results within groups of 24 h and 48 h.

4. RESULTS

4.1 Evaluation of ALP measurements on day 7

ALP enzyme activity was not observed in chitosan scaffolds containing Ti and TiB_2 where ALP enzyme activity was measured on the 7th day of incubation. When cell viability was examined, it was observed that there was no cell viability in chitosan scaffolds.

4.2 24-hour cytotoxicity assessment of Ar-TiB_2, V70-TiB_2 and V50-TiB_2

The results of cytotoxicity of control and 100%, 50%, 25% and 12,5% extract groups of Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extraction at 24 h are showed on Table 1. SaOs-2 cell viability on 24 h for control and each extract groups are showed on Fig 13,14,15. The mean value of cell viability of control was 1.656±0.009. For all extract groups of Ar-TiB_2 were 0.89 ±0.09 , 1.14 ±0.04, 1.24±0.03, 1.19±0.04 respectively, while for V70-TiB_2 were 0.70±0.23, 1.08±0.15, 1.29±0.10, 1.32±0.07 respectively, and for V50-TiB_2 were 0.91±0.07, 1.22±0.01, 1.34±0.003, 1.33±0.002 respectively. Statistical significance was found between the control and 100%, 50%, 25%, 12,5% extract groups ($p=0.00$, $p<0.001$).

Table 4.1 Cytotoxic effect results of control and Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 24 hours.

	Mean	Std.Deviation	Std.Error	Minimum	Maximum	Sig
CONTROL	1,6560	,00985	,00569	1,65	1,67	,000
Ar-TiB_2						
100%	,8900	,08920	,05150	,82	,99	,000
50%	1,1403	,04362	,02518	1,10	1,18	,000
25%	1,2443	,03580	,02067	1,21	1,28	,000
12,5%	1,1953	,04216	,02434	1,17	1,24	,000
V70-TiB_2						
100%	,7063	,23166	,13375	,52	,97	,002
50%	1,0860	,15846	,09149	,92	1,24	,003
25%	1,2957	,10744	,06203	1,18	1,39	,004
12,5%	1,3240	,07957	,04594	1,25	1,41	,002
V50-TiB_2						
100%	,9173	,07321	,04227	,87	1,003	,000
50%	1,2267	,01818	,01049	1,21	1,25	,000
25%	1,3437	,00321	,00186	1,34	1,35	,000
12,5%	1,3327	,00252	,00145	1,33	1,34	,000

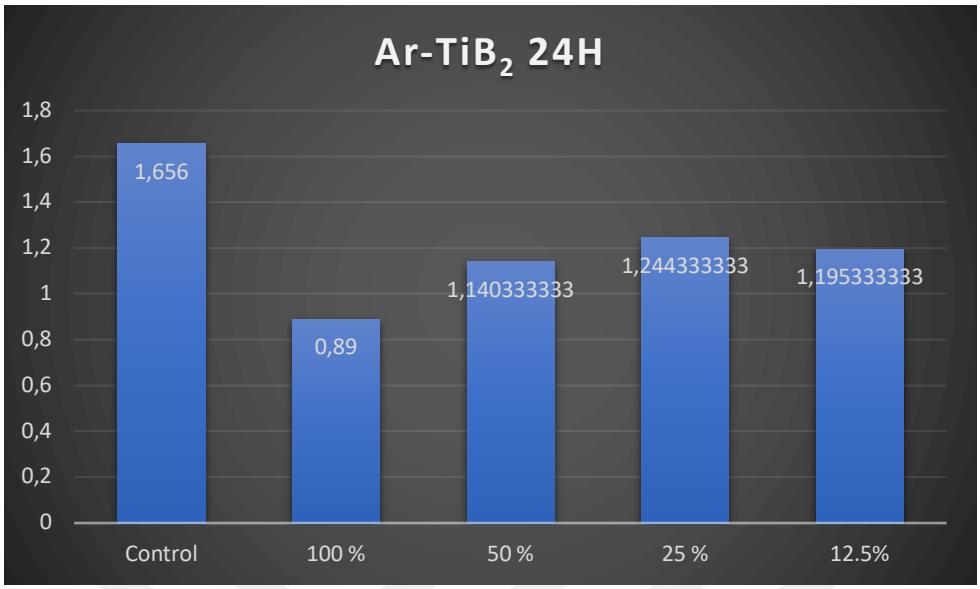

Figure 4.1 The mean SaOs-2 cell viability of control and Ar-TiB_2 group on 24 hours

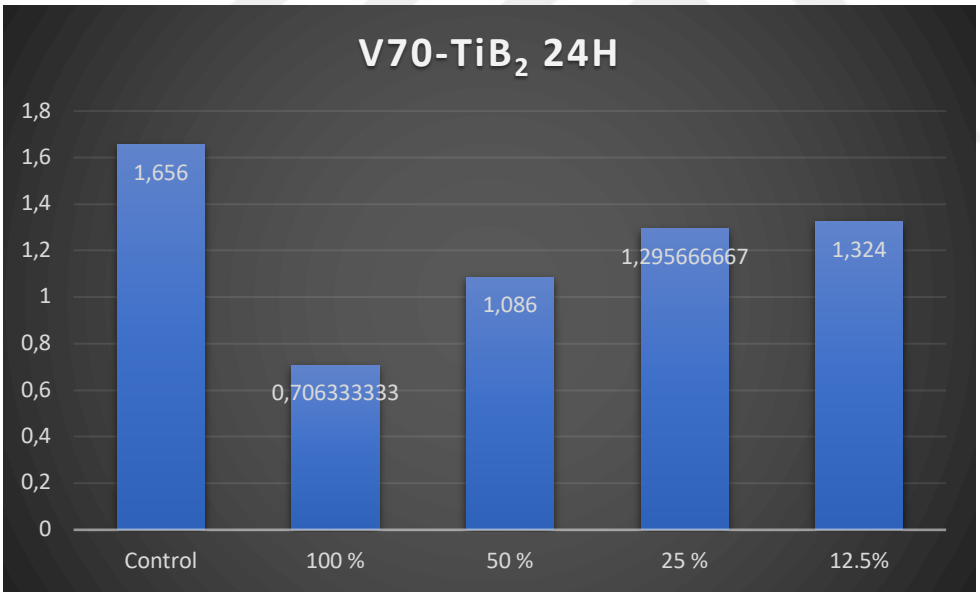

Figure 4.2 The mean SaOs-2 cell viability of control and V70-TiB_2 group on 24 hours

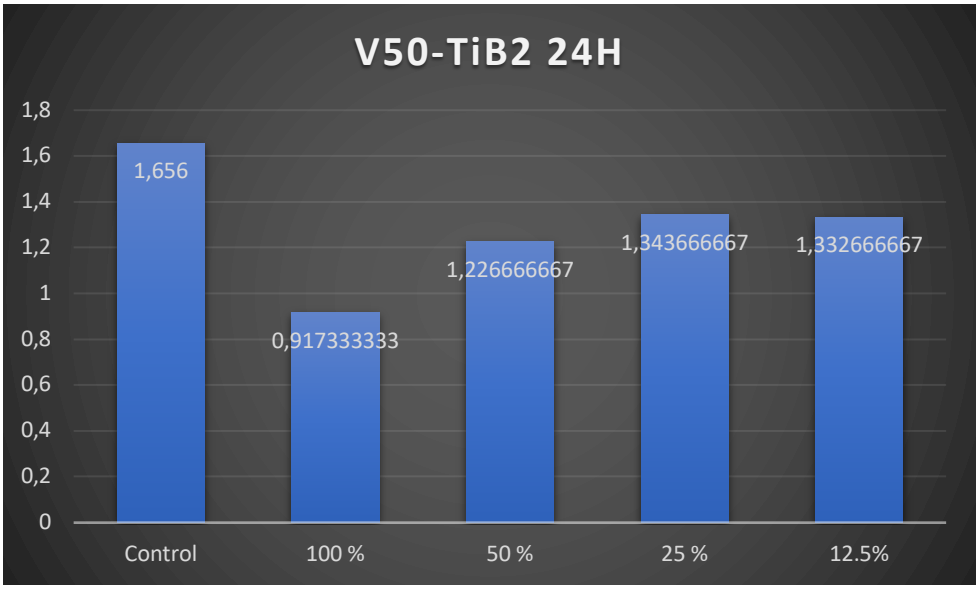

Figure 4.3 The mean SaOs-2 cell viability of control and V50-TiB_2 group on 24 hours

Post-hoc comparison between groups of 100%, 50%, 25% and 12,5% are showed on Table 2,3,4,5, respectively. Statistically significant differences were found between 100% extracts of Ar-TiB_2 ($p=0.024$), V70-TiB_2 ($p=0.009$), V50-TiB_2 ($p=0.017$) and control. There was no statistically significant difference in between each group ($p>0.05$) (Table 2)

Table 4.2 Post-hoc results of control and 100% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 24 hours.

	(I)	(J)	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval	
						Lower Bound	Upper Bound
Tamhane	Control (24h)	Ar-TiB_2	,76600000*	,05181377	,024	,2308080	1,3011920
		V70-TiB_2	,94966667*	,13386726	,009	-,4828828	2,3822162
		V50-TiB_2	,73866667*	,04265104	,017	,3065806	1,1707527
	Ar-TiB_2	Control (24h)	-,76600000*	,05181377	,024	-1,3011920	-,2308080
		V50-TiB_2	-,02733333	,06662665	,999	-,3574796	,3028129
		V70-TiB_2	,18366667	,14331938	,885	-,8699622	1,2372955
	V70-TiB_2	Control (24h)	-,94966667	,13386726	,009	-2,3822162	,4828828
		V50-TiB_2	-,21100000	,14026721	,824	-1,3490142	,9270142
		Ar-TiB_2	-,18366667	,14331938	,885	-1,2372955	,8699622
	V50-TiB_2	Control (24h)	-,73866667*	,04265104	,017	-1,1707527	-,3065806
		V70-TiB_2	,21100000	,14026721	,824	-,9270142	1,3490142
		Ar-TiB_2	,02733333	,06662665	,999	-,3028129	,3574796

Statistically significant difference were found between control and 50% extracts of Ar-TiB_2 ($p=0.00$, $p<0.001$), V70-TiB_2 ($p=0.00$, $p<0.001$), V50-TiB_2 ($p=0.001$). There was no statistically significant difference in between each group ($p>0.05$) (Table 3).

Table 4.3 Post-hoc results of control and 50% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 24 hours.

	(I)	(J)	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Control (24h)	Ar-TiB_2	,51566667*	,06762725	,000	,2991004	,7322329
		V70-TiB_2	,57000000*	,06762725	,000	,3534338	,7865662
		V50-TiB_2	,42933333*	,06762725	,001	,2127671	,6458996
	Ar-TiB_2	Control (24h)	-,51566667*	,06762725	,000	-,7322329	-,2991004
		V50-TiB_2	-,08633333	,06762725	,601	-,3028996	,1302329
		V70-TiB_2	,05433333	,06762725	,851	-,1622329	,2708996
	V70-TiB_2	Control (24h)	-,57000000*	,06762725	,000	-,7865662	-,3534338
		V50-TiB_2	-,14066667	,06762725	,238	-,3572329	,0758996
		Ar-TiB_2	-,05433333	,06762725	,851	-,2708996	,1622329
	V50-TiB_2	Control (24h)	-,42933333*	,06762725	,001	-,6458996	-,2127671
		V70-TiB_2	,14066667	,06762725	,238	-,0758996	,3572329
		Ar-TiB_2	,08633333	,06762725	,601	-,1302329	,3028996

Statistically significant difference were found between control and 25% extracts of Ar-TiB_2 (p=0.000, p<0.001), V70-TiB_2 (p=0.000, p<0.001), V50-TiB_2 (p=0.001). There was no statistically significant difference in between each group (p>0.05) (Table 4).

Table 4.4 Post-hoc results of control and 25% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 24 hours.

	(I)	(J)	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval Lower Bound	Upper Bound
Tukey HSD	Control (24h)	Ar-TiB_2	,41166667*	,04642377	,000	,2630014	,5603319
		V70-TiB_2	,36033333*	,04642377	,000	,2116681	,5089986
		V50-TiB_2	,31233333*	,04642377	,001	,1636681	,4609986
	Ar-TiB_2	Control (24h)	-,41166667*	,04642377	,000	-,5603319	-,2630014
		V50-TiB_2	-,09933333	,04642377	,220	-,2479986	,0493319
		V70-TiB_2	-,05133333	,04642377	,696	-,1999986	,0973319
	V70-TiB_2	Control (24h)	-,36033333*	,04642377	,000	-,5089986	-,2116681
		V50-TiB_2	-,04800000	,04642377	,736	-,1966653	,1006653
		Ar-TiB_2	,05133333	,04642377	,696	-,0973319	,1999986
	V50-TiB_2	Control (24h)	-,31233333*	,04642377	,001	-,4609986	-,1636681
		V70-TiB_2	,04800000	,04642377	,736	-,1006653	,1966653
		Ar-TiB_2	,09933333	,04642377	,220	-,0493319	,2479986

Statistically significant difference were found between control and 12,5% extracts of Ar-TiB_2 (p=0.000, p<0.001), V70-TiB_2 (p=0.000, p<0.001), V50-TiB_2 (p=0.000, p<0.001). Statistical significant difference were found between Ar-TiB_2 and V50-TiB_2 (p=0.025) and V70-TiB_2 and Ar-TiB_2 (p=0.034). There was no statistically significant difference between V70-TiB_2 and V50-TiB_2 (p>0.05). (Table 5)

Table 4.5 Post-hoc results of control and 12,5% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 24 hours.

	(I)	(J)	Mean Difference (	Std. Error	Sig	95% Confidence Interval Lower Bound	Upper Bound
Tukey HSD	Control (24h)	Ar-TiB_2	,46066667*	,03699474	,000	,3421965	,5791369
		V70-TiB_2	,33200000*	,03699474	,000	,2135298	,4504702
		V50-TiB_2	,32333333*	,03699474	,000	,2048631	,4418035
	Ar-TiB_2	Control (24h)	-,46066667*	,03699474	,000	-,5791369	-,3421965
		V50-TiB_2	-,13733333*	,03699474	,025	-,2558035	-,0188631
		V70-TiB_2	-,12866667*	,03699474	,034	-,2471369	-,0101965
	V70-TiB_2	Control (24h)	-,33200000*	,03699474	,000	-,4504702	-,2135298
		V50-TiB_2	-,00866667	,03699474	,995	-,1271369	,1098035
		Ar-TiB_2	,12866667*	,03699474	,034	,0101965	,2471369
	V50-TiB_2	Control (24h)	-,32333333*	,03699474	,000	-,4418035	-,2048631
		V70-TiB_2	,00866667	,03699474	,995	-,1098035	,1271369
		Ar-TiB_2	,13733333*	,03699474	,025	,0188631	,2558035

Cell viability of control and extraction groups on 24 h are shown on Fig 16. Cell viability of 100% extracts of Ar-TiB_2, V50-TiB_2 and V70-TiB_2 were found below 70% compared to control. More than 70% cell viability was observed in each extract groups of 50%, 25%and 12,5%. As the rate of TiB_2 content decreased, increased cell viability was observed, a negative correlation was found between cell viability and material content according to ISO guideline, which it is mandatory to possess cell viability above 70%. Ar-TiB_2 (r=-,713 , p=0.009), V70-TiB_2 (r=-,842 , p=0.001), V50-TiB_2 (r=-,773 , p=0.003).

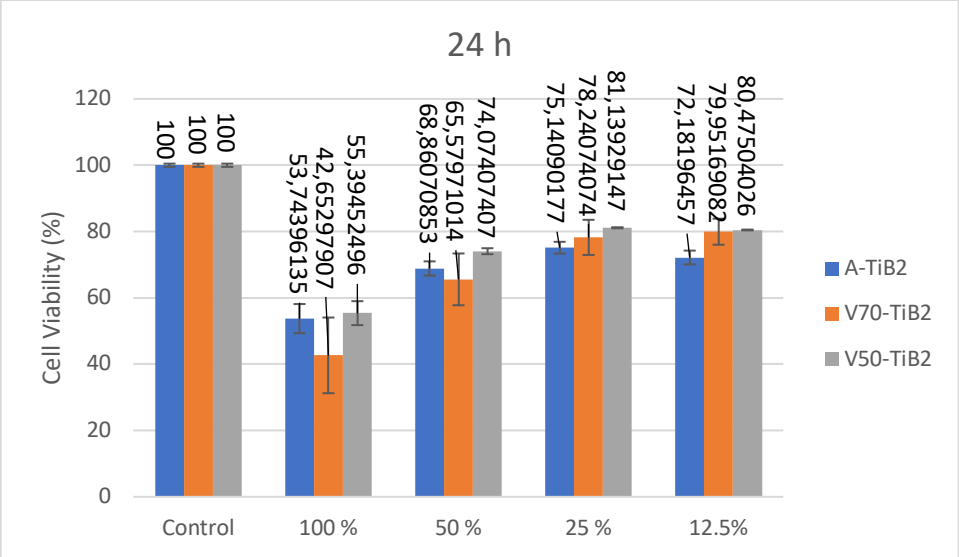

Figure 4.4 Cell viability (%) of Control, Ar-TiB_2, V70-TiB_2, V50-TiB_2 on every extract (%) after 24 hours

4.3 48-hour cytotoxicity assessment of Ar-TiB_2, V70-TiB_2 and V50-TiB_2

The results of cytotoxic effect results of control and 100%, 50%, 25% and 12,5% extract groups of Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extraction at 48 h are showed on Table 6. SaOs-2 cell viability on 48 h for control and each extract groups are showed on fig 17,18,19. The mean value of cell viability of control was 1.44±0.01. For all extract groups of Ar-TiB_2 were 0.41 ±0.03 , 0.54 ±0.01, 0.58±0.04, 0.67±0.02 respectively while for V70-TiB_2 were 0.27±0.14, 0.49±0.13, 0.64±0.05, 0.71±0.05 respectively and for V50-TiB_2 were 0.42±0.10, 0.52±0.04, 0.6±0.01, 0.6±0.006 respectively. Statistical significance was found between the control and 100%, 50%, 25%, 12,5% extract groups. ($p=0.000$, $p<0.001$)

Table 4.6 Cytotoxic effect results of control and Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 48 hours.

	Mean	Std.Deviation	Std.Error	Minimum	Maximum	Sig
CONTROL	1,4427	,01458	,02471	1,42	1,49	,000
Ar-TiB_2						
100%	,04157	,03259	,01882	,39	,45	,000
50%	,5423	,01877	,01084	,53	,56	,000
25%	,5873	,04895	,02826	,55	,64	,000
12,5%	,6777	,02701	,01559	,65	,71	,000
V70-TiB_2						
100%	,2733	,14079	,8129	,17	,43	,000
50%	,4930	,13156	,07596	,36	,62	,001
25%	,6433	,05064	,02924	,59	,68	,000
12,5%	,7103	,05599	,03232	,65	,75	,000
V50-TiB_2						
100%	,4233	,10540	,06085	,34	,54	,000
50%	,5267	,04980	,02875	,47	,57	,000
25%	,6000	,01442	,00833	,59	,62	,000
12,5%	,6030	,00624	,00361	,60	,61	,000

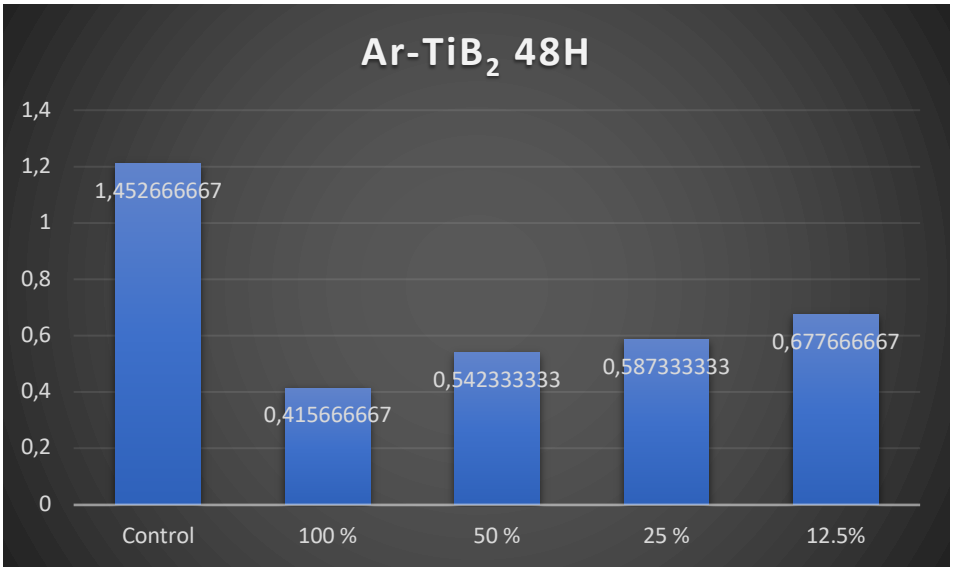

Figure 4.5 The mean SaOs-2 cell viability of control and Ar-TiB_2 group on 48 hours

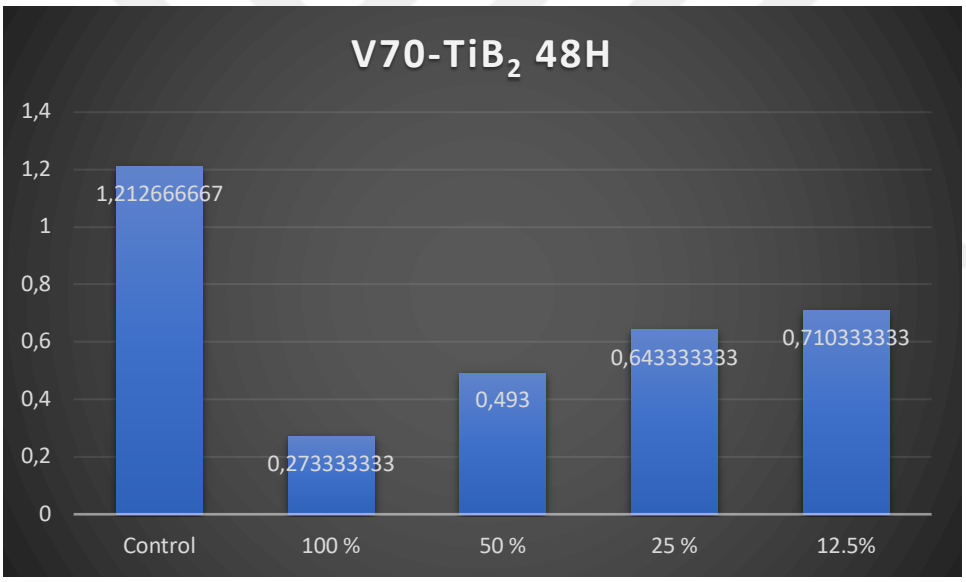

Figure 4.6 The mean SaOs-2 cell viability of control and V70-TiB_2 group on 48 hours

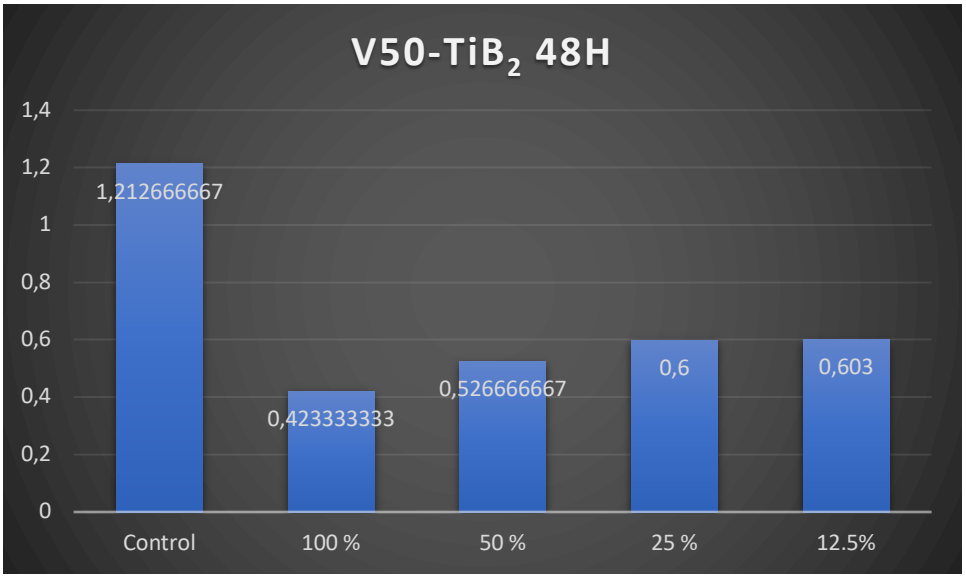

Figure 4.7 The mean SaOs-2 cell viability of control and V50-TiB_2 group on 48 hours

Post-hoc comparison between groups of 100%, 50%, 25% and 12,5% are showed at Table 7,8,9,10 respectively. Statistically significant differences were found between 100% extracts of Ar-TiB_2 ($p=0.000$, $p<0.001$), V70-TiB_2 ($p=0.040$), V50-TiB_2 ($p=0.030$) and control. There was no statistically significant difference in between each group ($p>0.05$) (Table 7).

Table 4.7 Post-hoc results of control and 100% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 48 hours.

	(I)	(J)	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval Lower Bound	Upper Bound
Tamhane	Control (48h)	Ar-TiB_2	,79700000*	,2101322	,000	,6638112	,9301888
		V70-TiB_2	,93933333*	,08182230	,040	-,0971031	1,7815636
		V50-TiB_2	,78933333*	,06156478	,030	,1765599	1,4021068
	Ar-TiB_2	Control (48h)	-,79700000	,02101322	,000	-,9301888	-,6638112
		V50-TiB_2	-,00766667	,06369371	1,000	-,5294541	,5141208
		V70-TiB_2	,142333333	,08343594	,771	-,6176744	,9023411
	V70-TiB_2	Control (48h)	-,93933333	,08182230	,040	-1,781563	-,0971031
		V50-TiB_2	-,15000000	,10153926	,773	-,6684805	,3684802
		Ar-TiB_2	-,14233333	,08343594	,771	-,9023411	,6176744
	V50-TiB_2	Control (48h)	,789333333*	,06156478	,030	-1,402106	-,1765599
		V70-TiB_2	,150000000	,10153926	,773	-,3684805	,668406
		Ar-TiB_2	,00766667	,06369371	1,000	-,5141208	,5294541

Statistically significant difference were found between control and 50% extracts of Ar-TiB_2 (p=0.042), V70-TiB_2 (p=0.033), V50-TiB_2 (p=0.027). There was no statistically significant difference in between each group. (p>0.05) (Table 8)

Table 4.8 Post-hoc results of control and 50% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 48 hours.

	(I)	(J)	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval Lower Bound	Upper Bound
Tamhane	Control (48h)	Ar-TiB_2	,67033333*	,05831381	,042	,4835920	,8570747
		V70-TiB_2	,71966667*	,05831381	,033	,5329253	,9064080
		V50-TiB_2	,68600000*	,05831381	,027	,4992587	,8727413
	Ar-TiB_2	Control (48h)	-,67033333*	,05831381	,042	-,857074	-,483592
		V50-TiB_2	,01566667	,05831381	,993	-,171074	,2024080
		V70-TiB_2	,04933333	,05831381	,832	-,137408	,2360747
	V70-TiB_2	Control (48h)	-,71966667*	,05831381	,033	-,906408	-,532925
		V50-TiB_2	-,03366667	,05831381	,936	-,220408	,1530747
		Ar-TiB_2	-,04933333	,05831381	,832	-,236074	,1374080
	V50-TiB_2	Control (48h)	-,68600000*	,05831381	,027	-,872741	-,499258
		V70-TiB_2	,03366667	,05831381	,936	-,153074	,2204080
		Ar-TiB_2	-,01566667	,05831381	,993	-,202408	,1710747

Statistically significant differences were found between control and 25% extracts of Ar-TiB_2 (p=0.043), V70-TiB_2 (p=0.038), V50-TiB_2 (p=0.035). There was no statistically significant difference in between each group (p>0.05) (Table 9).

Table 4.9 Post-hoc results of control and 25% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 48 hours.

	(I)	(J)	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval	
						Lower Bound	Upper Bound
Tamhane	Control (48h)	Ar-TiB_2	,62533333*	,03008599	,043	,5289874	,7216793
		V70-TiB_2	,56933333*	,03008599	,038	,4729874	,6656793
		V50-TiB_2	,61266667*	,03008599	,035	,5163207	,7090126
	Ar-TiB_2	Control (48h)	-,62533333*	,03008599	,043	-,7216793	-,5289874
		V50-TiB_2	-,01266667	,03008599	,973	-,1090126	,0836793
		V70-TiB_2	-,05600000	,03008599	,315	-,1523459	,0403459
	V70-TiB_2	Control (48h)	-,56933333*	,03008599	,038	-,6656793	-,4729874
		V50-TiB_2	,04333333	,03008599	,511	-,0530126	,1396793
		Ar-TiB_2	,05600000	,03008599	,315	-,0403459	,1523459
	V50-TiB_2	Control (48h)	-,61266667*	,03008599	,035	-,7090126	-,5163207
		V70-TiB_2	-,04333333	,03008599	,511	-,1396793	,0530126
		Ar-TiB_2	,01266667	,03008599	,973	-,0836793	,1090126

Statistically significant difference were found between control and 12,5% extracts of Ar-TiB_2 ($p=0.041$), V70-TiB_2 ($p=0.026$), V50-TiB_2 ($p=0.034$). Statistical significant difference were found between V70-TiB_2 and V50-TiB_2 ($p=0.015$). There was no statistically significant difference between Ar-TiB_2 and V70-TiB_2 ($p>0.05$) and between Ar-TiB_2 and V50-TiB_2 ($p>0.05$) (Table 10)

Table 4.10 Post-hoc test results of control and 12,5% Ar-TiB_2, V70-TiB_2 and V50-TiB_2 extracts on 48 hours.

	(I)	(J)	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval Lower Bound	Upper Bound
Tamhane	Control (48h)	Ar-TiB_2	,53500000*	,0263470 4	,04 1	,450627 5	,619372 5
		V70-TiB_2	,50233333*	,0263470 4	,02 6	,417960 8	,586705 8
		V50-TiB_2	,60966667*	,0263470 4	,03 4	,525294 2	,694039 2
	Ar-TiB_2	Control (48h)	-,5350000*	,0263470 4	,04 1	-,619372	-,450627
		V50-TiB_2	,07466667	,0263470 4	,08 4	-,009705	,159039
		V70-TiB_2	-,03266667	,0263470 4	,62 1	-,117039	,051705
	V70-TiB_2	Control (48h)	-,50233333*	,0263470 4	,02 6	-,586705 8	-,417960 8
		V50-TiB_2	,10733333*	,0263470 4	,01 5	,022960 8	,191705 8
		Ar-TiB_2	,03266667	,0263470 4	,62 1	-,051705 8	,117039 2
	V50-TiB_2	Control (48h)	-,60966667*	,0263470 4	,03 4	-,694039 2	-,525294 2
		V70-TiB_2	-,10733333*	,0263470 4	,01 5	-,191705 8	-,022960 8
		Ar-TiB_2	-,07466667	,0263470 4	,08 4	-,159039 2	,009705 8

Cell viability of control and extraction groups on 48 h are shown on Figure 20. Cell viability of 100%, 50%, 25% and 12,5% extracts of Ar-TiB_2, V50-TiB_2 and V70-TiB_2 were found below 70% compared to control. As the rate of TiB_2 content decreased, increased cell viability was observed, a negative correlation between cell viability and material content, according to the ISO guideline were found below 70%. Ar-TiB_2 (r=-,950 , p<0.001), V70-TiB_2 (r=-,885 , p<0.001), V50-TiB_2 (r=-,885 , p<0.001).

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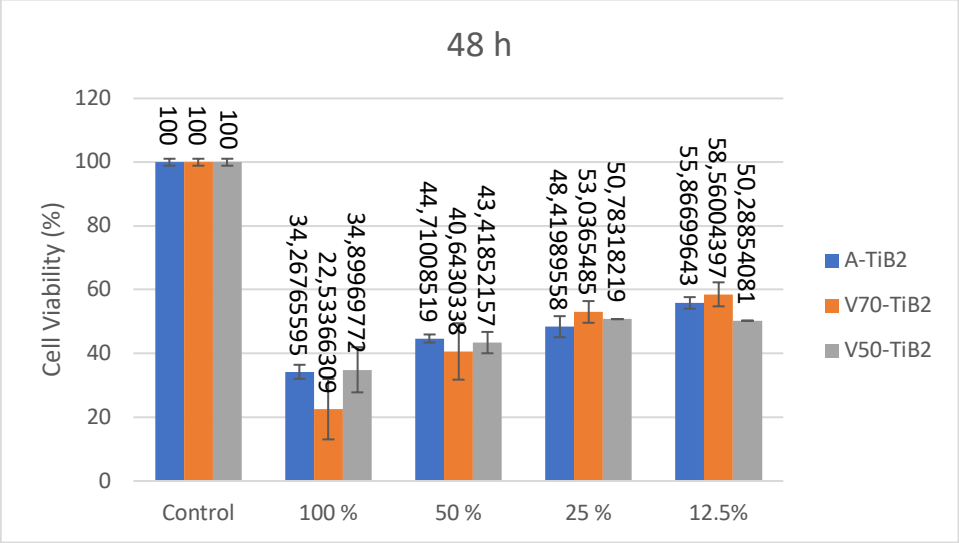

Figure 4.8 Cell viability (%) of Control, Ar-TiB_2, V70-TiB_2, V50-TiB_2 on every extract (%) after 48 hours

Table 11 shows the cell viability comparison results of 100%, 50%, 25% and 12,5% dilated extracts on 24 h and 48 h. Statistical differences were found between 24 h and 48 h on cell viability test results of 100%, 50%, 25%, 12,5% Ar-TiB_2, V70- TiB_2 and V50- TiB_2

Table 4.11 Cell viability test results between 24 hours and 48 hours.

	Pair	Mean	Std. Deviation	Sig.
A-TiB_2	100%	,47433	,06174	,006
	50%	,59800	,06090	,003
	25%	,65700	,05957	,003
	12,5%	,51767	,05001	,003
V70-TiB_2	100%	,43300	,09111	,014
	50%	,59300	,02700	,001
	25%	,65233	,06603	,003
	12,5%	,61367	,05630	,003
V50-TiB_2	%100	,49400	,03460	,002
	%50	,70000	,03551	,001
	%25	,74367	,01350	,000
	%12,5	,72967	,00404	,000

5. DISCUSSION

Correct selection of implant biomaterial is one of the important factors for long-term implant success because biological tissue does not allow every biomaterial used in implant production. Implants fabricated to fulfill their function should not have locally or systematically negative effects on the body, which the term biocompatibility has emerged (136). However the biocompatibility depends on many factors. The reaction of the cells to the material, resistance of the material to chemical and physical forces in the region and the amount of corrosion resistance of the material against body fluids (106).

Implants produced with different biomaterials have different physical, surface and biocompatible features, affecting their optimal application. Ti and Ti alloys are the most common used biomaterial for producing implants with good corrosion resistance, bone-like elasticity and strength with excellent biocompatibility. Although its use as a biomaterial has been proven to be highly safe and convenient studies have also seen complications with Ti implants.

When metals are long-term associated with body fluids, they can release particles to surrounding tissues, which can accumulate in tissues; or it can interfere with the circulatory system and affect the body systematically (137). Therefore, it is necessary to investigate metals that are minimal in ions secreted into the body in term of long exposure.

Kyeong Tae Kim et al, have stated that there was an increase in studies on titanium's cytotoxicity and allergy. They concluded implants with failed osteointegration may increase the ion release to the surrounding tissue, showing accumulation in blood circulation and organs. Increased ion levels in surrounding tissue shows toxic and allergic effects (138).

Luciana M. Safioti et al conducted a case control study by measuring Ti ion levels in peri-implantitis tissues. Researchers have reported that implants with peri-implantitis have more Ti ions in the surrounding tissues than healthy implants. They suggested that there may be a correlation between the spread of Ti particles to the surrounding tissues as a result of the corrosion of Ti, and peri-implantitis (139).

In the comparative study conducted by Okazaki Y. et al, studied on ion release levels of stainless steel, Co – Cr – Mo casting alloy, cpTi grade 2, Ti – 6Al– 4V, V-free Ti – 6Al – 7Nb and Ti – 15Zr-4Nb-4Ta, they reported Ti alloys with Ta-Zr-Nb

demonstrated reduced Ti ion release and increased cell growth compared to pure Ti grade 2 (140).

There are also studies showing inadequate physical resistance of Ti implants. By means of ion release, fractures or total breakdowns of orthopedic (141) or dental Ti implants (142). It is also a concern for improving the physical properties of Ti biomaterial. Alloys such as Zirconium, Niobium and Boron have been studied for their effect on improving the physical properties of Ti (2,102).

Recently, there are various studies showing the effect of boron in bone formation and regulation (11), wound healing (12), inflammation formation, magnesium absorption (95), estrogen, testosterone and vitamin D metabolism (96) Boron also plays an important role in regulating brain nerve-impulse transition (97).

Boron mineral and its derivatives have antibacterial, antifungal and antiseptic properties (9,10). Sayin Z. et al, studied the antibacterial effect and antibiofilm capacities of boron on staphylococcus aureus, aeromonashydophila, pseudomonas aeruginosa. Researchers monitored the biofilm production by scanning electron microscopy and concluded that boron show protective properties against bacteria and yeast by inhibiting biofilm formation (98).

Gorustovich A. et al. studied the effect of boron deficient diet on periodontal alveolar bone development on mice by determining the effects of boron-deficient diet on bone modelling and remodeling in the buccal and lingual layers of the alveolar bone (143). It has been showed that the activity of osteoblasts decreased in the buccal and lingual layers of alveolar bones, and cellular activities required for bone formation decreased in mice fed on boron deficient diet.

Since boron, as a mineral, is essential on bone regulation, nowadays it has been introduced as one of the Ti alloying elements in many fields.

TiB_2 are transitional metal-metalloid compounds (144). Titanium borides include TiB and its equation state, TiB_2. TiB and TiB_2 does not appear in nature and it can be synthesized by reduction of titanium oxides with boron carbide (145). TiB is not a stable compound and tends to convert into TiB_2, which is considered the most stable compound of titanium borides (144).

TiB_2 is a transitional metal with superior mechanical, chemical and physical properties with high melting point, durability and abrasion resistance. Because of its high corrosion resistance property, it is currently used in areas where high temperature and

pressure changes are experienced. TiB_2 is currently used as coating materials in parts where high corrosion resistance is needed such as in ballistic armors, cutting tools and aerospace applications (145).

In studies with boron it has been observed that boron has beneficial effects for the body, and it has been shown that the TiB material when alloyed with Ti increases the resistance of the Ti material against forces. In the light of these studies in present study it was aimed to test the suitability of TiB_2 material for use in the biomedical field by examining the biological effects of the TiB_2 material alone rather than forming a compound with Ti. The biocompatibility of a material is a parameter that can be determined by various tests. Prior to use metals or materials on living tissues, its toxicity should be evaluated. It is not ethically possible to use materials without knowing the local and systemic effects on human physiology (146). In vitro study model is the first step before conducting in vivo and clinical studies, in order to reduce possible complications seen in living creatures. As far as we know TiB material has been used to increase the physical strength of Ti alloys however no cytotoxicity study has been conducted on produced TiB_2 material. In present study it was aimed to investigate the cytotoxicity of TiB_2 material on osteoblastic cell line by following ISO guideline on 24 h and 48 h in a in vitro study.

Gorsse S. et al. compared the bending and force resistance of specimens obtained by mixing Ti and TiB materials with pure Ti. In the specimens subjected to bending tests they reported that Ti-TiB_2 products had higher strength against bending forces by suggesting that Ti can be reinforced with TiB (147).

F.M.Makau et al formed a Ti-TiB double matrix and investigated the cytotoxicity of this fabricated composite in an in vitro environment. Dual matrix system was created on bulk Ti by using TiB powder and Ti using spark plasma sintering, which created Ti-TiB dual matrix. The researchers reported that no cytotoxic behavior on Ti-TiB dual matrix system, and more study is needed for determining long term biocompatibility of this dual matrix system (5).

Producing bulk TiB_2 plates using powder metallurgy is a difficult process due to its high melting point and the properties of its covalently bonded atomic bonds. Conventional sintering methods for production of bulk TiB_2 is a costly procedure due to long duration and high energy needs (100).

Production methods, while reducing cost of production, can affect physical strength or corrosion resistance of products, causing increased ion release and showing toxic effects to cells in the area of use.

The physical properties of the materials to be used in implant production also affect their biocompatibility. The elasticity modulus, tensile, compressive, shear, yield and fatigue strength, ductility, hardness and toughness are well known (148). Many researchers have studied different aspects of physical properties of Ti and Ti alloyed implants, but corrosion resistance is considered one of the key factors for biocompatibility. For materials with low corrosion resistance, the release of ions can occur, which may have toxic, allergic or mutagenic effect on the body. At the same time metals with low corrosion resistance can cause implant failures (149).

Ti and its alloys are considered to have good corrosion resistance. However, implants produced using Ti and Ti alloys are considered to have poor fretting corrosion and low sliding wear resistance (150). Studies have been conducted in order to improve the wear resistance of Ti and Ti alloys such as use of different production methods or production of other non-toxic alloying elements such as zirconium and boron for production of Ti alloys (7,8)

Pascu C. I. et al studied the physical properties of bulk Ti, produced using Ti powder with spark plasma sintering method. They compared the physical properties of bulk Ti produced by SPS and conventional method. They concluded that using of powder metallurgy with SPS method can reduce the cost of production of Ti based biomaterials without sacrificing any physical features (151).

In vitro study conducted by Kassapidou M. et al investigated the cytotoxic effects of chromium-cobalt (Co-Cr) alloys produced using different production methods. They reported that Co-Cr alloys produced in cast and milled form release more ions into the environment. They observed increased ion release and stated that it might be because of its corrosion resistance being lower than other production methods (152).

Surface modification is considered as an alternative production method for Ti implants. Coating of Ti implants with non-toxic alloying elements has been researched for increasing the wear resistance of Ti implants. Recently, many studies on elements such as zirconium, niobium and boron have carried out, which can form alloys with Ti without compromising its biocompatible properties while increasing hardness of implants (7, 8,13,153).

Hardness effects the wear resistance of the implant material. Increasing the hardness reduces the wear of the material, thus reducing the amount of ion release of the material to the surrounding tissues and limiting its toxic effect on cells (148).

In order to increase the hardness and wear resistance of Ti implants, boron has been the subject of many studies. Researchers, studying the effect of surface coating of Ti and Ti alloys, producing Ti and boron alloyed implants have been observed to increase in hardness and enhancement of wear resistance of produced implants (154,155).

Sivakumar B. et al. studied on different thickness of TiB_2 on the Ti surfaces coated by boron with different temperature (850 °C, 910 °C, 1050 °C) and different time intervals (1, 3, 5 h) effecting the overall hardness and corrosion resistance of produced materials (145). They observed a positive correlation between thickness and temperature and time. When these parameters are increasing, the corrosion resistance of material increased as well.

Turan A. conducted a study on SPS system of TiB_2 material investigated the effect of pressure amount, temperature variables, vacuum and argon (Ar) pressures on the relative density and hardness of the material. He observed that the relative density of the products sintered under Ar atmosphere is higher than the products produced under vacuum atmosphere at same pressure and temperature. It has been concluded that hardness of TiB_2 material increased with increasing pressure and temperature at vacuum atmosphere, while Ar atmosphere was effective on higher temperatures, increasing the hardness of TiB_2 products with increased temperatures (100).

TiB_2 bulk metal produced by using powder metallurgy with SPS method was used in present study. Bulk metals were sintered under two different atmosphere (Argon, Vacuum), three different degrees (1800°C, 1780°C, 1600°C) and two different pressure (50mPa, 70mPa). Cytotoxic evaluations were made on 24 and 48h. V70-TiB_2, exhibited the most toxic effect of produced TiB_2 at 24 and 48 h by means of cell viability. The more amount of extract administered to the cells, the most cell decrease was seen in V70-TiB_2 (0.70±0.23 at 24h and 0.27±0.14 at 48h). Data obtained from present study were suggested that TiB_2 bulk material produced at 70 mPa and 1780°C showed higher toxic effect on cells, suggesting a similar conclusion those of Kassapidou M. et al. It might be explained that material produced from different production methods has different cytotoxicity by means of ion release.

In vitro studies provide researchers an environment in which environmental conditions can be completely under control where the physical and chemical properties of biomaterials can be analyzed for the purpose of the study. All the features of the working environment and biomaterial are under the control of the researcher and can be modified as desired. In vitro studies can be performed with high sample numbers with the advantage of decreased cost and without the need of ethical approval. It is an essential step for biomaterial research before conducting animal experiments and preclinical studies in order to determine the effects on animal cells (156). The biomaterial research conducted in present study followed ISO 10993 guidelines. Depending on ISO 10933 guidelines, no cytotoxicity study was conducted on TiB_2. Since it is mandatory to do in vitro study for determining the effects of TiB_2 on living cells prior to in vivo study, this was conducted on TiB_2 in order to proceed to animal study (157).

In vitro cytotoxicity tests can be done with direct, indirect contact test method or by using the extracts gathered from the desired material but it has been showed that these three cytotoxicity methods can effect the test result of the investigated material. All three different test methods are used in the biomedical field (158).

Reports on spontaneous blindness have been reported after using perfluoro-n-octane (PFO) material. Girish K. Sristava et al studied on cytotoxic effect of PFO material by using direct test method. It has stated that even if cytotoxicity of PFO determined with extraction method by manufacturer was found to be negative, the material was toxic in direct test method and they pointed out that each test method can alter the result of cytotoxicity of material (159).

In present study, both cytotoxic test were used for evaluating cell viability in different physical forms of TiB_2 following ISO guideline recommendations and the bulk and powder forms of TiB_2 was toxic.

Direct test method is an in vitro test method where the studied material is in direct contact with cultured cells. This test method is conducted by observing the morphological changes and studying the effect of studied material on viability of cells. Although direct test method is highly accurate it has some limitations. Biomaterials that can float in liquid or lose their physical properties in liquid cannot be studied using the direct test method (158). For studies on these materials, scaffolds that has positive affect cell morphology and cell viability can be recommended (160)

Chitosan is a natural polysaccharide derived from deacetylation of chitin found in exoskeletons of insects, crustaceans and fungi. It has low solubility properties in neutral and alkaline solutions (161). Chitosan is a material studied in different biomedical areas such as wound healing (70), scaffold and tissue engineering (71), and drug delivery systems (73).

Siew Shee Lim et al, studied the in vitro effects of chitosan scaffolds containing calcium ions on osteoblast adhesion, proliferation and differentiation. They fabricated chitosan scaffolds containing calcium ions via dry freezing method for increased physical integrity. They have suggested that the scaffolds produced had a positive effect on the osteoblastic activities of the cells (160).

Yan Chen et al, compared the effects of chitosan and chitosan/nano hydroxyapatite-collagen scaffold on osteoblastic activity by studying the effects of produced scaffolds on cell viability, cell morphology and the level of alkaline phosphatase enzyme in both. Scaffolds with chitosan containing hydroxyapatite-collagen was superior for facilitating osteoblastic mineralization compared to scaffolds containing chitosan only (162).

Since studies stated that chitosan has negligible minimal negative effect on osteogenic activity, in present study, chitosan was chosen as scaffolding material for TiB_2 powder form by using freeze-drying and lyophilization technique.

ALP is an enzyme expressed in early development of bone and cartilage. In early development of bone, ALP is found on cell surface and cell matrix. Studies on osteoblastic activity ALP enzyme activity is examined for early bone production results. In early bone formation ALP enzyme activity is high while bone formation continues ALP enzyme decreases and the amount of other proteins such as osteocalcin increases (50). The activity of the ALP enzyme has been recognized as defining the amount of early bone formation (163).

The physical form of studied material can also effect the outcome of cytotoxicity tests. In study with Ti and Ti alloying elements; tantalum, niobium, zirconium, molybdenum, tin and silicon, Yuncang Li et al investigated the biocompatibility of powder particles and bulk forms of these elements. They hypothesized that Ti and its alloys, ion emissions of materials differ with different physical forms. They speculated that Ti, silicon and molybdenum materials as powders are cytotoxic. They stated that the bulk Ti did not show cytotoxic properties and had no negative effect on cell proliferation.

It has been concluded that metals in powder form can produce more cytotoxic effects on cells by secreting more ions that their mass forms (143).

In present study our results were similar to the study of Yungcang Li et al. the level of in chitosan scaffolds containing TiB_2 powder, resulted in negative test results. When cell viability of these groups were examined, there was no cell viability was observed in chitosan scaffolds containing TiB_2 powder.

Considering previous studies showing higher ion release increasing cytotoxic effects of Ti, TiB_2 was produced into bulk form of TiB_2 by spark plasma sintering method which is a more stable structure.

Extraction tests, also known as 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide (methyl thiazolyl tetrazolium; MTT) assay, is the most commonly used method for determining materials effect on cell growth rate and cell viability. The working principle of this method can be summarized as follows; as a result of the mitochondrial activities of living cells, mitochondrial dehydrogenase breaks the tetrazole ring in the MTT, causing the MTT to turn from yellow to purple. The medium that turns purple is measured under the absorbance value reflecting the number of living cells (156).

In the study conducted by Baek H.S et al., they investigated the cytotoxic effects of latex gloves on mouse fibroblast (L929) by using the extraction method. They reported that latex gloves extracted with distilled water and saline had more cell viability than serum-free media and cell culture media, observing negative correlation with increased extract amount. The researchers also reported the increase of cell viability with decreased extract amount on cell media (164).

In a similar study conducted by Liu X. et al the effect of different extraction parameters on cytotoxicity test results of magnesium was investigated. They studied the effects of extraction time and volume on cytotoxicity. It has been showed a negative correlation between extract amount on cell media and cell viability, concluding shorter extraction time and smaller extract medium results in less ion release of material with decreased cytotoxic effect of material (165).

In present study the scaffolds containing TiB_2 powder and chitosan showed no cell viability measuring by ALP levels. It was observed that as the rate of TiB_2 in extracts 100%, 50%, 25%, 12,5% increased, its cytotoxicity was increased showing decrease cell viability. After 48 h cell viability was observed to decrease below 70% for all the plates and extracts. As the rate of TiB_2 decreased, the increased cell viability was observed. A

negative correlation between the cell viability and material content were found ($p<0.001$). These results are supporting the results of Liu X. et al.

One of the biggest factors affecting the biocompatibility of Ti and Ti alloys is the titanium dioxide (TiO_2) layer formed on the surface of the Ti material in contact with oxygen (166). This layer formed on the surface helps the adhesion of the cells in the surrounding tissue while preventing the spread of metallic ions to the surrounding tissue (140).

In the interaction of TiB_2 with oxygen at low temperatures the surface forms two layers. Its inner layer is covered with TiO_2 while its outer layer is covered with B_2O_3 (167). In the study conducted by Albuz et al in 2019 it was described that B_2O_3 showed antiproliferative properties on L929 and DLD-1 colorectal adenocarcinoma cells (168).

Another factor affecting the results of cytotoxicity while examining the biocompatibility of newly developed materials is the exposure time of the examined material on the cells. Various researches have reported that there is tendency to increase in cell viability and number with longer exposure time on material with proliferative effect on cells (169,170). On the other hand materials with toxic properties have showed negative effects between cell viability and longer exposure time with the material (171).

Albuz et al, have reported that B_2O_3 has an antiproliferative effect on L929 and DLD-1 cells, inducing apoptotic and necrotic effect. They have stated a positive correlation between the increased apoptotic effect on cells with increased exposure time.

Studies have suggested that there are possibilities for TiB_2 to show the same biocompatibility abilities as pure Ti material if B_2O_3 layer covering the outer layer of TiB_2 can be discharged. When TiB_2 is exposed to temperatures of 1000 degrees and above in an oxygenated environment, the B_2O_3 layer on its surface vaporizes and the TiO_2 layer emerges (167,172)

In present study, cell viability of 100% extracts of Ar-TiB_2, V50-TiB_2 and V70-TiB_2 were found below 70% compared to control. More than 70% cell viability was observed in each extract groups of 50%, 25%and 12,5% at 24 h. Cell viability of 100%, 50%, 25% and 12,5% extracts of Ar-TiB_2, V50-TiB_2 and V70-TiB_2 were found below 70% compared to control at 48 h. As the material content decreased, increased cell viability was observed, a negative correlation was found between cell viability and material content. Our results were similar with the study of Baek H.S et al.and Liu X et

al. It might be explained by ion release which it has antiproliferative effect and time exposure and also consider the B_2O_3 layer on surface.

In addition to method used in cytotoxicity test, it has shown that the cell type can also affect the outcome of the cytotoxicity (170,171).

In the comparative study of Maria Alcaide et al on hydroxyapatite-βTCP/agarose with L929 and SaOs-2, the material had an apoptotic effect on SaOs-2 cell line; while it has no such effect on L929 cell line (173).

Çobankara F.K et al have studied on L-929 and SaOs-2 cells, for determining the cytotoxicity effect of 5 different resin-based sealers used in endodontic treatment. They reported some of the sealers studied showed high cytotoxic effect on L-929 cell line, while showing less toxic effect on SaOs-2 cell line. They stated that, different cells origins used in studies might have an effect on cytotoxicity results (174).

Based on the interaction with the cell type and cytotoxicity we considered to use SaOs-2 cell line which is similar to osteoblast cells with its physiological and biological properties. We aimed to eliminate the effect of cell type on cytotoxicity.

In conclusion, based on ISO 10933 guideline, our results are showed that the cytotoxicity of TiB_2 on 24 h, cell viability being above 70% in all bulk material and only 100% extract of all materials showed cytotoxic effect whereas all extracted material below 70% on 48 h. It might be explained by the presence of B_2O_3 on surface of material which toxic effect on cells was observed in previous studies. Further studies are needed exposed to temperatures after produced bulk material for evaluating the cytotoxicity. We recommend further studies on TiB_2 for implant production after removing the B_2O_3 layer covering the outer layer of TiB_2 bulk metal in terms of surface coating for titanium implants in order to increase their physical properties.

6. CONCLUSION

Powder and bulk forms were used for evaluation of TiB_2 cytotoxicity

- Osteosarcoma cell line (SaOs-2) with normal cell medium was used as control
- Chitosan scaffolds containing 0.0025% and 0.005% TiB_2 powder were produced by freeze drying method in 4 samples each.
- Periodontal ligament stem cells were used for detecting cell viability in the powder form of TiB_2.
- Powdered TiB_2 metal was fabricated by spark plasma sintering method at various temperatures (1600°C, 1780°C, 1800°C) under different atmospheric pressure (50mPA and 70mPA), under vacuum and argon gas atmospheres. Extracts of produce TiB_2 metals were taken and dilated with cell culture media at 100%, 50%, 25% and 12,5%.
- ALP enzyme activity was not observed in chitosan scaffolds containing TiB_2
- Cell viability were measured according to ISO
- The extracts of TiB_2 bulk metal did not show cytotoxic effect in 50%, 25% and 12,5% at 24 h.
- V70-TiB_2 plate was found to be more cytotoxic on 24 h ($p<0.001$) and statistical significant difference were found between 12,5% extracts of Ar-TiB_2 and V50-TiB_2 ($p=0.025$), and between V70-TiB_2 and Ar-TiB_2 ($p=0.034$).
- Statistical significant differences were found between 12,5% extracts of Ar-TiB_2 and V50-TiB_2 ($p=0.025$), and between V70-TiB_2 and Ar-TiB_2 ($p=0.034$).
- %100 dilation of TiB_2 bulk metals showed cytotoxicity at 24 h.
- All extracts of all TiB_2 plates showed cytotoxicity at 48 h.
- Negative correlation was found between cell viability and material content ($p<0.001$)
- Our results were showed that both powder and bulk plate forms of TiB_2 had a cytotoxic effect on cell viability according to ISO guideline, which it is mandatory to achieve cell viability above 70%.
- Data obtained from present study could explained by the ions released from B_2O_3 formed on the surface of bulk TiB_2 metal which it prevents cells maintenance of vitality.

- We recommend further studies on TiB_2 for implant production after removing the B_2O_3 layer covering the outer layer of TiB_2 bulk metal in terms of surface coating for titanium implants in order to increase their physical properties.

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Education

Degree	Department	The name of the Institution Graduated From	Graduation Year
PhD	Oral and Maxillofacial Surgery	**Yeditepe University**	2020
University	Faculty of Dentisty	**Yeditepe University**	2015
High School	-	Üsküdar Fen Lisesi	2010

Languages	Grades
English	88,7500

Work Experience

Position	Institute	Duration (Year-Year)
Dentist	Private Practice	2018-2020
		-

Computer Skills

Program	Level
IBM SPSS Statistics	basic
Microsoft Office	average

Scientific Works

The articles published in the journals indexed by SCI, SSCI, AHCI

Evaluation of Significant Radiographic Findings and Their Impact on the Oral Health-Related Quality of Life of Patients with Complete Dentures, Int J Prosthodont. 2018;31(6):594-600. doi:10.11607/ijp.5717

Articles published in other journals

Proceedings presented in international scientific meetings and published proceedings books

Others (Projects / Certificates / Rewards)
