Test of Biomaterials in Biological Systems

By

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ABSTRACT

Ceramic, metallic, polymeric and composite materials are generally used as biomaterials in order to improve human health. In addition to desired mechanical properties of biomaterials, biocompatibility is important in the treatment or replacement of body parts. Prior to the introduction of new biomaterials to the market, detailed biological tests are carried out to prevent any undesired side effects in the body. Both *in vitro* and *in vivo* tests are applied initially which is followed by the evaluation with clinical trials of the biological safety and performance.

The aim of this study was to examine some biomaterials in biological systems. For this purpose, the effects of ceramic, metallic, polymeric and ceramic composite materials with different chemical and surface properties on the viability of peripheral blood mononuclear cells (PBMC) by trypan blue exclusion method, on the proliferation of PBMC by incorporation of bromodeoxyuridine to DNA in the proliferating cells and the activation of PBMC by MTT test were investigated. Furthermore, the effects of biomaterials on the secretion of proinflammatory cytokines (IL-1α and IL-6) from PBMC were examined by using ELISA kits. The alteration of conductivity and pH in different solutions were determined to elucidate dissolution properties of ceramic pellets. In addition, AMES test (*Salmonella typhimurium* reverse mutation test) for the determination of mutagenic potentials and the agar diffusion method for examination of anti-bacterial effects of biomaterials were applied. Adhesion of pathogenic bacteria to the surface of biomaterials was investigated by staining bacteria and examining under the optical microscope.

Except for HA 800 °C pellets, all samples showed positive results for biocompatibility compared to the controls without biomaterials. The dissolution of HA 800 °C pellets in the culture medium changed the ionic environment that led to a decrease in the viability, proliferation and activation of PBMC. However, BSA-coated HA 800 °C pellets increased the cell viability with respect to uncoated HA 800 °C pellets. Polished metallic samples and other metallic and polymeric samples showed high percent cell viabilities during 48 hours. After 72 hours, most probably because of released ions and particles to the environment, a decline in the viabilities of PBMC was determined. A negative correlation between increasing extract concentration and the cell

viability was observed for all ceramic samples especially after 48 and 72 hours treatments.

Cytokine secretion analysis after treatment of PBMC with biomaterials indicated that HA 800 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C pellets led to a decrease in IL-1α secretion and BSA-coating of HA 1250 °C, alumina 1450 °C and zirconia 1450 °C resulted in higher levels of IL-1α with respect to the control and BSA-coated HA 800 °C pellets in the presence of LPS. Stainless steel, titanium alloy and cirulene pellets caused low levels of IL-1α secretion. In addition, BSA-coated HA 800 °C pellets increased IL-6 secretion compared to uncoated pellets. In metallic samples, low IL-6 levels were obtained with and without LPS stimulation.

Moreover, the proliferation and activation of PBMC in the presence of biomaterials were evaluated. HA 800 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C samples had inhibitory effects on the proliferation of PBMC in the presence of Con A. In the activation of PBMC, HA 800 °C and HA 900 °C samples showed the lowest values at all incubation periods. Moreover, other ceramic samples showed lower cell activation than the control cultures after 48 and 72 hours treatments. In addition, the cell activation was observed at 24 hours after treatment with the ceramic extracts at low concentrations.

Furthermore, investigation of dissolution properties of ceramic samples indicated that only HA 800 °C pellets led to significant increases in the conductivity of cell culture medium and deionized water. Moreover, a slight increase in the pH levels of solutions was obtained in the presence of HA 800 °C samples, but not in the other samples.

Finally, none of the extracts of biomaterials in PBS had mutagenic effect on *Salmonella typhimurium* TA100 strain when they were compared to the mutagenic material (sodium azide) and the negative controls. In addition, all tested ceramic powders, ceramic pellets, metallic and polymeric materials had no anti-bacterial effects on both gram-negative strains (*E. coli, P. aeruginosa, K. pneumonia, Proteus spp*.) and gram-positive strains (*S. aureus* and *S. pyogenes*). In bacterial adhesion studies, it was found that surface roughness and other surface properties play important roles for attachment, adhesion and formation of biofilm by bacteria on the surface of material. As a result, although the ceramic samples sintered at low temperatures resulted in a

decrease in the viability of PBMC, all tested biomaterials showed positive results for *in vitro* biocompatibility evaluation.

ÖZ

Seramik, metalik, polimerik ve kompozit malzemeler genellikle biyomalzeme olarak kullanılmakta ve insan sağlığına önemli katkılarda bulunmaktadır. Biyomalzemelerin istenilen mekanik özellikleri yanı sıra, bu malzemelerin biyouyumluluğu vücut parçalarının tedavisinde ve değiştirilmesine oldukça önemlidir. Yeni biyomalzemeler piyasaya çıkarılmadan önce, vücut içerisinde istenilmeyen bir etkiye neden olmamaları amacıyla detaylı biyolojik testlere tabi tutulmaktadırlar. İlk olarak vücut dışında daha sonra vücut içerisindeki testler yapılmalı, bunu takiben klinik denemelerle malzemenin biyolojik güvenilirliği ve performansı tespit edilmelidir.

Bu çalışmadaki amacımız bazı biyomalzemeleri biyolojik sistemlerde incelemekti. Bu amaçla ilk olarak değişik kimyasal ve yüzey özelliklerine sahip seramik, metalik, polimerik ve seramik kompozit malzemelerin insan periferik kan mononüklear hücrelerinin canlılığı üzerindeki etkileri trypan mavisiyle boyama metodu ile, periferik kan mononüklear hücrelerinin çoğalmaları üzerine etkileri bromodeoksiuridine'nin DNA'ya bağlanmasının tespiti ile ve mononüklear hücrelerin aktivasyonu MTT metoduyla incelenmiştir. Ayrıca, biyomalzemelerin inflamasyonda rol oynayan IL-1α ve IL-6 sitokinlerinin kan hücrelerinden salgılanmasını nasıl etkilediği ELISA kitleri kullanılarak araştırılmıştır. Seramik örneklerin değişik solüsyonlar içindeki çözünürlük özelliklerinin aydınlatılmasında elektriksel iletkenlik ve pH değerlerindeki değişim baz alınmıştır. Test edilen biyomalzemelerin mutasyona neden olup olmadığı AMES testi ile *Salmonella typhimurium* suşu kullanılarak ve anti-bakteriyel etkileri agar difüzyon yöntemi kullanılarak incelenmiştir. Patojen bakterilerin biyomalzeme yüzeylerine adezyonunun tespiti için yüzeydeki bakteriler boyanmış ve optik mikroskop altında incelenmiştir.

HA 800 °C peletleri dışında bütün test edilen malzemeler biyomalzeme içermeyen kontrollerle karşılaştırıldığında pozitif sonuçlar göstermişlerdir. HA 800 °C peletleri hücre besiyeri içerisindeki çözünürlüğü ortamdaki iyon dengelerini etkileyerek mononüklear hücrelerin canlılığında, çoğalmasında ve aktivasyonunda azalmaya neden olmuştur. BSA ile kaplanmış HA 800 °C peletleri kaplanmamış HA 800 °C peletlerine oranla hücre canlılığını arttırmıştır. Parlatılmış metalik örnekler ve diğer metalik örneklerle polimerik örnekler 48 saat inkübasyonda yüksek hücre canlılığı oranları

göstermişlerdir. 72 saatten sonra, büyük olasılıkla ortama salınan iyon ve partiküllerden dolayı hücre canlılığında bir azalma tespit edilmiştir. Özellikle 48 ve 72 saat inkübasyonlardan sonra, artan ekstrakt konsantrasyonu ile azalan oranda hücre canlılığı arasında bir korelasyon gözlemlenmiştir.

Periferik kan mononüklear hücrelerinden sitokin salgılanması analizlerine göre HA 800 °C, HA-Alumina 1250 °C and HA-Zirkonya 1250 °C peletleri IL-1α salgılanmasında azalmaya neden olmuş, diğer yandan LPS ile tetiklenmiş hücrelerde BSA ile kaplanmış HA 1250 °C, alumina 1450 °C ve zirkonya 1450 °C peletleri kontrol kültürlerine ve BSA ile kaplanmış HA 800 °C peletlerine nazaran yüksek oranda IL-1α salgılanmasına yol açmışlardır. Paslanmaz çelik, titanyum alaşımı ve cirulene örneklerinde düşük oranlarda IL-1α tespit edilmiştir. BSA ile kaplanmış HA 800 °C peletleri kaplanmamış HA 800 °C peletlerine oranla daha yüksek miktarda IL-6 salgılanmasına neden olmuşlardır. Metalik örneklerle inkübe edilen hücrelerde (LPS varlığında ve yokluğunda) düşük seviyede IL-6 salgılanması tespit edilmiştir.

Mononüklear hücrelerin biyomalzemelerin varlığında çoğalmaları ve aktivasyonu incelenmiştir. Con A ile tetiklenen mononüklear hücrelerin çoğalması üzerinde HA 800 °C, HA-Alumina 1250 °C ve HA-Zirkonya 1250 °C örneklerinin inhibisyon etkileri olduğu belirlenmiştir. Mononüklear hücrelerin aktivasyonunda HA 800 °C ve HA 900 °C örnekleri bütün inkübasyon sürelerinde en düşük seviyede aktivasyona neden olmuşlardır. Bunun yanı sıra, diğer seramik örnekler de 48 ve 72 saat inkübasyonlarda kontrollerden daha düşük oranda hücre aktivasyonu göstermişlerdir. Sadece 24 saatte ve düşük konsantrasyonlarda seramik ekstraktları hücreleri aktive etmişlerdir.

Çalışmamızda seramik örneklerin çözünürlük özellikleri belirlenmiştir. Sadece HA 800 °C peletleri hücre besiyerinin ve deiyonize suyun elektriksel iletkenliğinde yükselmeye neden olmuştur. Ayrıca, diğer örneklerde görülmemesine rağmen, HA 800 °C peletlerinin varlığında hücre besiyerinin ve deiyonize suyun pH değerlerinde az oranda yükselme tespit edilmiştir.

Biyomalzemelerin PBS içerisindeki ekstraksiyonlarında *Salmonella typhimurium* TA100 suşu üzerinde herhangi bir mutajenik etki mutajen madde (sodyum azid) ve negatif kontrol ile karşılaştırıldığında gözlemlenmemiştir. Ayrıca, seramik, metalik ve polimerik örneklerin hem toz hem de pelet formlarının gram-negatif suşlar

(*E. coli, P. aeruginosa, K. pneumonia, Proteus spp*.) ve gram-pozitif suşlar (*S. aureus* and *S. pyogenes*) üzerinde herhangi bir anti-bakteriyel etkisi bulunmamıştır. Bakteri adezyonu çalışmalarında yüzey pürüzlülüğünün ve diğer yüzey özelliklerinin bakterilerin bağlanması, adezyonu ve biyofilm oluşturmalarında önemli roller oynadığı sonucuna varılmıştır. Sonuç olarak, düşük sıcaklıkta sinterlenmiş örnekler hücre canlılığında azalmaya neden olmasına rağmen bütün test edilen malzemeler vücut dışındaki biyouyumluluk değerlendirmeleri için pozitif sonuçlar vermişlerdir.

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ABBREVIATIONS

HA	: Hydroxyapatite
BrdU	: Bromodeoxyuridine
BSA	: Bovine Serum Albumin
Cirulene	: Ultra High Molecular Weight Polyethylene
Con A	: Concanavalin A
dH_2O	: Deionized Water
DLC	: Diamond-like Carbon
DMSO	: Dimethyl Sulfoxide
ELISA	: Enzyme-Linked Immonusorbent Assay
ePTFE	: Expanded Polytetrafluoroethylene
FBS	: Fetal Bovine Serum
IL	: Interleukin
LPS	: Lipopolysaccharide
PBMC	: Peripheral Blood Mononuclear Cells
PBS	: Phosphate-Buffered Saline
RPMI-1640	: Roswell Park Memorial Institute-1640 Culture Medium
TNF	: Tumour Necrosis Factor

Chapter 1

INTRODUCTION

The use of natural biomaterials dates back to antique times. Hair, cotton, animal sinew, tree bark and leather of animals have been used as natural suture materials for almost 4000 years. Plates made up with gold were used for skull repair in 1000 B.C., and gold-wire sutures as early as 1550 [1]. Today, almost nobody exists without one or more biomaterials in his/her body. Dental filling materials, hip prosthesis, cardiac pacemakers, catheters, kidney dialysers, vascular grafts, eyeglasses and lenses are some examples of biomaterials. Ceramic, metallic, polymeric and composite materials produced by high technology are used in order to improve or treat human health and to increase welfare of the society. One of the important advantages of biomaterials to the society is to increase survival rate through the end of 20^{th} century. In fact, after age of 30, human body starts to deteriote, connective tissues begin to decrease, volume of bones and their strength decrease and the probability of fracture increases [2]. In this point, the importance of biomaterials is inevitable. Not only older members of society but also the young need biomaterials for more effectiveness and welfare in their old ages. Therefore, production of new materials with optimum physical strength and biocompatibility has being the subject of many studies.

Newly synthesized or produced biomaterials have to be tested for their physical, chemical and mechanical properties. Desired biological responses and performance of biomaterials should be examined by *in vitro* and *in vivo* tests first. Afterward, the evaluation has to be concluded with clinical trials. An ideal biomaterial does not cause inflammation, undesired immunological responses, cancer, cytotoxicity and mutagenicity. During implantation of biomaterials, they contact with blood especially mononuclear cells having roles in the immunological responses and inflammation. Therefore, the purpose of this study was to examine the effects of ceramic, metallic and polymeric biomaterials and their extracts with different surface properties on the viability, proliferation, activation of peripheral blood mononuclear cells (PBMC) and the release of inflammatory cytokines IL-1α and IL-6 from PBMC. In addition, the mutagenic potential of extracts of biomaterials, anti-bacterial effects and adhesion of

pathogenic bacteria to the surface of biomaterials were investigated. Finally, the dissolution properties of ceramic pellets especially at low and high sintered temperatures were studied on the basis of conductivity and pH alterations in different solutions.

Chapter 2

BIOMATERIALS AND BIOCOMPATIBILITY

2. 1. Biomaterials

A biomaterial is a non-viable material that interacts with biological systems which is used in the production of a medical device [3]. As a field of study, biomaterials combine different disciplines. As a material science, biomaterials are related with the physical properties, chemical compositions and structures. As an interdisciplinary science, biomaterials are considered the interactions between living and non-living materials. As a medical science, their goal is the improvement of the human health and quality of life [4]. Some of the commonly used biomaterials are explained in Table 2.1. According to biological responses, biomaterials can be classified as biotolerant, e.g. bone cement, stainless steel, and cobalt-chrome alloy; bioinert, e.g. alumina, zirconia, carbon materials and titanium; and bioactive materials, e.g. calcium phosphate ceramics, hydroxyapatite (HA), and glass ceramics. Between a biotolerant material and surrounding bone a connective tissue layer is observed. In fact, tissue encapsulates these inert materials in fibrous tissue. A direct contact between implant material and surrounding bone is observed in bioinert materials and a stable oxide layer on the surface is the main characteristic of bioinert materials. A direct chemical bond is formed in bioactive materials between implant and surrounding bone [5]. Chemical composition and structure of biomaterials is the main reference for the production of medical devices. Ceramics, metals, polymers and composite materials are classified on the basis of their chemical composition.

Table 2.1. Examples of biomaterials applications [6]

Cardiovascular implants	Heart and valves
	Vascular grafts
	Pacemakers
	Stents
Plastic and reconstructive implants	Breast augmentation or reconstruction
	Maxillofacial reconstruction
	Penile implant
Orthopedic prostheses	Knee joint
	Hip joint
	Fracture fixation
Opthalmic systems	Contact lenses
	Intraocular lenses
Dental implants	
Neural implants	Hydrocephalus shunt
	Cochlear implant
Extracorporeal	Oxygenators
	Dialyzers
	Plasmapheresis
Catheters	
Devices for controlled drug delivery	Coatings for tablets or capsules
	Transdermal systems
	Microcapsules
	Implants
General surgery	Sutures
	Staples
	Adhesives
	Blood substitutes
Diagnostics	Fiber optics for endoscopy

2. 1. 1. Ceramics

Specially designed ceramic materials, in other words, bioceramics have being used for the repair, reconstruction, and replacement of diseased or damaged parts of the body since 1960s. Polycrystalline (Alumina or HA), bioactive glass, bioactive glass-ceramic or bioactive composite (Polyethylene-HA) are the main examples of bioceramics. Principal characteristics of bio-inert ceramics are wear resistance, minimal biological response, stiffness, strength and toughness especially in total joint replacements. Bioactive ceramics have porous and crystalline structures and they are also biocompatible. However, ceramic materials are brittle, difficult to produce and they have no resilience properties. In fact, low tensile strength and fracture toughness limit the usage of bioactive ceramics [7].

The ingrowth of tissues into pores of ceramic materials on the surface or throughout the material takes place. The increased interfacial area between the implant and surrounding tissue causes an increase in the resistance to the movement of device [5]. The growth of tissue into pores of ceramic material is termed “biological fixation”. For the viability of tissues in the pores, the size of pores must be >100-150 μm in diameter. This large interfacial area is essential for blood supply of connective tissue. In the pores with <100 μm in diameter, no vascular tissue was observed. In some cases micromovement takes place at the interface of a porous implant that results in damage to the tissue. The tissue dies, inflammation ensues and the interfacial stability can be destroyed. Types of bioceramic tissue attachment mechanisms are explained in Table 2.2.

Surface activity of bioactive ceramics especially bioactive glasses and HA is responsible for the formation of the interfacial bond. The formation of a hydroxycarbonate apatite (HCA) surface layer is another characteristics of bioactive ceramics. The first stage in surface reactivity of bioactive glasses is the loss of sodium ions from the surface of the glass via ion exchange with hydrogen (H^+ or H_3O^+). The reaction takes place rapidly, within minutes of material exposure to body fluids. A dealkalinization of the surface layer with a negative charge is created. The removal of sodium ions through aqueous environment results in a localized breakdown of the silica network with the resultant formation of silanol ($Si(OH)_4$) groups. After that, repolymerization into silica-rich surface layer occurs. This surface layer is highly porous with average pore diameter of 30-50 Å.

Table 2.2. Types of bioceramic tissue attachments [5]

Type of attachment	Type of bioceramic
Dense, nonporous, almost inert ceramics attach by bone growth into surface irregularities by cementing the device into the tissue, or by press-fitting into a defect (morphological fixation).	Al_2O_3 ZrO_2
For porous implants, bone ingrowth occurs, which mechanically attaches the bone to the material (biological fixation).	Porous HA HA-coated porous metals
Surface reactive ceramics, glasses, and glass-ceramics attach directly by chemical bonding with the bone (bioactive fixation).	Bioactive glasses Bioactive glass-ceramics Dense HA
Resorbable ceramics and glasses in bulk or powder form designed to be slowly replaced by bone.	Calcium sulfate Tricalcium phosphate Calcium phosphate salts Bioactive glasses

It was proposed that the loss of soluble silica from the surface of bioactive glasses might be at least partially responsible for stimulating the proliferation of bone-forming cells on the glass surface. After the formation of the silica-rich surface layer, an amorphous calcium phosphate layer is formed on the glass surface and incorporation of biological molecules, for example, blood proteins, growth factors and collagen takes place. Adsorption of biological molecules occurs beginning from the surface reactions. Within 3 to 6 hours *in vitro*, calcium phosphate layer crystallize into hydroxycarbonate apatite layer that is the bonding layer. This surface, which is structurally and chemically similar to bone mineral and tissues in the body can attach to this layer directly. The reactivity goes on with time and HCA layer grows in thickness for the formation of a bonding zone with approximately 100-150 μm. This layer is mechanically compliant interface to maintain the bioactive bonding of the implant material to natural tissue. These surface reactions take place within the first 12 to 24 hours of implantation. Osteogenic cells, such as osteoblasts or mesenchymal stem cells, infiltrate a bony defect in 24-72 hours. Cells encounter a bonelike surface that is not foreign for cells. At the end of these sequential reactions, a direct bond of the material to tissue was produced. Bioactive glasses minimize the actions of macrophages and inflammatory responses [8].

In addition to biological response of bioceramics, mechanical and physical properties are crucial for their performance in the body (Table 2.3). Bioceramics have been used in non-load bearing parts of the body. In the restoration of middle ear, HA in

a combination of porous and dense forms has been used successfully. Moreover, HA has been applied onto mechanically stronger materials as a coating. HA coating was carried out on porous stainless steel as a tooth replacement. HA coating allows bone to be incorporated within the porous metal [9].

Table 2.3. Physical and mechanical properties of bioceramics [5,7]

Material	Porosity (%)	Density (mg/m^3)	Young's Modulus (GPa)	Compressive Strength (MPa)	Tensile Strength (MPa)	Flexural Strength (MPa)
Bioactive ceramics and glass ceramics	-	-	-	-	56-83	-
	-	2.8	-	500	-	100-150
	31-76	0.65-1.86	2.2-21.8	-	-	4-35
Hydroxyapatite	0.1-3	3.05-3.15	7-13	350-450	38-48	100-120
	10	2.7	-	-	-	-
	30	-	-	120-170	-	-
	40	-	-	60-120	-	15-35
	2.8-19.4	2.55-3.07	44-48	310-510	-	60-115
	2.5-26.5	-	55-110	≤ 800	-	50-115
Tetracalcium-phosphate	"Dense"	3.1	-	120-200	-	-
Tricalcium-phosphate	"Dense"	3.14	-	120	-	-
		-	-	7-21	5	-
Al_2O_3	0	3.93-3.95	380-400	4000-5000	350	400-560
	25	2.8-3.0	150	500	-	70
	35	-	-	200	-	55
	50-75	-	-	80	-	6-11.4
ZrO_2, stabilized	0	4.9-5.56	150-190	1750	-	150-700
	1.5	5.75	210-240	-	-	280-450
	5	-	150-200	-	-	50-500
	28	3.9-4.1	-	< 400	-	50-65
Cortical bone	-	1.6-2.1 (g/cm^3)	7-30	100-230		
Cancellous bone	-	-	0.05-0.5	2-12	-	-

2. 1. 2. Metals

Metallic implants possess the property of being able to endure tensile stresses. Metals are mainly used in load bearing parts of the body. Typical examples of highly loaded implants are hip and knee endoprostheses, plates, screws, nails and dental implants. In addition, metallic biomaterials are applied to unloaded and functional devices such as cages for pumps, valves and heart pacemakers, conducting wires, etc.[10]. Generally, metals are characterized with toughness and ductility (Table 2.4). On the other hand, the main drawbacks of these materials are corrosion and density. The corrosion process of a metal implant produces a flow of electrons and ions through the surrounding tissue. This flow may affect the physiological ion movement of the nerve cells. An inorganic reaction of corrosion products is caused by the solution of metal ions in the body fluid. These products are transported into various organs where they are concentrated and lead to systemic or hypersensitive responses if the limit of toxicity for a certain metal is exceeded [10].

Table 2.4. Mechanical properties of metallic biomaterials [11]

Metal Alloys	Modulus (GPa)	Tensile Strength (MPa)
Stainless steel	190	586
Co-Cr alloy	210	1085
Titanium alloy	116	965
Amalgam	30	58

The most commonly used metallic biomaterials in orthopedics are stainless steel, cobalt based “super-alloys”, titanium and titanium based alloy systems. Stainless steel (20% chromium, 17% nickel) mostly 316L has appropriate mechanical properties for the production of medical devices. Cobalt-chrome alloys (30% chromium, 70% cobalt) are the most corrosion and fatigue resistant implant alloys. Commercially pure (c.p.) titanium is characterized with high corrosion resistance and biocompatibility. Elastic modulus of titanium is closer to cortical bone as compared to other metallic implant materials. Titanium alloy (Ti-6Al-4V) has superior mechanical properties with the same corrosion resistance and elastic modulus of c.p. titanium [12]. Titanium is rapidly oxidized when exposed to oxygen and this oxide layer minimizes the corrosion of

titanium and allows for the attachment of biological molecules in physiological environments. Serum proteins can adsorb to the surface oxide and produce a protein layer. Some of these proteins serve as ligands for the receptors of cell membrane. Appropriate interactions of ligand-receptor complexes lead to signals for intracellular chemical reactions and consequently cell functions such as adhesion and differentiation of cells on the material surface [13]. In addition to protein adsorption, significant changes take place on the surface of material. Oxidation of metallic samples was described both *in vivo* and *in vitro* systems [14,15]. Although metallic materials have a stable oxide layer, they undergo electrochemical changes in the physiological environments. Depending on the type of sterilization, c.p. titanium implants have an oxide thickness of 2-6 nm before implantation [16]. Films on implants removed from human tissues are 2-3 times thicker [14,16,17]. Moreover, the chemical composition of oxide layer changes by incorporating Ca, P, and S [16,17].

Metallic materials are coated with films and other materials to increase their biocompatibility. Diamond-like carbon (DLC) coatings did not result in toxicity toward living cells, inflammatory response or loss of cell integrity and any cellular damage [18]. Ceramic materials especially HA are suitable porous coating materials on the surface of metals for tissue ingrowth [12,19]. Furthermore, the molecules with known biological activity such as alkaline phosphatase or albumin were covalently linked to oxidized titanium surfaces to improve integration of implants to the host tissues [20].

2. 1. 3. Polymers

Polymers are used mainly in tissue engineering, implantation of medical devices, production of artificial organs and prostheses, ophthalmology, dentistry, repair of bones and drug delivery systems. Polymethylmethacrylate (PMMA), polyethylene (PE), polypropylene (PP), polytetrafluoroethylene (PTFE) and Teflon are some examples of polymers used in the medical applications (Figure 2.1). The main advantages of polymers are elasticity and easiness in the production. Whereas, mechanical properties and some deformation-degradation problems have been observed in the medical applications. The mechanical properties of commonly used polymers in the medical applications are described in Table 2.5.

Polymeric materials must be biocompatible at least on the surface. Many polymeric systems used as biomaterials were rejected by the body and were isolated

after implantation with collagenous encapsulation. However, due to encapsulation of biofilm generated on the surface, materials do not induce any harmful effects. Moreover, a thrombus formation is seen when polymers contact with blood cells. In this respect, polymers with non-thrombogenic blood compatible surfaces are used to interact directly with blood stream [21]. Expanded polytetrafluoroethylene (ePTFE) or woven Dacron used as vascular grafts inert to white blood cell activation and thrombogenicity [22]. Functional groups on surface are responsible for biocompatibility, biodegradation and other therapeutic actions.

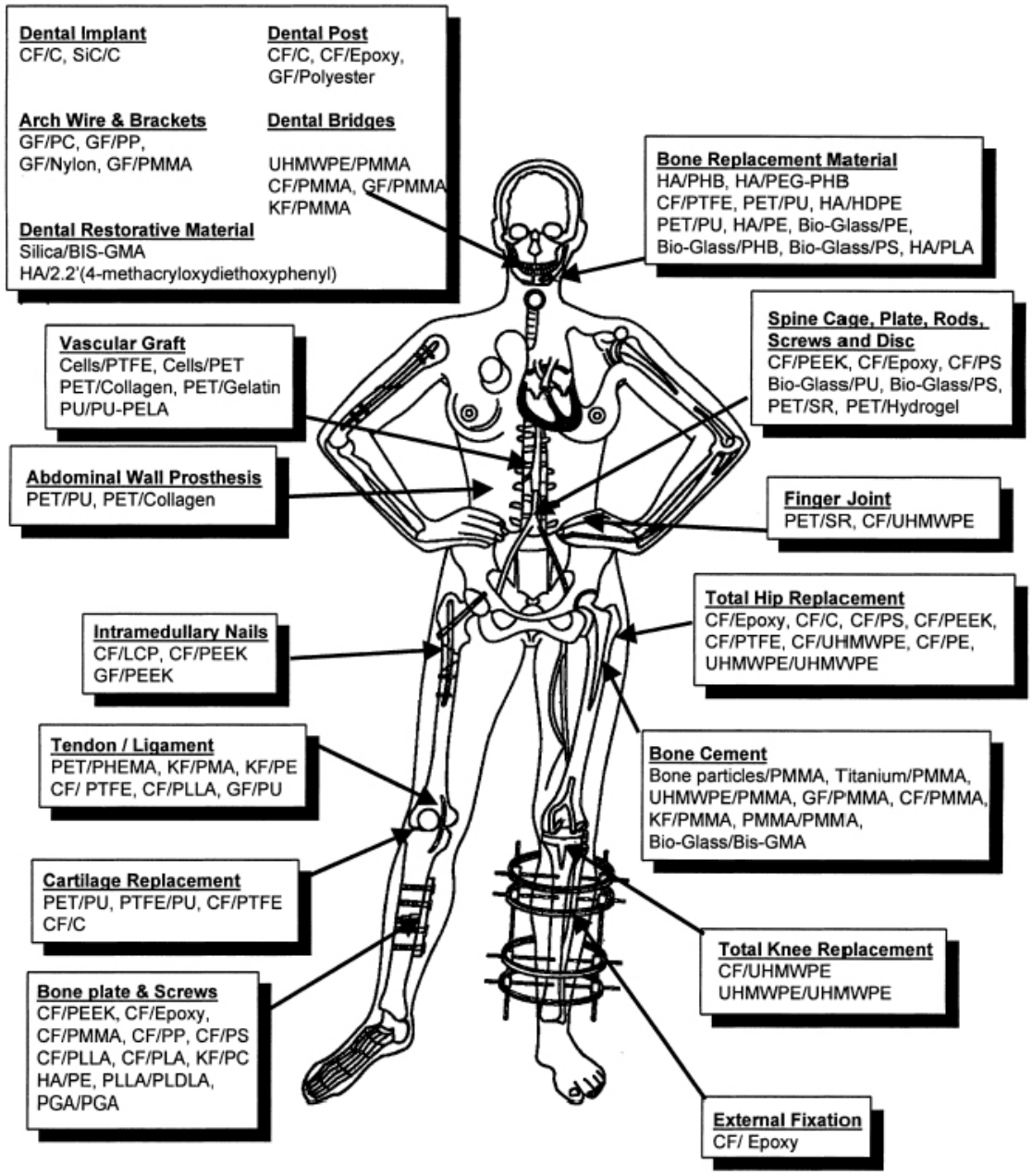
Table 2.5. Mechanical properties of polymeric biomaterials [11]

Material	Modulus (GPa)	Tensile Strength (MPa)
Polyethylene (PE)	0.88	35
Polyurethane (PU)	0.02	35
Polytetrafluoroethylene (PTFE)	0.5	27.5
Polyacetal (PA)	2.1	67
Polymethylmethacrylate (PMMA)	2.55	59
Polyethylene terepthalate (PET)	2.85	61
Polyetheretherketone (PEEK)	8.3	139
Silicone rubber (SR)	0.008	7.6
Polysulfone (PS)	2.65	75

Biodegradable polymers also called bioerodible or bioresorbable are produced from synthetic or natural origins. As a result of hydrolysis, the body eliminates non-toxic alcohols, acids and other low molecular weight products. High molecular weight polymers with hydrolytically unstable crosslinks may be degraded through crosslinked chains. Water insoluble polymers may be converted into water soluble ones because of ionization, protonation or hydrolysis of side chains. These events do not affect the molecular weight of materials, but in the topical applications they may be responsible for bioerosion [21].

Implantation of a non-biological material leads to an interaction with proteins in the body fluids. Mainly, adsorption of proteins to polymer surfaces is responsible for cell-biomaterial interactions [23]. Coating of polymers with albumin, high-density

lipoprotein and immunoglobulin G inhibited the adhesion of endothelial cells. On the contrary, fibronectin coating onto polyethyleneterephthalate, fluoroethylenepropylene copolymer and Teflon promoted the cell adhesion [24].



CF: carbon fibers, **C**: carbon, **GF**: glass fibers, **KF**: kevlar fibers, **PMMA**: Polymethylmethacrylate, **PS**: polysulfone, **PP**: Polypropylene, **UHMWPE**: ultra-high-molecular weight polyethylene, **PLDLA**: poly(L-DL-lactide), **PLLA**: poly (L-lactic acid), **PGA**: polglycolic acid, **PC**: polycarbonate, **PEEK**: polyetheretherketone; **HA**: hydroxyapatite, **PMA**: polymethylacrylate, **BIS-GMA**: bis-phenol A glycidyl methacrylate, **PU**: polyurethane, **PTFE**: polytetrafluoroethylene, **PET**: polyethyleneterephthalate, **PEA**: poltethylacrylate, **SR**: silicone rubber, **PELA**: Block co-polymer of lactic acid and polyethylene glycol, **LCP**: liquid crystalline polymer, **PHB**: polyhydroxybutyrate, **PEG**: polyethyleneglycol, **PHEMA**: poly(20hydroxyethyl methacrylate)

Figure 2.1. Various applications of polymer composite biomaterials in the human body [11].

2. 1. 4. Composites

Composite materials are made from two or more materials to obtain stronger, tougher, stiffer and long lasting materials. Mechanical properties of composite materials depend on the properties of both phases (matrix and strengthening phase or phases) as well as on the nature of phase interfaces, their volume fractions, and the local and global arrangement of the reinforcing phase(s) [25]. For example, ceramic-polymer composites (HA-Polyethylene) are used for the production of bone analogue rather than in load bearing bone implants. The main drawback of these materials is the difficulty in production. Composites are mostly used in dentistry.

2. 2. Tissue-Biomaterial Interactions

The tissue-biomaterial interface is established on implantation process that lead to contact of implant materials with blood. These initial events are taken place by the adsorption of proteins from the blood onto the surface of materials. At least three different driving forces play a role in the adsorption of proteins onto polymer surfaces according to previous work. Firstly, thermodynamically either enthalpy or entropy changes may be sufficient to provide negative free energy change for protein adsorption under physiological conditions. Secondly, the ambivalent polar/non-polar characteristics of proteins favour a concentration of proteins at interfaces; and thirdly, adsorption of proteins increases as the solubility decreases [26]. The nature of implanted material or device, including surface chemistry, surface morphology, net charge, porosity and degradation rate are important for tissue-biomaterial interactions [27]. Adsorption of proteins and other biologically active molecules onto implant materials with appropriate interactions is followed by attachment and proliferation of cells. In the case of bone implants, following the implantation a sequence of events take place including the development of vascularized granulation tissue and collagenous materials (fibrous callus), deposition of non-mineralized bone matrix, deposition of mineralized bone around bone cells and matrix (bony callus) and, finally, remodeling of woven bone to lamellar bone (Figure 2.2). Tissue ingrowth of mesenchymal cells (fibroblasts, endothelial cells and bone cells) occur through the implanted material which are especially porous material implants and bone cements [28].

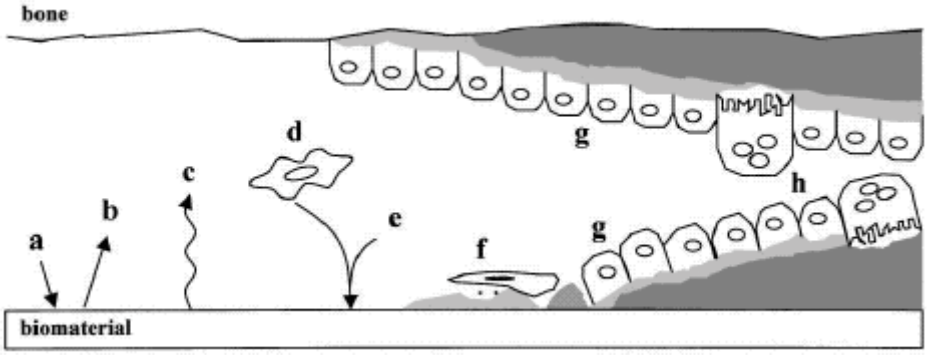


Figure 2.2. Sequential events in the bone-implant interface. (a) Protein adsorption from blood and tissue fluids, (b) protein desorption, (c) surface changes and material release, (d) movement of inflammatory and connective tissue cells through the implant, (e) release of matrix proteins and selected adsorption of proteins such as noncollagenous proteins (bone sialoprotein and osteopontin), (f) formation of *lamina limitans* and adhesion of osteogenic cells, (g) bone deposition on both the exposed bone and the implant surfaces, (h) remodelling of newly formed bone [29].

2. 3. Biocompatibility

Biocompatibility can be defined as "the ability of a material to perform with an appropriate host response in a specific application" [30]. Tissue compatibility and blood compatibility constitute biocompatibility of a test material. Ideally, a blood compatible material does not lead to thrombosis (formation of a solid mass from the molecular and cellular constituent of blood), destruction of cellular components, alteration of plasma proteins and enzymes. Tissue compatible material does not cause undesired immune responses, inflammation, mutation, cancer, and toxic reactions in the body [31].

2. 3. 1. The Inflammatory Responses

Most of the biomaterials are believed that they are inert, non-toxic and non-immunogenic. However, most of implants prompt acute inflammatory responses in the host and the main characteristic of this response is the accumulation of phagocytes on

implant surfaces. Pain, redness, heat and swelling at the site of infection or physical injury are the major features of inflammatory response. The magnitude of these effects depends on the intensity and extent of inflammatory process. The presence of an implant does not lead to additional signs or symptoms, but may alter their severity and duration [32]. The size, shape, chemical and physical properties of biomaterials may be responsible for severity and duration of inflammation and wound healing processes. In the inflammatory response, predominant cell types depend on the age of injury, i.e., the time since the implant was inserted. During the first several days following the implantation, neutrophils are predominant cell type of acute inflammation. Then, neutrophils are replaced by monocytes as the predominant cell type. According to the extent of injury, acute inflammation occurs in minutes to days. Acute inflammation is characterized with the exudation of fluid and plasma proteins (edema) and the immigration of leukocytes (predominantly neutrophils) [33]. Localization of leukocytes at the site of implant material causes phagocytosis, release of enzymes and reactive oxygen intermediates and other agents by the activation of neutrophils and macrophages. Phagocytosis takes place when the cell membrane interacts with a foreign material, which is either inorganic or organic. In fact, chemical composition, local pH differences and electrochemical factors associated with foreign material affect attraction of neutrophils. The released hydrogen ions, enzymes, reactive oxygen intermediates and other agents affect the biodegradation of biomaterials [34,35]. Ions or particles such as cobalt, chromium and nickel are released from metallic alloys into tissue around the implant. These particles can activate neutrophils and release of lysozyme from these cells. Furthermore, if implant material is toxic for tissue, it leads to death and lysis of cells. Recent studies showed that ceramic powders, such as HA, stimulate neutrophils to secrete lysosomal enzymes that digest proteins on the surface of ceramic powders. In addition, stimulated neutrophils phagocytose the ceramic powders *in vivo* [36]. Frequently, acute inflammatory responses are followed by chronic inflammation. Furthermore, fibrosis around implants is observed. Chronic inflammation causes the degradation and failure of many types of implants, such as pacemaker leads, mammary prosthesis and joint implants. In fact, accumulation of host proteins and adsorption of fibrinogen play important roles in the induction of inflammation [37].

2. 3. 2. The Immunological Responses

A biomaterial is a foreign substance to the body; therefore, it is not surprising that it may cause the production of immune cells, mediator molecules and proteins in the biomaterial applications. Inflammatory responses are the first line of defence mechanism. The second line of defence possesses both specificity and memory that is called the immunological response. Only certain types of materials result in an immune response and extent of this response depends on the nature of materials. The memory is developed when the materials are first applied. This recognition pattern is brought into play when the tissues are exposed a second or subsequent times. Most of biomaterials are non-antigenic. However, biomaterials may activate the immune responses due to binding of products with some appropriate carrier molecules in the tissues. The humoral response of the body against biomaterials occurs via production of antibodies to bind and inactivate foreign bodies and antigens. In the cell-mediated response, especially lymphocytes are able to recognize invaders with receptors to kill or inactivate in the body [26].

Complement activation system containing 13 serum proteins is a part of the immune response and plays an important role in acute inflammation. Mainly C3a, C5a and C3b proteins of complement system result in alterations in vascular permeability, histamine release from mast cells, activation of leukocytes and phagocytosis of pathogens. C3a and C5a activate monocytes and macrophages to secrete interleukin-1 (IL-1) that has an ability to stimulate the proliferation of fibroblasts and pyrogenic activity [36]. In rats, inflammation can be observed due to activation of complement after intramuscular implantation of cobalt and nickel discs [38]. Ceramic powders such as $CaHPO_4$, $Ca_3(PO_4)_2$ and coral can trigger complement system due to resorption of ceramic implants. The interaction of blood and biomaterial involves adsorption of plasma proteins that mediates cellular adhesion. Surface of macrophages has receptor molecules for C3b. These receptors lead to the cells to adhere to other cells or biomaterial surfaces bearing C3b. Adhesion of phagocytic cells on the surface of biomaterials causes release of degradative enzymes and high-energy oxygen species. Phagocytosis of both HA and β-whitlockite by macrophages results in resorption of ceramic biomaterials [36].

2. 3. 3. Tumour Induction by Biomaterials

Tumours are the result of excessive and uncontrolled proliferation of cells. Benign tumours are localized to their site of origin. On the other hand, malignant tumours are able to spread other organs and tissues via the body fluids. In addition to the chemical composition of biomaterials, physical factors play roles in the formation of tumours. Tumour induction properties of biomaterials were tested in the laboratory animals. Examples of polymeric, metallic, ceramic and mineral materials have been shown to be carcinogenic under some conditions in the experimental animals, especially rodents [39]. The size of implant material is important for the tumour formation in the case of polymeric implants. Large monolithic materials tend to be most effective agents, whereas small particles are generally less active tumourigenic agents. However, in metallic implants, small particles with enhanced surface area are able to act as a source of ions. In fact, metal ions such as nickel are known to be potential chemical carcinogens; therefore, chemical composition dominates particle size effects in metals [26].

2. 3. 4. Cytokine Secretion From Cells

Cytokines are soluble proteins that regulate proliferation, differentiation and functions of many kinds of cells. Cytokine producing cells and target cells form a complex cellular network and signalling within the immune system. Low concentrations (nano or picomolar) of cytokines act very effective. Growth factors, colony stimulating factors, interleukins, lymphokines, monokines and interferons are described as cytokines. In the inflammatory responses of body, cytokines such as IL-1, IL-6 and TNF-α play important roles.

2. 3. 4. 1. Interleukin-1α

IL-1 consists of IL-1α and IL-1β types. The cells of healthy individuals do not secrete IL-1 with the exception of skin keratinocytes, some epithelial cells and certain cells of the central nervous system. In the stimulus of inflammatory agents, infections and microbial endotoxins, an increase in the production of IL-1 by macrophages and other cell types has been observed [40,41]. IL-1 plays important roles in the immune functions. IL-1 has effects on macrophages/monocytes, T lymphocytes, B lymphocytes, and Natural Killer Cells. IL-1 activates secretion of other cytokines, e.g. IL-6 and

Tumor Necrosis Factor (TNF) [42,43]. Moreover, induction of fever and increasing the levels of C Reactive Protein expression, production of inflammatory responses and prostaglandin are some biological responses of IL-1.

Secretion of IL-1 in the presence of some biomedical polymers (polyethylene, silica-free polydimethylsiloxane (PDMS), woven Dacron fabric, ePTFE and segmented polyurethane (Biomer)) was examined. Statistically significant differences were obtained between tested polymeric materials. High secretions of IL-1 from monocytes in Dacron and PE, intermediate secretion in ePTFE and low secretions in Biomer and PDMS were obtained [44].

2. 3. 4. 2. Interleukin-6

IL-6, a multifunctional protein, produced by lymphoid and non-lymphoid cells, normal and transformed cells, including T cells, monocytes/macrophages, fibroblasts, hepatocytes, vascular endothelial cells, cardiac myxomas, bladder cell carcinomas, myelomas, astrogliomas and glioblastomas. Secretion of IL-6 from these cells are regulated either positively or negatively by mitogens, antigenic stimulation, lipopolysaccharides (LPS), IL-1, TNF, platelet derived growth factor and viruses [45-47]. IL-6 has several biological functions on the cells. For example, it stimulates differentiation and secretion of antibodies on B cells [48-52]; IL-2 production and IL-2 receptor expression on T cells. Furthermore, IL-6 shows a growth factor activity for mature thymic or peripheral T-cells and stimulates differentiation of cytotoxic T-cells in the presence of IL-2 or interferon-γ [53-55]. In addition to described roles of IL-6, it has a major role in the mediation of inflammatory and immune responses initiated by an infection or injury.

Proinflammatory effects of some metals have been reported. For example, test specimens fabricated from copper, nickel, zinc, cobalt, palladium, tin, indium, a high noble cast alloy and a dental ceramic gave rise to significant increase in IL-6 levels in fibroblast-keratinocyte cultures [56]. Woven Dacron used as a vascular graft induced significantly higher levels of IL-6 and TNF-α from white blood cells compared to another vascular graft material, ePTFE [57].

2. 3. 5. Blood Compatibility

The evaluation of blood compatibility has been gained importance by the widespread use of cardiovascular devices and grafts. Catheters, cannulae, guide wires, stents, shunts, heart valves, heart and ventricular assist devices, oxygenators, and dialysers are commonly used devices. The variables influencing blood-biomaterial interactions are explained in Table 2.6. Some events can lead to serious clinical complications in the body. These complications include: (1) thrombosis, (2) thromboembolism, (3) consumption (ongoing destruction) or activation of circulating blood elements, and (4) activation of inflammatory and immunologic pathways. Thrombus forms as the localized accumulation of blood elements on, within, associated with a vascular device. Formation of thrombus results in device dysfunction or blood vessel occlusion. Interruption of normal blood flow may cause ischemia (relative lack of oxygen) and infarction (tissue death due to total oxygen deprivation) in distal circulatory beds leading to heart attacks and strokes. Blood clots are relatively homogenous containing red blood cells and platelets trapped in a mesh of polymerised protein (fibrin). However, thrombus composed of layers of fibrin and platelets can be formed under arterial flow conditions and high fluid shear rates. Thromboembolism is the blocking vessel by a thrombus in the blood flow. Thromboembolism is the common cause of stroke (cerebrovascular infarction) and peripheral limb ischemia [58]. When an artificial surface interacts with blood, it causes platelet adhesion and aggregation in the body. Adsorption of gamma globulin and fibrinogen on the surface of materials increase platelet adhesion, but this adhesion is reduced by albumin. Incomplete heterosaccharides of these proteins and glycosyl transferases on the platelet membrane constitute the adsorption mechanism on the surface. In fact, the absence of such saccharide chains in albumin structure leads to its inhibitory effect [31].

Table 2.6. Variables influencing blood-biomaterial interactions [58]

Device Properties	Size and shape
	Surface composition
	Texture and roughness
	Mechanical properties
Blood Flow Phenomena	Shear factors
	Convection and diffusion of reactants, products, cofactors and inhibitors
	Disturbed flow and turbulence
Blood-Chemistry Related Effects	Coagulation status
	Antithrombotic and other therapies
	Contrast media
Other Variables	Duration of device blood exposure
	Tissue injury
	Infection

2. 4. *In vitro* Biocompatibility Tests

In vitro biocompatibility tests were developed to simulate and anticipate biological reactions to implant materials in the host when operated into or on tissues [59]. Some of the *in vitro* tests are described below.

2. 4. 1. The Viability of Cells

The viability of cells is a crucial parameter for biocompatibility of materials that is determined by staining cells with neutral red, trypan blue, amido black or propidium iodide (PI) after incubation of cells with test materials in the optimum cell culture conditions. The lysosomes of viable cells engulf neutral red and these cells stained red. Cellular proteins within non-viable cells take up trypan blue and dead cells appear as blue under light microscope. Live and dead cells are counted and proportion of the cells gives the percent cell viability. In another method, after fixation of cells, amido black weakly binds to cell proteins and dye can be extracted from cells by alkaline treatment. By measuring the optical density of released neutral red, trypan blue or amido black by a spectrophotometer, a reliable index of the number of viable cells in the sample is

obtained. In addition to vital dyes, PI stains the necrotic cells with destructed cell membranes by toxic materials. PI intercalates with double stranded DNA and/or RNA in these cells and the proportion of dead cells can be quantified by flow cytometric analysis [60]. The effects of alkyl-2-cyanoacrylate esters which are used as adhesive materials with good mechanical properties on L 929 mouse fibroblasts were examined by neutral red and trypan blue staining. The tested cyanoacrylate adhesive materials were found to be cytotoxic and to inhibit cell proliferation [61].

2. 4. 2. The Cytotoxicity of Materials

Cytotoxicity is the evaluation of toxic effects of materials using cell culture *in vitro*. Cytotoxicity assays measure only limited effects on cells during the first 12-24 hours after being exposed to toxic substances. The host cells either recover from or accept their chemical injury. However, many biological reactions are not simply as *in vitro* cytotoxicity [59]. Toxic or undesired effects such as inflammation and immune reactions can be seen in long period of time. *In vitro* cytotoxicity tests are used to determine the response of cells in the culture by direct contact with devices or with their extracts in DMSO, physiological saline or cell culture medium. The toxicity is assayed by loss of cell viability. MTT assay was used for the quantitation of lymphokines, cell mediated cytotoxicity, cell chemosensitivity testing of anti-tumour agents and generally for cell growth and activation. Recently, the assay was applied for the determination of cytotoxicity of biomaterials in *in vitro* tissue culture systems. MTT assay is a rapid and sensitive colorimetric assay. The assay is an alternative method to the conventional ^{3}H-thymidine uptake and other assays for measurement of cell viability and proliferation. MTT assay is based on the capacity of the mitochondrial dehydrogenase enzymes to convert a yellow-water soluble tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a purple insoluble formazan product by a reduction reaction. The insoluble crystals are dissolved in DMSO or acidic isopropanol for the measurement of absorbance values in a spectrophotometer. Active mitochondrial dehydrogenases of living cells can cause conversion of the dye. Dead cells do not lead to this change [62-65]. The biocompatibility of cast non-alloyed titanium and castable alloys were examined by using human primary oral fibroblasts. All tested metallic materials showed cytotoxic properties on primary cultures on the basis of MTT assay [66].

2. 4. 3. The Mutagenicity of Materials

The mutagenicity tests show the ability of a material to induce mutation or chromosomal damage and tumour formation. Mutation is inheritable genetic change that occurs either in genes or in chromosome number or structure. In *in vitro* mutagenicity tests, prokaryotic organisms are mostly used due to their simple genetic structure [67]. AMES test is a sensitive, rapid and accurate method for the identification of mutagens. The test is based on histidine dependence and mutations in *Salmonella typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535. The Salmonella reverse mutation test is carried out by mixing test sample with *Salmonella* strains in a soft agar solution containing only small amounts of histidine that permits the bacteria to undergo a limited number of divisions, but it is not sufficient for normal growth of the bacteria. If the strain undergoes a reverse mutation in the histidine gene induced by test substance or material, the bacteria do not require histidine anymore to grow and they can be observed as visible colonies. In addition, a spot test is performed by spotting test substance on a plate containing the bacteria with a small amount of histidine. Mutagenicity of test substance is determined by the inhibition of bacterial growth on the plate that is the result of diffusion of test substance through agar. The tests are carried out both with and without S-9 activation, which is used to simulate mammalian liver enzyme systems. The purpose is to detect substances undergoing metabolic activation from non-mutagenic forms [68]. Moreover, detection of mutagens on the basis of specific mutations in tryptophan operon is carried out with WP2 strain of *E. coli* [67].

In addition to AMES and *E. coli* tests, there are other mutagenicity tests. One of them is mouse lymphoma test. In this test procedure, cells containing the enzyme thymidine kinase (TK) are susceptible to a toxic effect of trifluorothymidine (TFT), which is metabolized in a false metabolite incorporated in DNA and RNA. The cells with mutated TK gene cannot form the false metabolite. These cells can survive in the presence of TFT [67]. The other mutagenicity test is quantitative mammalian cell gene mutation assay. Mutations are detected at hprt locus in the genome of V79 fibroblasts of Chinese hamster. V79 fibroblasts are grown at optimum conditions and each day cell number is determined for acute toxicity of materials. After 10 days subculturing, cells are replated in a selective medium containing 6-thioguanine in order to isolate mutated

cells. The cell colonies are stained with methylene blue and mutation frequency is calculated [69].

Mutagenic effects of various dentin bonding agents were examined by using AMES test. Elution of test samples were carried out in DMSO and physiological saline and aliquots of serially diluted elutes were used for the assay. Only glutaraldehyde containing agent showed a mutagenic effect on *S. typhimurium* TA102 strain [70]. In another study, the mutagenic potentials of 12 commercially available dental cements used in root canal filling were determined by AMES test. Only one test sample showed mutagenic effect on both TA98 and TA100 strains of *S. typhimurium* [71].

2. 5. Biomaterial-Bacterial Interactions

Biofilms can be described as microecosystems in which different microbial strains and species efficiently cooperate to protect themselves against environmental factors and to facilitate more efficient nutrient uptake [72]. Formation of biofilms is seen in some biomaterial applications and that causes biomaterial-centred infections in humans. These infections are troublesome because biofilm organisms are protected against the host immune system and antibiotics cannot eradicate easily. As a result, an infection around or on biomaterial leads to degradation or loosening of the implant, reoperation, osteomyelitis, amputation or death [73].

Adhesion of pathogenic bacteria to material surfaces is the initial stage in the pathogenesis of prosthetic infections. In these infections, bacteria come from two sources. The first one is direct contamination of the wound during surgery from patient's skin and air. The second type of contamination is hematogenous or lymphatic seeding from infections present in the other parts of body [74-76].

Staphylococcus aureus, one of the causative agents of ostemyelitis, is a usual pathogen associated with damaged tissue. *S. epidermidis*, a nonpathogenic skin saprophyte, attach to the surface of vascular grafts and catheters and becomes an important pathogen in the presence of biomaterials. *S. epidermidis* is responsible for one half or more orthopaedic infections. *S. epidermidis* preferentially adhere to polymer surfaces and *S. aureus* to metal surfaces [77]. Additional microorganisms mostly seen in biomaterial-centred infections are *E. coli*, *Peptococcus*, *P. aeruginosa*, *Proteus mirabilis*, *β-hemolytic streptococcus*, *Klebsiella* group and *Micrococcaceae* [77,78].

Bacterial adhesion to biomaterial surfaces takes place in a few stages. The initial stage is the bacterial attachment. This is a physical contact between bacteria and implant materials and usually is reversible. In the following step, bacteria adhere firmly to the surface by a complete interaction, including a time-dependent phase of irreversible chemical and cellular adherence [77]. In bacterial adhesion, physicochemical interactions between bacteria and material surface take place. By the effects of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, the effect of surface electrostatic charge and hydrophobic interactions bacteria move to a material surface. In the case of short-range interactions in which cell and surface come into close contact (<3 nm), chemical bonds, such as hydrogen bonds, ionic and dipole interactions or hydrophobic interactions occur [77]. Bacteria in aqueous suspensions are always negatively charged. High surface charge is represented by a hydrophilic character of bacteria, but a hydrophobic bacteria may still have a high surface charge [79]. Surface charge of bacteria depends on bacterial species and is affected by growth medium, bacteria age and bacterial surface structure [80]. Bacteria with hydrophobic properties prefer hydrophobic surfaces and hydrophilic bacteria tend to adhere hydrophilic material surfaces. In fact, hydrophobic bacteria adhere to a greater extent than hydrophilic bacteria [81-83].

After the physicochemical interactions, molecular interactions between bacteria and substratum surfaces are seen. In this case, bacterial surface polymeric structures, which include capsules, fimbriae or pili and slime, play important roles. Capsules can be seen in stained and unstained preparations as a clear zone surrounding both gram-positive and gram-negative bacteria. Capsules are composed of polysaccharides and proteins that act as bacterial adhesins. Fibrillar structures were observed at the surface of various streptococci and these structures help to bacteria for better adhesion onto HA substrata [84]. Slime is an extracellular substance of bacteria that may be partially free from the bacteria after dispersion in a liquid medium [85]. Slime is made up with polysaccharides and it is important for intercellular interaction during surface colonization [86,87]. Bacteria washed to remove slime showed a similar amount of adhesion to poly(tetrafluorethylene-co-hexafluorprophylene) compared to non-washed bacteria [88]. Therefore, bacterial strains producing slime are more adherent and more pathogenic than non-producing strains [89-92].

The characteristics of material surfaces influence the bacterial adhesion process. Chemical composition, surface charge, hydrophobicity, surface roughness and physical configuration of material surfaces are the main factors in the adhesion. In addition, binding of serum or tissue proteins to material surfaces may change adherent behavior of bacteria. For example, fibronectin promotes adhesion of *S. aureus* to substratum [93-97] and fibrinogen increases especially adherence of staphylococci to biomaterials. The promoting effect of laminin on *S. aureus* is in lesser extent than fibronectin and fibrinogen [98]. However, surface-adsorbed albumin inhibits adhesion of bacteria to polymer, ceramic and metal surfaces. Furthermore, pre-incubation of Teflon catheters in human serum led to 80-90% decrease in the adhesion of *S. epidermidis* [77].

Inhibition of biofilm formation is achieved by using broad-spectrum antibiotics, such as penicillins or cephalosporins, before and after surgery [77]. In addition, slow-release of antibiotics from surface or by changing physiochemical properties of surfaces are the other strategies to prevent bacterial adherence. Combinations of alkyl phosphates, a disodium phosphate of 1-octadecanol and non-ionic surfactant coating of HA led to inhibition of *S. mutans* adhesion by 98% [99]. Another potential coating material is silver. Polyurethane discs with silver coating resulted in 10-100-fold reduction in the number of adherent *E. coli* compared with untreated control samples [100]. Protein coating was applied successfully for preventing bacterial adhesion. Many biologically active proteins including albumin, fibrinogen, fibronectin, laminin and denatured collagen were studied. Among them, albumin showed inhibitory effects on the adhesion of *S. aureus* and *S. epiderimidis*. In addition to inhibitory effects of albumin, it prevents platelet aggregation and activation as well as improving biocompatibility of biomaterials [77].

Chapter 3

MATERIALS AND METHODS

3. 1. Materials

For mammalian cell culture experiments, Roswell Park Memorial Institute (RPMI)-1640 medium, Fetal Bovine Serum (FBS), gentamycin sulfate, concanavalin A (Con A), lipopolysaccharide (LPS), trypan blue, Bovine Serum Albumin (BSA), MTT and DMSO were purchased from Sigma Chemical Company. Heparin-sodium (Lequimine) was from Roche. Biocoll cell separation solution (Ficoll with density 1.071 g/ml) was obtained from Biochrom Chemical Company. Potassium monohydrogen phosphate, potassium dihydrogen phosphate, sodium chloride were obtained from Mérck Chemical Company. Tissue culture plates were from Corning Costar, polypropylene tubes and disposable pipettes were obtained from Greiner. Cell proliferation assay kit was purchased from Amersham Life Sciences. Quantikine® Human IL-1α and IL-6 ELISA kits were from R&D Systems.

In bacterial culture studies, Luria broth and Luria agar were purchased from Sigma Chemical Company. Mueller-Hinton agar, Mueller-Hinton broth, nutrient broth no:2, meropenem and vancomycin antibiotic discs were from Oxoid. The culture media for Viridans *spp*. BacT was supplied by Organon Teknika. Bacterial stains; methylene blue from Mérck, acid fuchsin and bacteriological agar were obtained from Difco. *Salmonella typhimurium* strains were obtained from Prof. Bruce Ames (Biochemistry Department, University of California, Berkeley). *E.coli*, *S. aureus*, Viridans (*S. mitis* and *S. mutans*), *S. pyogenes, P. aeruginosa*, *Proteus spp.* and *Enterobacter spp.* strains were kindly provided by Prof. Dr. Altınay Bilgiç (Dept. of Microbiology and Clinical Microbiology, Faculty of Medicine, Ege University, İzmir).

Calcium phosphate (tri basic) was obtained from Aldrich Chemical Company. Alumina powder was from Sumitoma AKP-50 and zirconia powder was from Tosoh TZ-3Y. Stainless steel (316L), titanium alloy (Ti-6Al-4V) and cirulene (ultra high molecular weight polyethylene) discs were kindly supplied by Dr. Cevdet Alptekin (Hipokrat Tıbbi Malzemeler İmalat ve Pazarlama A.Ş., İzmir).

3. 2. Methods

3. 2. 1. Preparation of Biomaterials and Test Samples

Ceramic and composite pellets were prepared in İzmir Institute of Technology, Faculty of Engineering Laboratories. In order to prepare HA pellets, 1.5 g of polyvinylalcohol (PVA) was dissolved in 25 ml deionized water (dH_2O) on a hot plate by stirring and mixed with 50 g of calcium phosphate (tri basic) (HA). After drying the mixture at 100 °C by mixing at 10-15 minutes intervals, the powder form was obtained by grinding in a mortar.

Table 3.1. The weight and compaction pressure of ceramic pellets

Ceramic Material	15 mm diameter die		5 mm diameter die	
	Powder (gram)	Pressure (MPa)	Powder (gram)	Pressure (MPa)
Hydroxyapatite	0.70	180	0.070	240
Alumina	0.90	180	0.090	240
Zirconia	1.20	180	0.140	240
HA-Alumina	0.75	180	0.085	240
HA-Zirconia	0.80	180	0.090	240

The HA green pellets were sintered at mainly 800 °C and 1250 °C by using a high temperature oven (Carbolite RHF 1600). In addition, for some tests HA pellets were sintered at 900 °C, 1000 °C, 1100 °C and 1400 °C. Alumina and zirconia pellets were prepared in a similar way and sintered at 1450 °C. HA-Alumina composites were prepared by mixing 4 g HA and 1 g alumina powder. Similarly, HA-Zirconia composites were prepared by mixing 4 g of HA and 1 g of zirconia powder. In the preparation of samples, 1 g alumina powder was suspended in 2 ml dH_2O and sonicated for 8 minutes. Then, 4 g HA powder was added and ultrasonically treated for 8 more minutes. 1.25 ml dissolved PVA in dH_2O was added and dried at 100 °C by mixing at 10-15 minutes intervals. The powder form was obtained by grinding dried composite

mixture. HA-Zirconia composite powders were also prepared similarly. Composite pellets were sintered at 1250 °C.

Metallic biomaterials (stainless steel (316L) and titanium alloy (Ti-6Al-4V)) were supplied by Hipokrat A.Ş. with 10-14 mm in diameter and 2 mm in thickness. For polishing process, the molds were prepared by using polymer resin material in Simplimet 2 mounting press (Buehler). The samples were polished by using 1200 grit SiC, 6 microns diamond suspension, 1 microns alumina and 0.5 microns alumina suspensions on Metaserv 2000 grinder/polisher (Buehler). The surface roughness of polished samples was examined by using an optical microscope (Olympus BX60M reflected light version). In addition, polymeric sample (ultra high molecular weight polyethylene) was obtained from Hipokrat A.Ş. with 10-14 mm in diameter and 2 mm in thickness.

All ceramic samples were washed 4-5 times with dH_2O for cleaning. Metallic discs were cleaned with methanol and DLC coated AISI 52100 disc was cleaned by sonication 2 times for 15 minutes in acetone. Metallic samples were washed with dH_2O several times and dried at 100 °C for 1-1.5 hours. After covering with aluminium foil, they were sterilized by dry heat at 200 °C for 2 hours. However, cirulene samples were wrapped with cheesecloth and autoclaved at 121 °C for 15 minutes.

3. 2. 1. 1. Protein Adsorption to Ceramic Pellets

Bovine Serum Albumin (BSA) adsorption studies were carried out by incubating ceramic pellets (15 mm in diameter) in 10 ml protein solution with a concentration of 20 mg/ml BSA in sodium phosphate buffer (pH 7.3). The samples were incubated at 37 °C with shaking at 110 rpm for 3 hours in a thermal shaker (Gerhardt Thermoshake). The pellets were immediately used or stored at +4 °C until use.

3. 2. 2. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood is the primary source of lymphoid cells for the examination of immune responses in humans. Density gradient centrifugation with Ficoll is a simple and rapid method for the purification of PBMC on the basis of difference in the densities of leukocytes and other blood elements [101]. While Ficoll sucrose polymer aggregates the erythrocytes in the bottom, low density PBMC and platelets form a layer on the top of gradient. Platelets are removed by washing with PBS. The prepared

PBMC population contains 5% B-lymphocytes, 5-15% monocytes, 60-70% T-lymphocytes, and 5-15% Natural Killer Cells [102].

PBMC were freshly isolated from healthy, non-smoking men and women donors with ages between 24 and 30 as described by Hokland M. *et al*. [103]. Isolation procedure is described in Figure A1. In summary, 10 ml blood was taken into heparinized needle with 400 µl sterile heparin-sodium solution (50 I.U.). The blood was transferred into 2 sterile tubes and equal volume of sterile 0.9% NaCl was added. 5 ml of NaCl-blood mixture was taken by sterile, pasteur pipette and layered onto 3 ml Ficoll solution (density 1.071 g/ml) very slowly to prevent disruption of the layer in a conical bottom falcon tube. The tubes were centrifuged at 1600 rpm (Hettich 30RF Centrifuge), at room temperature for 25 minutes. At the end of centrifugation, a buffy coat layer of PBMC was obtained. The layer between plasma and Ficoll was carefully removed into new tubes and 4-5 volumes of PBS (pH 7.4) containing 2% FBS were added. The cells were centrifuged at 800 rpm for 10 minutes. After removing the supernatant, the pellet was resuspended with 10 ml PBS containing 2% FBS and centrifuged at 500 rpm for 8 minutes. This washing was repeated once more to remove thrombocytes and platelets. The pellet was resuspended in RPMI-1640 medium (pH 7.4) containing 15% FBS and 50 µg/ml gentamycin sulfate. Isolated cell number was determined by mixing 50 µl cell suspension with 50 µl trypan blue solution (0.4 %) and the cells were counted immediately in 25 squares of hemocytometer by using light microscope. The following formula gives the number of cells per ml:

$$\text{Number of cells/ml} = \text{Number of cells counted in 25 square} \times \text{Dilution factor} \times 10^4$$

3. 2. 3. The Viability of PBMC

The effects of biomaterials on the viability of PBMC were determined by using trypan blue exclusion method. For this purpose, HA pellets (sintered at 800 °C, 900 °C, 1000 °C, 1100 °C, 1250 °C), alumina pellets (1000 °C, 1450 °C), zirconia pellets (1450 °C), their extracts in PBS, stainless steel (316L), titanium alloy (Ti-6Al-4V), cirulene (ultra high molecular weight polyethylene) pellets and BSA-coated samples were used. PBMC at 2.5×10^5 cells/ml were incubated in the presence of sterile samples at 15 mm in diameter in sterile 24-well polystyrene tissue culture treated plates. Some of the cells at

1×10^5 cells/well were incubated with the extracts of biomaterials in 96-well flat bottom tissue culture treated plates. Incubation was carried out for 24, 48 and 72 hours at 37 °C with 5% CO_2, humidified incubator. The procedure of the method is described in Figure A2. At the end of incubation periods, the cells were collected into eppendorf tubes and centrifuged at 100 rpm for 10 minutes for settlement of the cells. The supernatant was removed until 100-200 µl remained in eppendorf tubes. The cells were resuspended slowly with a micropipette and 50 µl of the cell suspension was mixed with 50 µl trypan blue solution (0.4 %). Live and dead cells were counted immediately by using hemocytometer under light microscope. Dead cells absorbed the blue dye due to the damage in the cell membranes and they appeared in blue color. The viability of cells was calculated by the following formula:

Percent Cell Viability = [Number of live cells / Number of total cells (live+dead)] × 100

3. 2. 4. IL-1α and IL-6 Secretion from PBMC

The secretions of IL-1α and IL-6 in the presence of biomaterials were determined by using Quantikine® ELISA kits (R&D Systems). Briefly, PBMC (2.5×10^5 cells/well) were incubated with sterile samples at 15 mm in diameter in 24-well tissue culture plates at 37 °C with 5% CO_2, humidified incubator for 17 hours with and without LPS (10 µg/ml). The cell suspensions were collected into eppendorf tubes and centrifuged at 15,000 rpm for 30 seconds by using a microcentrifuge (Techne Genofuge 16M). The supernatants were collected into new eppendorf tubes and stored at –20 °C to conduct ELISA experiments.

Human IL-1α secretion in the cell culture supernatants was determined by the quantitative sandwich enzyme immunoassay technique as described by the manufacturer. Briefly, 200 µl standards and sample supernatants were pipetted into a microtiter plate, which was pre-coated with a monoclonal antibody specific for IL-1α. After 2 hours incubation at room temperature, the microtiter plate was washed 3 times to get rid of unbound substances. Horseradish peroxidase conjugated polyclonal antibody specific for IL-1α was added to the wells. Following 1-hour incubation period at room temperature, unbound antibody-enzyme reagent was removed and a substrate solution was added to the wells. After 20 minutes incubation, the color development

was stopped with stop solution. Absorbance of the color development in proportion to the amount of IL-1α bound in initial step was measured at 450 nm with an ELISA plate reader (ELX 800 Universal Plate Reader, Bio-Tek Instruments).

The same strategy was applied to determine IL-6 secretion in the supernatants from PBMC culture incubated with indicated biomaterials.

3. 2. 5. The Proliferation of PBMC

The proliferation of PBMC was determined as described in the Biotrak cellular communication assays, cell proliferation ELISA system version 2 kit (Amersham Life Sciences). The test is based on the incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells. PBMC (8×10^4 cells/well) were incubated in 96-well flat bottom tissue culture treated plate with the biomaterials (5 mm in diameter) at 37 °C with 5% CO_2, humidified incubator for 2 days. Con A (5 μg/ml) was added into the cell culture as a positive control for cell proliferation. After that, BrdU was added to the cells and incubation was carried out for additional 24 hours to label DNA. The cells were centrifuged at 1200 rpm for 15 minutes at room temperature in a plate rotor of Hettich 30 RF Centrifuge. After removing the culture medium, the cells were dried and fixed with hair dryer for 15 minutes. DNA was denatured with fixative solution for 60 minutes incubation. Fixative was removed by tapping and 200 μl/well blocking reagent was added (blocking stock solution was diluted 1:10 with dH_2O) and incubated for 30 minutes at room temperature. Blocking reagent was removed and 100μl/well peroxidase-labelled anti-BrdU working solution was added and incubated for 2 hours at room temperature. Peroxidase-labelled anti-BrdU working solution was prepared just before use and it is not stored. Peroxidase-labelled anti-BrdU stock solution was diluted 2:100 with antibody dilution solution. In this period, peroxidase-labelled anti-BrdU binds to the BrdU incorporated in newly synthesized, cellular DNA. Antibody solution was removed by tapping and the wells were washed 3 times with 200-300 μl wash buffer. Immediately, 100 μl room temperature equilibrated TMB substrate solution was added and incubated for 5-30 minutes at room temperature by covering aluminium foil and by shaking on an orbital shaker until color development. When color development was achieved, reaction was stopped by pipetting 25 μl of 1M sulphuric acid into each well

and mixed slowly. Optical density at 450 nm was determined within 5 minutes using an automated ELISA plate reader (ELX 800 Universal Plate Reader, Bio-Tek Instruments).

3. 2. 6. The Activation of PBMC (MTT Assay)

The activation of PBMC was determined by MTT assay. This assay also represents cytotoxic effects of biomaterials. The test is based on the conversion of yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple water insoluble formazan crystals by mitochondrial enzymes of metabolically active cells.

Sterile HA 800 °C, HA 900 °C, HA 1100 °C, HA 1250 °C, HA-Alumina 1250 °C, HA-Zirconia 1250 °C pellets (5 mm in diameter) and the extracts of HA 800 °C, HA 900 °C, HA 1000 °C, HA 1250 °C, alumina 1450 °C and zirconia 1450 °C in PBS (pH 7.4) were tested for the activation of PBMC. The test procedure is described in Figure A3. The samples were incubated with 1×10^5 cells/well in 96-well tissue culture treated plates for 24 hours, 48 hours and 72 hours at 37 °C with 5% CO_2, humidified incubator. MTT stock solution (5 mg/ml) was added into each well at 1:10 ratio for last 3 hours of incubation period. At the end of each incubation period, the cells incubated with ceramic pellets were highlighted and transferred into blank wells. The plate was centrifuged at 1800 rpm for 10 minutes at room temperature in a plate rotor of Hettich 30 RF Centrifuge to avoid accidental removal of formazan crystals. Supernatants were removed by tapping slowly on a paper towel. DMSO (150 µl) was added into each well and mixed on an orbital shaker at 150 rpm for 5 minutes. The absorbance values were determined at 540 nm (690 nm reference wavelength) by using an automated plate reader (Organon Teknika Reader 230 S, Version 1.22).

3. 2. 7. AMES Mutagenicity Test

Mutagenic potential of biomaterials was determined by using AMES test [68]. Reverse mutations of tester *Salmonella* strains are detected for their histidine dependence or independence.

The extracts for mutagenicity test were prepared by incubating sterile ceramic samples with 5 ml PBS (pH 7.4) and sterile metallic samples with 7 ml PBS (pH 7.4) in

sterile 6-well polystyrene tissue culture treated plates at 37 °C with 5% CO_2, humidified incubator for 120 hours without shaking. The extracts were stored at –20 °C.

The procedure of AMES mutagenicity test is described in Figure A4. Briefly, *Salmonella typhimurium* strain TA 100 was grown in nutrient broth no:2 in test tubes or in 50 ml flasks at 37 °C with shaking overnight in a thermal shaker (Gerhardt Thermoshake). To prevent effects of light on the viability of bacteria, tubes or flasks were covered with a aluminium foil during incubation and experiment. Next day, 0.5 ml PBS (pH: 7.4), 0.1 ml of bacterial culture and 0.1 ml biomaterial extracts were mixed slowly by vortex and incubated for 1 hour at 37 °C with shaking. After preincubation of the bacteria with the extracts, 2 ml top agar containing 0.5 mM Histidine-Biotin was added into tubes, mixed slowly by vortexing at low speed for 3 seconds and poured onto glucose agar plates immediately. Uniform distribution of top agar on glucose plates was achieved. Duplicate plates were incubated at 37 °C for 48 hours and the revertant colonies with background lawn were counted. The revertant colonies in the negative control (no extract), in the positive control (5 µl sodium azide (17.2 mg/ml)) plates and in the plates with top agar containing only biotin were compared for the mutagenic potentials of biomaterials.

3. 2. 8. Bacterial Adhesion to Biomaterial Surfaces

For the determination of bacterial adhesion to biomaterial surfaces, *S. aureus*, *E. coli*, Viridans (*Streptococcus mutans* and *Streptococcus mitis*) and KNS (Koagulase negative S*taphylococcus*) bacterial strains were used. Briefly, the test procedure is explained in Figure A5. Dry heat sterilized metallic and ceramic pellets were placed in polystyrene, 90 mm in diameter, sterile, 4 partitioned plates. 6 ml of LB broth (pH: 7.4) for *S. aureus*, *E.coli* and KNS strains, 6 ml of BacT Medium for Viridans strains were added into each partition of plates. Dilutions of bacterial suspensions in sterile dH_2O were prepared according to McFarland 0.5 (10^8 CFU/ml) and 0.1 ml was added into each part. The plates with *S. aureus* and *E. coli* were incubated for 6 hours and the plates with Viridans and KNS for overnight at 37 °C with shaking at 40 rpm in a thermal shaker (Gerhardt Thermoshake). The pellets were washed with sterile dH_2O 4-5 times to remove unbound bacteria from the surface and transferred into new plates. After air drying, bacteria on the pellets were fixed on hot plate for 30 seconds or at 60

°C for 1 hour and stained with methylene blue or acid fuchsin (Bacto TB carbolfuchsin KF) for 30 seconds. The pellets were washed with dH_2O to remove excess stains and they were air dried before examination under an optical microscope (Olympus BX60M reflected light version). Photographs were taken by using Olympus semiautomatic exposure photomicrography system.

3. 2. 9. Anti-Bacterial Effects of Biomaterials

The agar diffusion method was used to determine anti-bacterial effects of biomaterials. Gram-negative bacterial strains (*E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Proteus spp*.) and gram-positive strains (*S. aureus* and *Streptococcus pyogenes*) were used for this assay. One colony was picked up from the bacteria grown on blood agar plates for overnight and it was dissolved in 1 ml sterile dH_2O with a concentration of McFarland 1.0 (10^9 CFU/ml). Each bacterial suspension was spread onto separate Mueller-Hinton agar plates. Powder forms of ceramic biomaterials (0.05 g), sintered ceramic pellets, metallic and polymeric biomaterials were applied on the plates aseptically. Meropenem (10 μg) and vancomycin (30 μg) were used as the positive controls. The plates were incubated at 37 °C with 5% CO_2, humidified incubator. The zone diameters around test materials and the positive controls were measured everyday.

3. 2. 10. Dissolution Properties of Biomaterials

Dissolution properties of ceramic materials (HA, alumina and zirconia at different sintering temperatures) in the culture medium (RPMI-1640 with 15% FBS and 50 μg/ml gentamycin sulfate) with and without PBMC and in deionized water were examined. Sterile HA 800 °C, HA 1250 °C, alumina 1450 °C and zirconia 1450 °C pellets (15 mm in diameter) were used in this assay. The pellets were incubated with 1 ml of 2.5×10^5 cells/well in sterile 24-well tissue culture treated plates at 37 °C with 5% CO_2, humidified incubator for 4 hours and 72 hours. In 4 hours period, every hour, in 72 hours period, in 4 hours intervals and in 10 days period, everyday the samples were taken. At the beginning (time 0) and at the end of each time point, 1 ml cell suspension and 1 ml culture medium were collected into 50 ml sterile falcon tubes and the volume was completed to 25 ml with sterile dH_2O. For 10 days period, 2 ml dH_2O in the wells

were collected and brought to the total volume of 24 ml with dH_2O. The conductivity and pH of solutions were measured by using WTW Inolab Cond Level 2P Conductivity Meter and Hanna Instruments HI 9321 Microprocessor pH Meter.

Chapter 4

RESULTS AND DISCUSSION

4. 1. Effects of Biomaterials on The Viability of PBMC

Ceramic materials with different sintering temperatures, their extracts in PBS, BSA adsorbed ceramic materials, polished and unpolished metallic samples and polymeric samples were examined to see if they have any effects on the viability of PBMC culture.

Hydroxyapatite pellets 15 mm in diameter were sintered at 800 °C and 1250 °C, alumina pellets at 1450 °C and zirconia pellets at 1450 °C. Ceramic materials were incubated with freshly isolated human PBMC (2.5×10^5 cells/ml) for 24, 48 and 72 hours. The cells were collected and stained with trypan blue. Percent cell viabilities were calculated as described in Materials and Methods Section. In the evaluation of the effects of ceramic materials on the viability of cells, data were compared with the control culture which contains no biomaterial. After 24 hours incubation, the cell viability was not affected by HA 1250 °C, alumina 1450 °C and zirconia 1450 °C samples. However, HA 800 °C sample caused 1.3-fold decrease in the cell viability compared to the control culture (Figure 4.1). In addition, the viability of cells treated with HA 800 °C sample continued to decrease 3.3-fold and 3.9-fold when compared to the control cultures after 48 hours and 72 hours treatments, respectively. In contrast to HA 800 °C sample, other biomaterials tested did not cause a significant change in the cell viabilities after 48 and 72 hours treatments.

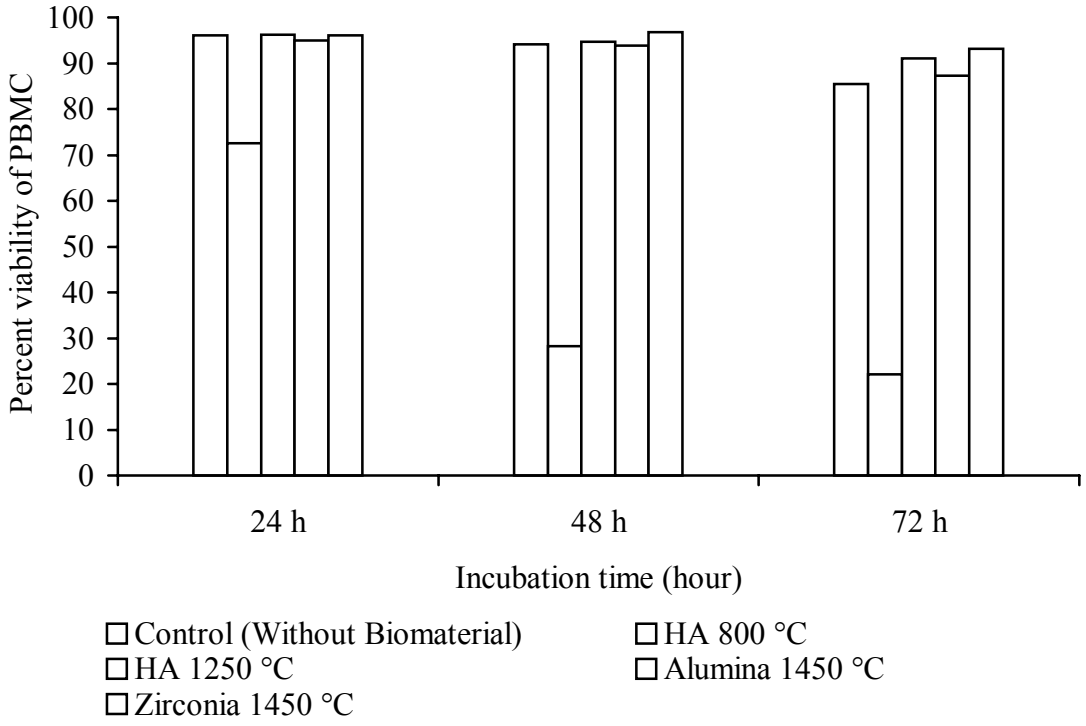


Figure 4.1. Effects of ceramic biomaterials on the viability of PBMC. PBMC at 2.5×10^5 cells/well were incubated with ceramic samples for 24, 48 and 72 hours. The cells were collected by centrifugation at 100 rpm for 10 minutes and stained with trypan blue. Live and dead cells were counted by hemocytometer and percent cell viability was determined as explained in Materials and Methods Section. Data are the average of duplicate samples from separate experiments.

In the first set of experiments, HA 800 °C pellets led to a change in the red color of culture medium to purple. Purple color in medium represents a basic solution. Because of the pH change of the medium, number of live cells might decrease. The reason of the pH change could be due to the dissolution of HA 800 °C pellets in the culture medium. Unlike HA 1250 °C and other ceramic samples, HA 800 °C pellets have porous structure, so that they have a relatively high surface area. In order to determine the negative effect of low sintering temperature, HA pellets with different sintering temperatures between 800 °C and 1250 °C were prepared. Alumina pellets at low sintering temperatures (1000 °C) were also prepared for the investigation of the

effects of low sintering temperature (porous structure of pellets) or chemical composition on the cell viability. PBMC 2×10^5 cells/well were incubated with indicated ceramic samples sintered at different temperatures for 24, 48 and 72 hours (Figure 4.2). After 24 hours, the viability of cells treated with HA 800 °C, HA 900 °C and HA 1000 °C pellets was 1.6-, 1.7- and 1.4-fold less than that of cells without any ceramic samples. HA pellets sintered at 1100 °C and 1250 °C did not affect the cell viability at all time points examined. After 48 hours incubation, the viability of cells decreased to 30% and 35% in the cells treated with HA 800 °C and HA 900 °C; however, there was a slight decrease in the viability of cells treated with HA 1000 °C pellet. After 72 hours, the viability reduced to 30% and stayed around at the same level for all HA pellets sintered at low temperatures: 800 °C, 900 °C and 1000 °C (Table B2).

In addition, the effect of alumina pellets sintered at different temperatures on the viability of PBMC was examined. After 24 hours incubation, alumina sintered at 1000 °C caused 1.11-fold decrease in the cell viability compared to the control culture. The cell viability decreased to 50% at 48 hours; the last time point examined (Figure 4.2; Table B2). The effect of alumina pellet sintered at 1450 °C on the viability of PBMC was tested only at 48 hours incubation period and the percent cell viability (85%) was close to that obtained from the control culture (95% out of maximum level). On the basis of these data, both porous structure and chemical composition of ceramics seem to affect the viability of PBMC. The chemical composition of ceramics changes with sintering temperature. At high temperatures, they are very dense. However, at low temperatures they have highly porous structure and low density.

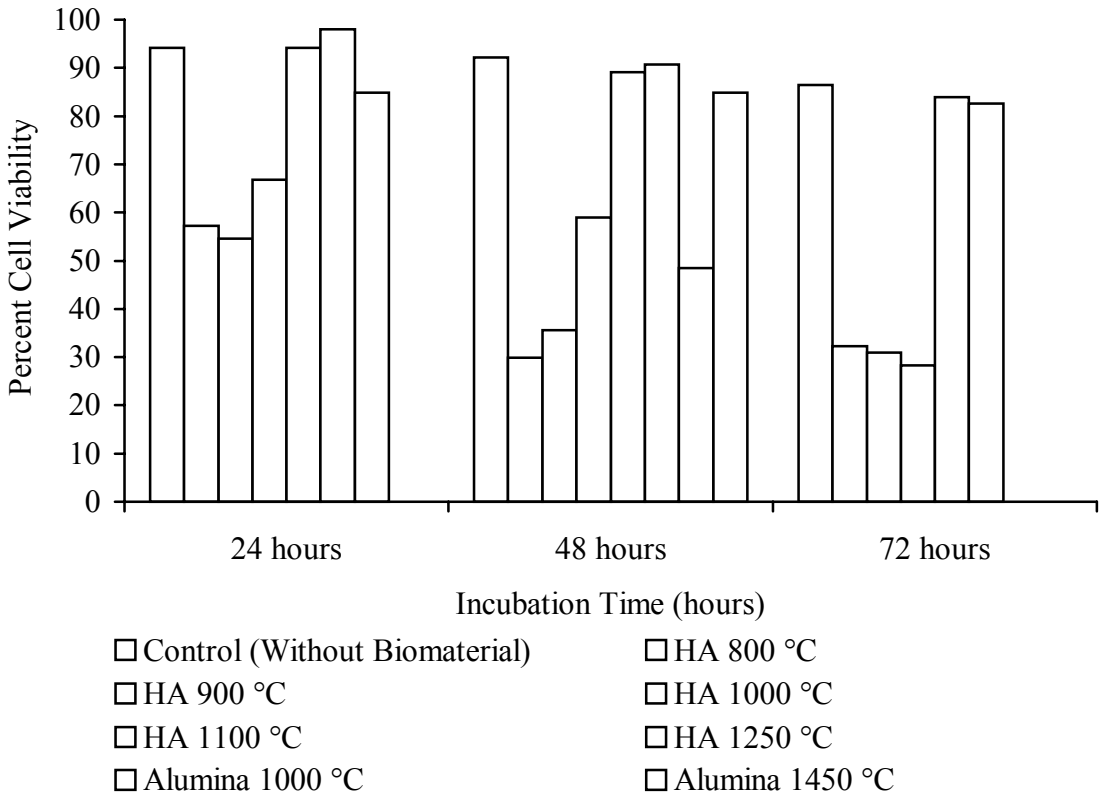
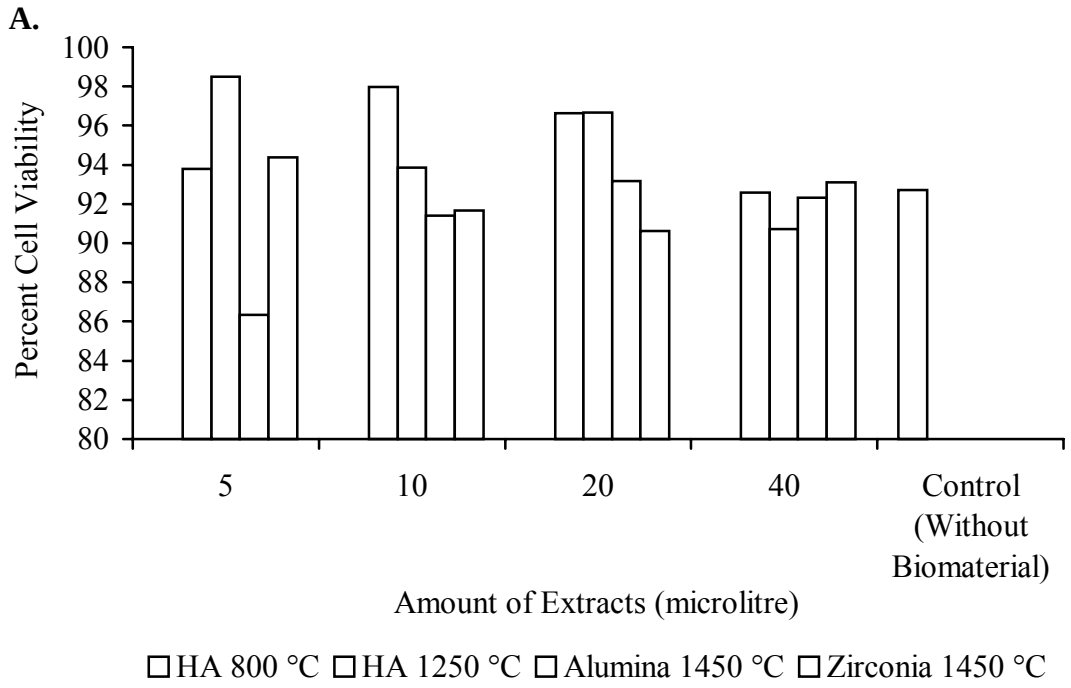


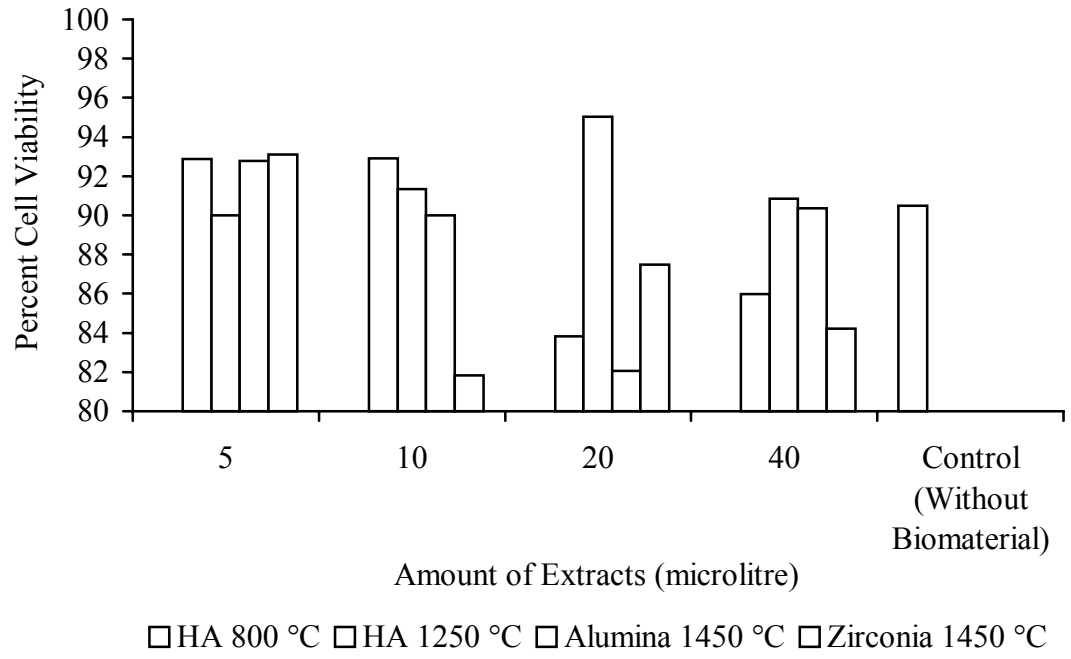
Figure 4.2. Effects of sintering temperatures on the viability of PBMC. 2×10^5 cells/well were incubated with ceramic pellets (15 mm in diameter) with different sintering temperatures between 800 °C and 1450 °C for 24, 48 and 72 hours. Percent cell viabilities were calculated as legend to Figure 4.1. Data are the average of duplicate samples from separate experiments.

The extracts of ceramic samples were obtained by incubating sterile pellets in 5 ml PBS (pH 7.4) in sterile 6-well polystyrene tissue culture treated plates at 37 °C with 5% CO_2, humidified incubator for 120 hours without shaking. The extracts were stored at -20 °C until used. PBMC (1×10^5 cells/well in 96-well tissue culture treated flat bottom plates) were incubated with the extracts of HA 800 °C, HA 1250 °C, alumina 1450 °C and zirconia 1450 °C pellets (5 µl, 10 µl, 20 µl, 40 µl) for 24 hours, 48 hours and 72 hours. Percent cell viabilities were calculated as shown in Table B3. After 24 hours incubation, the percent cell viabilities did not show significant differences in comparison to the control culture (Figure 4.3-A). Moreover, the increase of extract concentration in the cell culture did not affect the cell viability. After 48 hours

incubation, no effect on the cell viability was observed after treatment of the cells with 5 µl of all extracts (Figure 4.3-B). However, increased concentration of zirconia 1450 °C extracts (10 µl, 20 µl and 40 µl) resulted in 10%, 4% and 7% decrease in the cell viability, respectively. Similarly, 8% and 6% decline in the cell viabilities was observed in the cells treated with 20 µl and 40 µl HA 800 °C extracts. Alumina 1450 °C extract (20 µl) caused 10% decrease in the cell viability, but it had no effect at other concentrations. At the end of 72 hours, slight fluctuation was investigated in the cell viabilities after treatment with all the extracts at different concentrations. However, only HA 800 °C extracts at 10 µl and 40 µl concentration gave rise to a 14% and 25% decrease, respectively in the cell viability (Figure 4.3-C; Table B3). As a result of these data, a negative correlation between increasing extract concentration and the cell viability was observed for all samples.



B.



C.

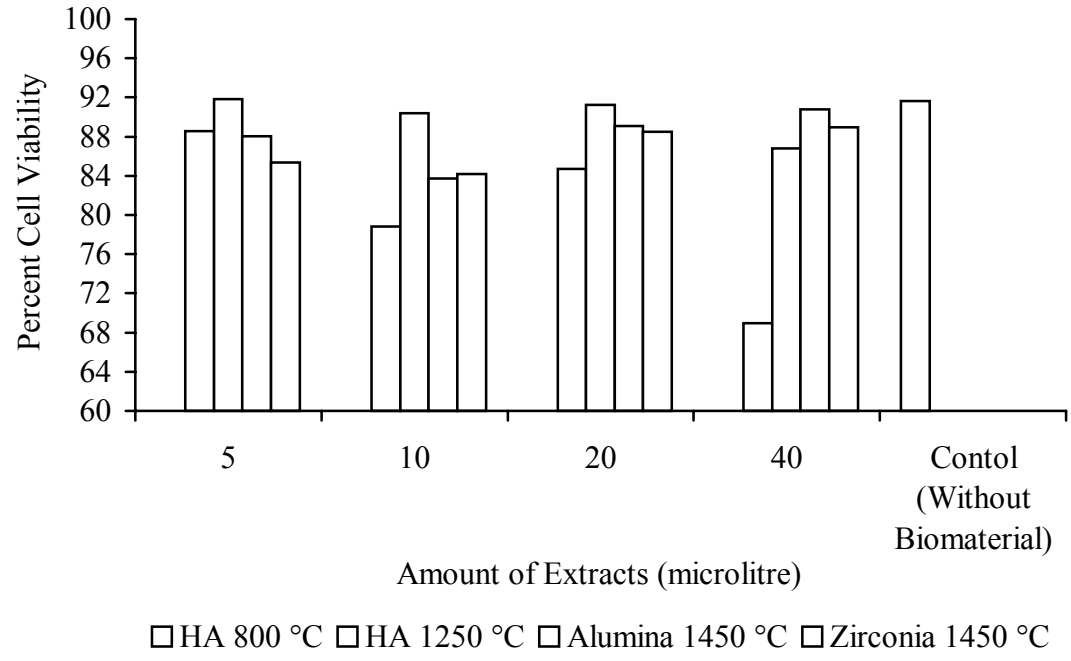


Figure 4.3. Effects of ceramic extracts on the viability of PBMC.

Extracts of ceramic samples were obtained by incubating pellets (15 mm in diameter) in PBS (pH 7.4) at 37 °C for 120 hours without shaking. 200 µl PBMC (1×10^5 cells/well in 96-well plate) were incubated in the presence of 5 µl, 10 µl, 20 µl, 40 µl extracts. Percent cell viabilities were determined at the end of 24 hours (A), 48 hours (B) and 72 hours incubation (C). Data are the average of duplicate samples from separate experiments.

In order to determine the effect of protein (BSA) adsorption onto ceramic samples on the cell viability, PBMC (2.5×10^5 cells/well) were incubated with BSA-adsorbed ceramic samples for 24 hours, 48 hours and 72 hours. The data obtained from protein adsorbed samples (Table B4) were compared with previously obtained data from uncoated ceramic samples (Table B1). Except for protein adsorbed HA 800 °C pellets, all samples showed similar results with the controls at all time points. In the case of protein coated HA 800 °C pellets, after 24 hours 13%, after 48 hours 102% and after 72 hours 62% increase in the viability of PBMC were obtained with respect to uncoated HA 800 °C pellets (Figure 4.4).

In the literature, Deligianni *et al*. reported that cell adhesion, proliferation and detachment strength were surface roughness sensitive and increased as the roughness of HA increased. In addition, the adhesion of cells such as human bone marrow cells on HA surfaces and the cell detachment strength may be explained by the selective adsorption of proteins present in serum [104]. These adsorbed proteins play important roles in the modulation of cellular interactions. For example, adsorption of serum fibronectin to the surface of calcium phosphate may be responsible for enhanced osteoblast adhesion [105]. The high surface area because of porous structure of HA 800 °C led to the adsorption of more protein. Therefore, the protein adsorption, which probably prevents the dissolution of HA pellets or leads to a decrease in the negative effect of HA 800 °C on the cell viability by attachment of the cells.

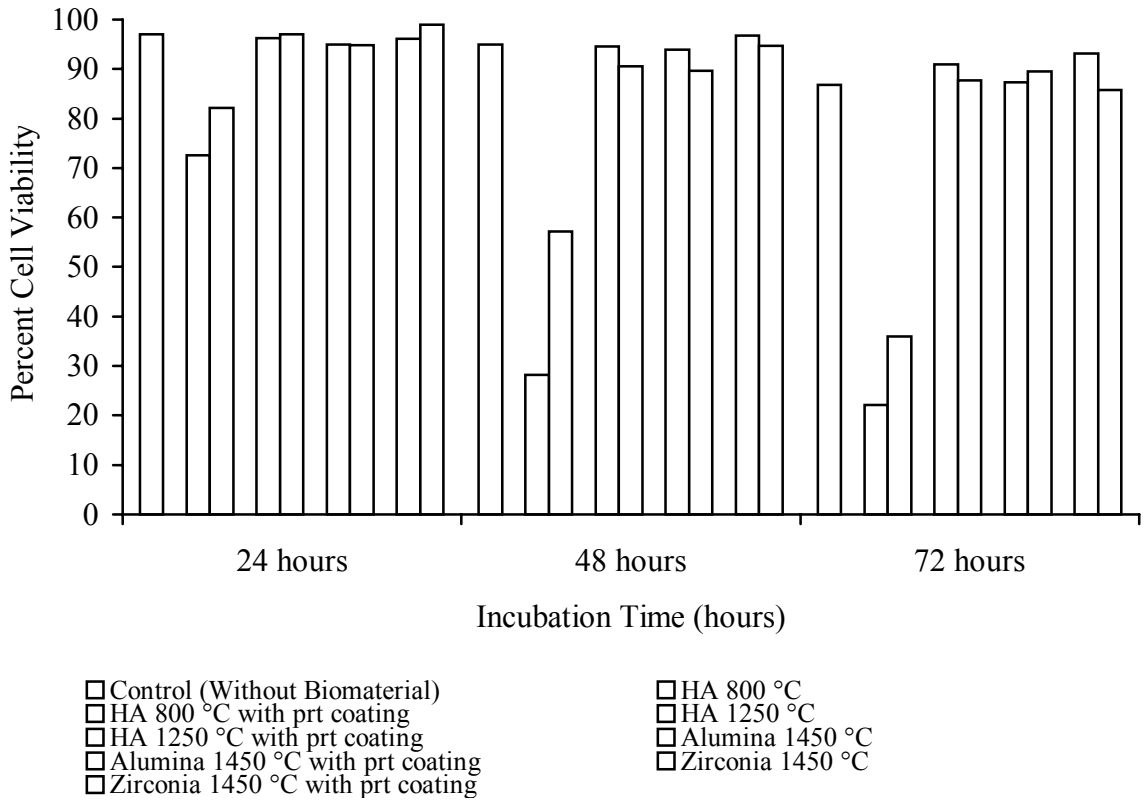


Figure 4.4. Effects of protein adsorption onto ceramic pellets on the viability of PBMC. BSA adsorbed ceramic pellets (15 mm in diameter) were incubated in 10 ml BSA (20 mg/ml) in sodium phosphate buffer (pH 7.3) at 37 °C with shaking at 110 rpm for 3 hours. Coated ceramic pellets were incubated with PBMC (2.5×10^5 cells/well) for 24, 48 and 72 hours. The percent cell viabilities were obtained by trypan blue exclusion method. Data for uncoated samples were adapted from Table B1. Data for protein adsorbed ceramic samples represent results of one experiment.

The effects of metallic biomaterials (polished and unpolished stainless steel (316L), titanium alloy (Ti-6Al-4V)) and polymeric biomaterial (cirulene, (ultra high molecular weight polyethylene)) 10 mm in diameter and 2 mm in thickness on the viability of PBMC were tested. Freshly isolated PBMC (2×10^5 cells/well in 24-well plates) were incubated with the samples at 37 °C with 5% CO_2 for 24, 48 and 72 hours. In polished stainless steel samples after 24 and 48 hours, no dead cells were observed and percent cell viabilities were calculated as 100%. After 24 hours and 48 hours, there

were no more than 3% decreases in the cell viabilities in the presence of metallic and polymeric samples with respect to the control cultures (Figure 4.5). Whereas, after 72 hours, a decrease in the viability of PBMC was obtained. Stainless steel, polished stainless steel, titanium alloy, polished titanium alloy and cirulene resulted in 22%, 5%, 10%, 4% and 16% respectively, decrease in the cell viability (Table B5). After 72 hours incubation with the cells, metallic samples and polymeric sample started to decrease the cell viabilities. Before 72 hours, they showed similar results with the control culture. In fact, release of some ions might change the ionic environment of the culture medium. These results suggested that, surface properties of biomaterials are important for the viability of cells. Especially in polished samples, almost 100% cell viabilities were obtained. In optical microscope observation, very rough surface including lines and scratches was observed in unpolished metallic samples, but not in polished samples. Polished surfaces are more suitable for suspension cells, such as PBMC. However, anchorage dependent cells, such as bone marrow cells and osteoblasts, prefer rough surfaces for adhesion and proliferation.

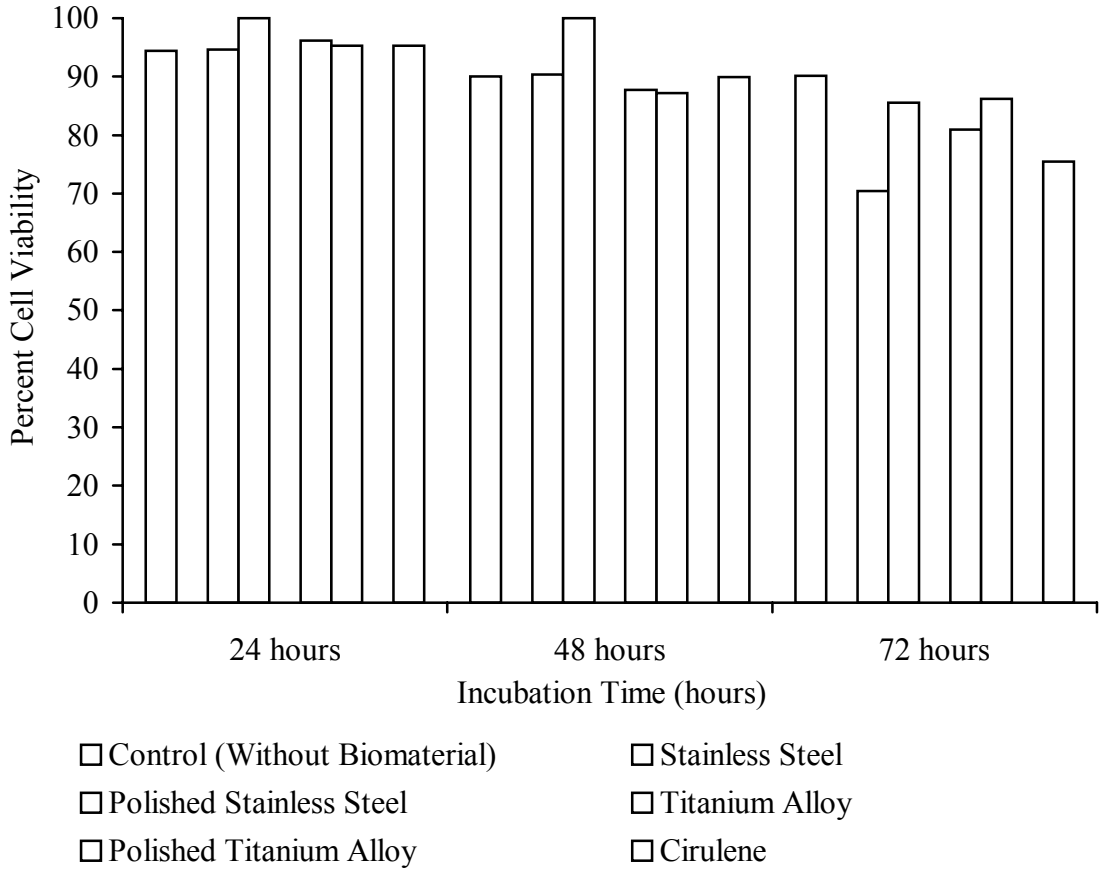


Figure 4.5. Effects of metallic and polymeric samples on the viability of PBMC.

Metallic samples (stainless steel and titanium alloy and their polished forms) and polymeric sample (cirulene) 10 mm in diameter were incubated with PBMC (2×10^5 cells/well) for 24, 48 and 72 hours. Percent cell viabilities were determined as legend to Figure 4.1. Data are the average of duplicate samples from separate experiments.

In vitro cell culture systems simulate the biological environment of the body. The samples were incubated for maximum 72 hours period with the cells. One disadvantage in these experiments was the use of primary cells for the viability measurements. Especially after 72-96 hours, these primary mononuclear cells start to die due to waste products and depletion of food supply. These cells are also non-adherent cells. For this reason, to change the culture medium is not possible. On the other hand, PBMC play important roles in immune system, inflammation and biomaterial-host interactions. All implant materials contact with blood consequently mononuclear cells. To determine effects of biomaterials on the viability of PBMC is crucial. The effects of these samples in long period of time should be tested to complete their biocompatibility evaluation.

In the viability tests, porous HA pellets showed low response in *in vitro* culture systems. However, the number of periosteal fibroblasts, osteoblasts and chondrocytes increased 29-, 23- and 17-fold, respectively on to porous calcium phosphate ceramic (sintered at 1100 °C for 8 hours) during 10 weeks period [106]. In fact, porous HA showed appropriate results and biocompatibility for the adherent cells. In addition, porous HA ceramics are widely used in medicine in the form of blocks or granules. Some of the medical applications are filling bone defects, drug delivery systems, alveolar ridge augmentation and orthognatic reconstruction [107].

4. 2. Effects of Biomaterials on The Secretion of Cytokines

IL-1α and IL-6 were quantitatively determined by using Quantikine® ELISA kits. Firstly, the calibration curves of IL-1α and IL-6 standards were plotted from standard IL-1α and IL-6 solutions (Figure B1 and Figure B2). Test was repeated with cells from different donors and in the presence and absence of mitogenic agent lipopolysaccharide from *E.coli* 055:B5 (LPS) (Table B6). After 17 hours incubation of

ceramic pellets (15 mm in diameter) with PBMC (2.5×10^5 cells/well), the secretion of IL-1α was detected. In the absence of LPS, there was no significant difference in IL-1α secretion between all the samples and the control culture. In the presence of LPS, the level of IL-1α secretion was highest in the control culture (Figure 4.6). There was a slight decrease in IL-1α secretion of the cells treated with HA 1250 °C, alumina 1450 °C and zirconia 1450 °C samples. Unlike other samples, HA 800 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C samples decreased the IL-1α secretion 5-fold, 3-fold and 2-fold, respectively when compared to the control culture.

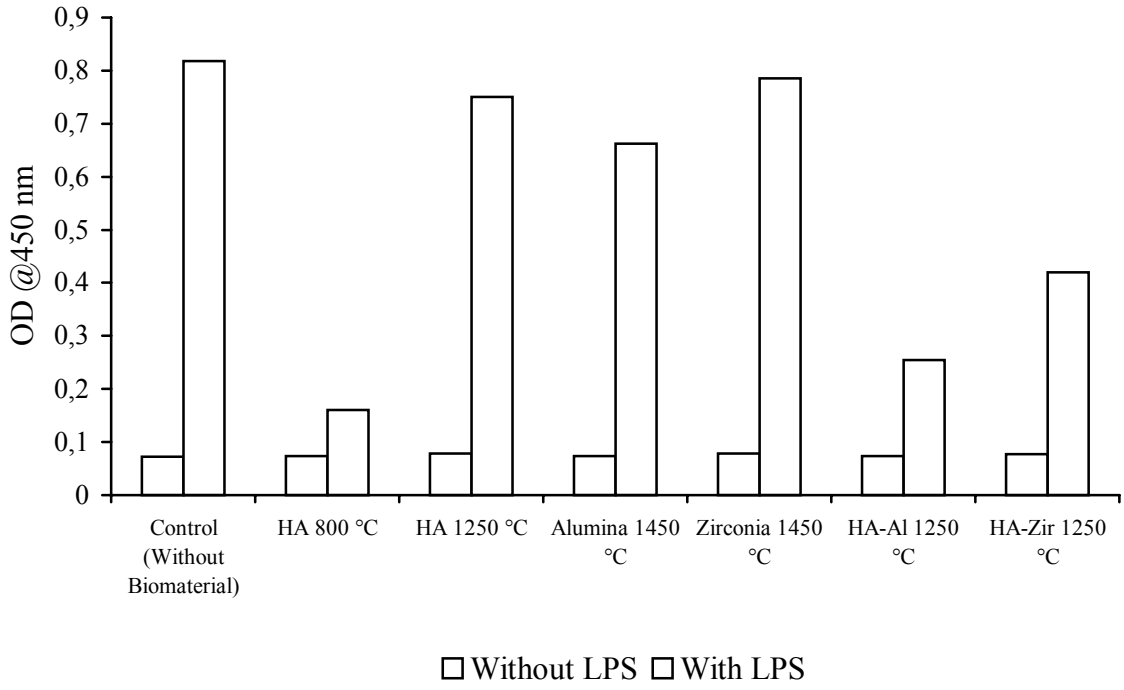


Figure 4.6. IL-1α secretion from PBMC in the presence of ceramics. PBMC (2.5×10^5 cells/well in 24-well plates) were treated with ceramic samples and ceramic-ceramic composite (HA-Alumina, HA-Zirconia) samples (15 mm in diameter) for 17 hours with and without LPS (10 μg/ml). Supernatants were collected after centrifugation. The level of IL-1α secretion from PBMC in the presence of test samples were determined as described in Materials and Methods Section. OD values directly represents IL-1α secretions. Data are the average of duplicate samples from the same experiment.

The effects of protein adsorbed ceramic, metallic and polymeric materials on the secretion of IL-1α from PBMC were examined with and without LPS stimulation and the cells from different donors (Table B7). Because BSA adsorption onto ceramic samples especially HA 800 °C pellets increased the viability of PBMC when compared to uncoated HA 800 °C pellets, the influence of BSA adsorption on HA 800 °C pellets on the IL-1α secretion from PBMC was investigated. In the absence of LPS, BSA coated HA 800 °C pellets led to 3-fold higher secretion of IL-1α than the control culture. IL-1α secretion in BSA-coated HA 1250 °C, alumina 1450 °C and zirconia 1450 °C pellets was 1.7-, 1.6- and 1.8-fold higher than in the control, respectively. However, in the presence of LPS, BSA-coated HA 800 °C pellets led to 30% decrease in the secretion of IL-1α. BSA-coated HA 1250 °C, alumina 1450 °C and zirconia 1450 °C pellets caused 1.1-, 1.1- and 1.2-fold increase, respectively, in the secretion of IL-1α (Figure 4.7). The results from BSA-coated and uncoated ceramic samples indicated that in the absence of LPS, IL-1α secretion was higher in the cells treated with HA 800 °C than the control. However, in the presence of LPS, IL-1α secretion in HA 800 °C treated cells was less than the control. It is probable that the dissolution of HA particles or ions affected the stimulatory effects of LPS on PMBC.

Metallic and polymeric samples were incubated with the cells as described above. In the absence of LPS, there was no significant change in IL-1α secretion compared to the control culture. Whereas, in the presence of LPS, these materials resulted in 30%, 10% and 23% decrease respectively, in IL-1α levels (Table B7 and Figure 4.7). As in the case of ceramic samples, ions or other factors might affect the secretion of IL-1α from PBMC in the presence of metallic samples and LPS.

The amount of IL-1α secretions (pg/ml) was calculated from the formula of calibration curve (Figure B1). Uncoated HA 800 °C pellets showed the lowest secretion and BSA-coated ceramic pellets (HA 1250 °C, alumina 1450 °C and zirconia 1450 °C pellets) showed the highest IL-1α secretions from PBMC (Table B8). Low secretion in HA 800 °C pellets might result from death of cells. Because, low cell viabilities in HA 800 °C pellets were observed (Figure 4.1,Table B1). IL-1 plays important roles in immune functions especially on monocyte/macrophages and lymphocytes. IL-1 induce

its own synthesis as well as secretion of TNF and IL-6 secretions from monocyte/macrophages. Normal production of IL-1 is obviously critical to mediation of normal host responses to injury and infection. However, inappropriate or prolonged secretions of IL-1 play roles in the formation of pathological conditions including sepsis, rheumatoid arthritis, inflammatory bowel disease, acute and chronic myelogenous leukemia, insulin-dependent diabetes mellitus, and atherosclerosis. Therefore, low levels of IL-1 secretion in the presence of biomaterials in the body is the desired condition. Biomaterials tested in the present study did not cause high production of IL-1α secretions compared to the control cultures indicating that these biomaterials are biologically compatible in this regard.

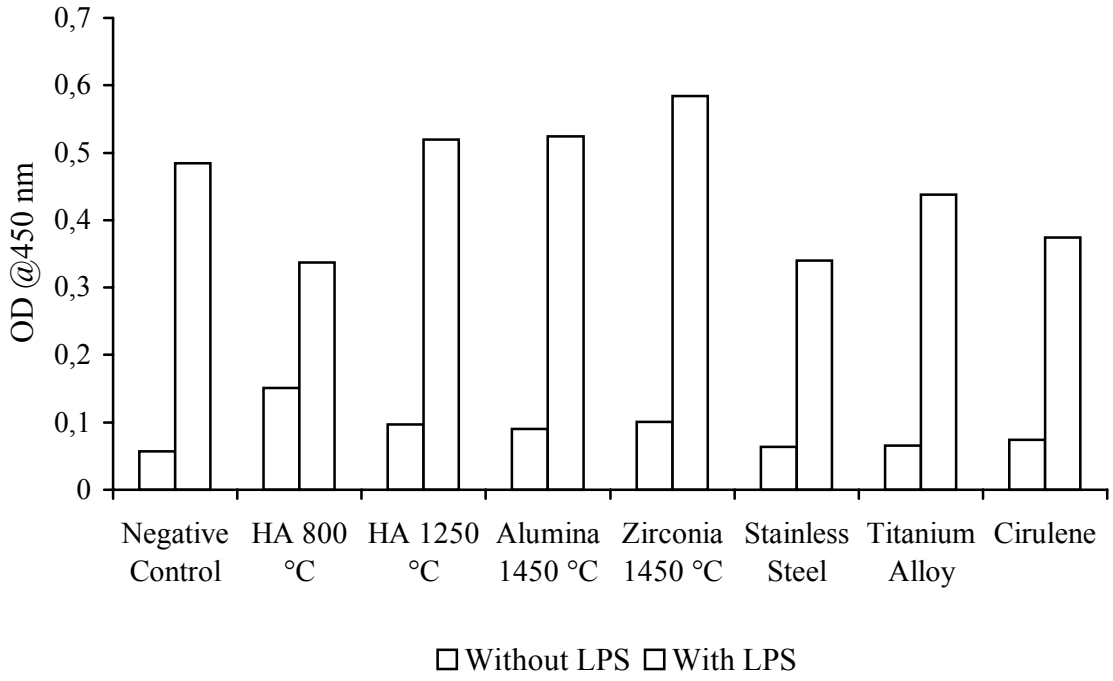


Figure 4.7. IL-1α secretion from PBMC in the presence of protein coated ceramic, metallic and polymeric samples. Ceramic samples were coated with BSA (20 mg/ml in sodium phosphate buffer (pH 7.3)). PBMC (2×10^5 cells/well in 24-well plate) were incubated for 17 hours with 15 mm in diameter BSA-coated ceramic pellets and 14 mm in diameter metallic and polymeric samples. LPS (10μg/ml) was added to one set of samples. The level of IL-1α secretion from cells was determined by using Quantikine® human IL-1α ELISA kit. Data are the average of duplicate samples from the same experiment.

In addition, IL-6 secretions from PBMC treated with biomaterials in the presence and absence of LPS were determined. IL-6 production of cells after treatment with ceramic pellets or ceramic-ceramic composites is shown in Figure 4.8 and Table B9. The level of IL-6 was the lowest in HA 800 °C treated cells (2.8-fold less than the control culture). In comparison with other biomaterials and the control culture, zirconia 1450 °C pellet gave rise to the highest IL-6 production, which is 1.14-fold higher than that of the control culture. In stimulated cells with LPS, the absorbance values for IL-6 production were higher than the limit of automated plate reader at 450 nm. Therefore, any data could not be taken from these samples.

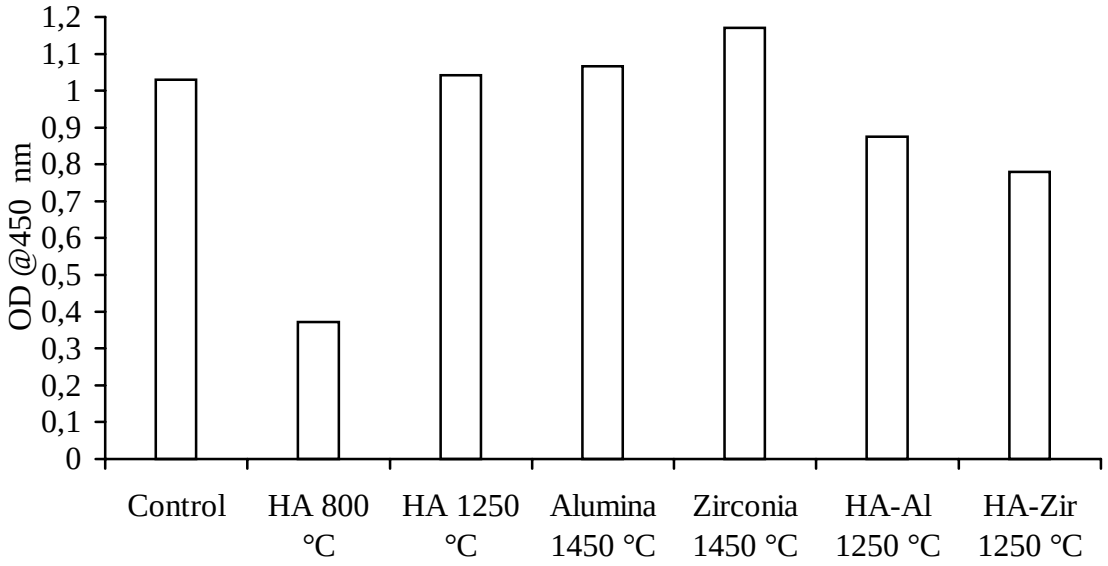


Figure 4.8. Effects of ceramic biomaterials on the secretion of IL-6 from PBMC. PBMC (2.5×10^5 cells/well) (in the absence of LPS) were incubated with the ceramic pellets for 17 hours. The cells were precipitated by centrifugation at 15,000 rpm for 30 seconds. The supernatants were collected and tested for IL-6 secretion. IL-6 levels were determined by using Quantikine® human IL-6 ELISA kit. Optical densities at 450 nm directly represent IL-6 secretions. Data are the average of two separate experiments.

Moreover, the effects of BSA adsorption on ceramic samples on IL-6 production were tested. In the absence of LPS, both BSA-coated HA 800 °C and alumina 1450 °C pellets led to IL-6 production 7.5-fold higher than the control culture (Figure 4.9 and Table B10). In BSA-coated HA 1250°C and zirconia 1450 °C pellets, automated plate reader could not read absorbance values at 450 nm because they were out of range. In the presence of LPS, all samples were diluted with a factor of 4 in order to be able to obtain data. When IL-6 secretion was examined for the cells treated with BSA-coated ceramic samples in the presence of LPS, no significant differences were detected between the control culture and BSA-coated ceramic treated cultures. However, only in BSA-coated HA 800 °C pellet, IL-6 production was 26% less than the control culture in the presence of LPS. This result could be due to a decrease in the cell viability or the positive effect of protein coating.

Metallic samples (stainless steel, titanium alloy and DLC coated AISI 52100) and polymeric material (cirulene) were tested for the effects on IL-6 secretion from PBMC. In stainless steel and cirulene samples, 21% and 8% less IL-6 production than the control culture; in titanium alloy and DLC coated sample 16% and 144% higher IL-6 production than the control were obtained. In stimulated cells with LPS, samples were diluted with the factor of 4. In stainless steel, titanium alloy and cirulene samples 12%, 9% and 9% less IL-6 levels were detected in duplicate samples, respectively (Figure 4.9 and Table B10).

The amount of IL-6 (pg/ml) in the presence of biomaterials and LPS was calculated from the formula of calibration curve (Figure B2). The results are presented in Table B11.

As a result, BSA-coated HA 800 °C pellets increased IL-6 secretion compared to uncoated pellets. However, in HA 800 °C pellets released ions or particles affected the stimulatory role of LPS on PBMC. In metallic samples, low IL-6 levels were obtained with and without LPS. Only in DLC coated sample, higher IL-6 secretion than the other metallic samples and the control culture. Therefore, DLC coating might stimulate IL-6 secretion from the cells. In biomaterial applications, low levels of IL-6 are the desired condition, because IL-6 plays important roles in inflammation. High levels of IL-6 may indicate the inflammatory responses in the body. IL-6 has stimulatory effects on other

cells such as T-cells. High or prolonged secretion of this cytokine is seen in many diseases including cancers.

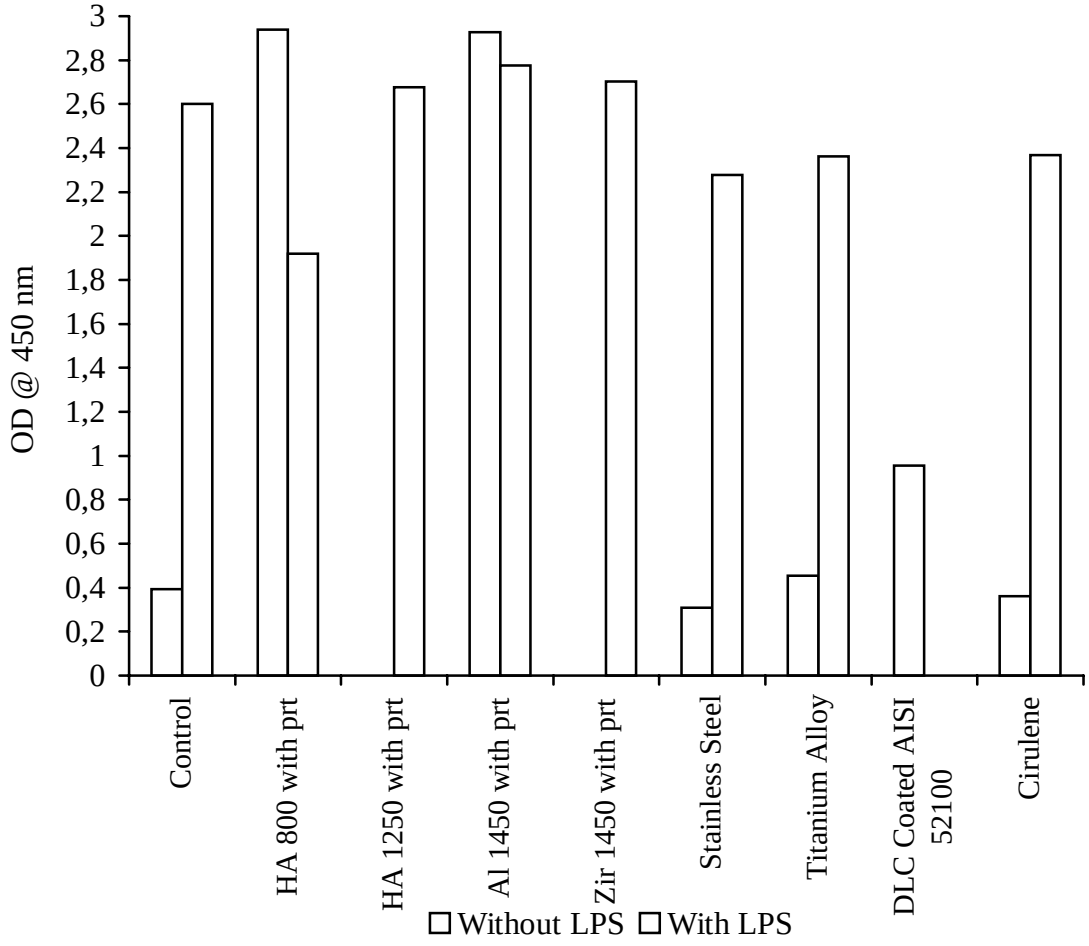


Figure 4.9. The effects of BSA-coated ceramic pellets, metallic and polymeric biomaterials on the secretion of IL-6 from PBMC. Protein coated ceramic pellets, metallic samples and polymeric sample were incubated with 1ml PBMC (2.5×10^5 cells/well in 24-well plates) with and without LPS (10 μg/ml) for 17 hours. The cells were precipitated by centrifugation and supernatants were collected. IL-6 secretion was determined by using Quantikine® human IL-6 ELISA kit. Data are the average of duplicate samples from one experiment. The samples with LPS diluted with a factor of 4.

Mononuclear cell cultures are used to determine the immune specific and aspecific responses to implant materials. Some materials can evoke immune reactions and it is required to remove implant from the body [108]. A variety of cytokines released from lymphocytes and monocytes/macrophages around an implant affect the response of tissues to the implant. In fact, bone remodelling is regulated through the release of cytokines stimulating bone resorption by the mononuclear cells [109,110]. Chiba *et al.* reported an enhanced activity of IL-1, IL-6 and TNF in tissues surrounding the femoral component of failed cementless total hip arthroplasties [111]. Chromium is a major alloying element in cobalt-chrome alloys and stainless steel. Granchi *et al*. showed that in unstimulated PBMC chromium ions slightly increased the release of IL-6 and TNF-α, even though the increase was not significant. However, the different concentrations of chromium extract significantly inhibited the response to phytohemagglutinin (PHA) stimulation, as shown by the decrease in IL-6 and sIL-2r release, and by the influence on cell viability and DNA synthesis [112]. These results are in agreement with our results in the metallic samples.

4. 3. Effects of Biomaterials on The Proliferation of PBMC

The proliferation of PBMC was determined by using Cell proliferation ELISA kit (Amersham Life Sciences). Concanavalin A (Con A) was used as a mitogen in the assay. For the detection of effective concentration of Con A on PBMC, different concentrations of Con A was incubated with the cells. At 200 μg/ml concentration of Con A, the proliferation of PBMC was maximum (Figure 4.10). Higher concentrations resulted in a decrease in the proliferation of cells. Therefore, in the test of ceramic materials, the optimum Con A concentration, 200 μg/ml, was used.

PBMC were incubated with bioceramic pellets (5 mm in diameter) in the presence and absence of Con A. When compared to the control culture, there was a slight increase (1.12-fold) in the proliferation of PBMC incubated with HA 800 °C (Figure 4.11;Table B12). On the other hand, HA-Alumina 1250 °C and HA-Zirconia 1250 °C caused 1.8-fold and 2.2-fold decrease, respectively in the cell proliferation. Other ceramic samples resulted in the cell proliferation at about the same level of the control culture. In the presence of Con A, all ceramic samples gave rise to a decrease in the proliferation of cells. Especially in the cells treated with HA 800 °C, HA-Alumina

1250 °C and HA-Zirconia 1250 °C samples, there was 3.1-fold, 5.7-fold and 5.7-fold decrease, respectively, in the cell proliferation compared to the control culture (Figure 4.11;Table B12). In addition, in the presence of Con A, the proliferation of HA 1250 °C, alumina 1450 °C and zirconia 1450 °C treated cells was 1.12-fold, 1.44-fold and 1.31-fold less than that of the control culture. Interestingly, in the presence of mitogenic agent Con A, lower cell proliferations than the control culture in the ceramic samples were obtained. As in the case of cytokine secretions in the presence of LPS, dissolution of ceramics, released particles or ions might affect the action of these stimulatory agents. Low cell proliferation in HA 800 °C pellets could be explained as death of cells, in other words, low cell viability. However, in other ceramic samples appropriate cell viabilities were obtained with respect to the control cultures. These results indicated that HA 800 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C samples have inhibitory effects on the proliferation of PBMC. Therefore, these samples are not biocompatible on the parameter of cell proliferation. Because the proliferation of cells after mitogen stimulation is indication of immune cell function.

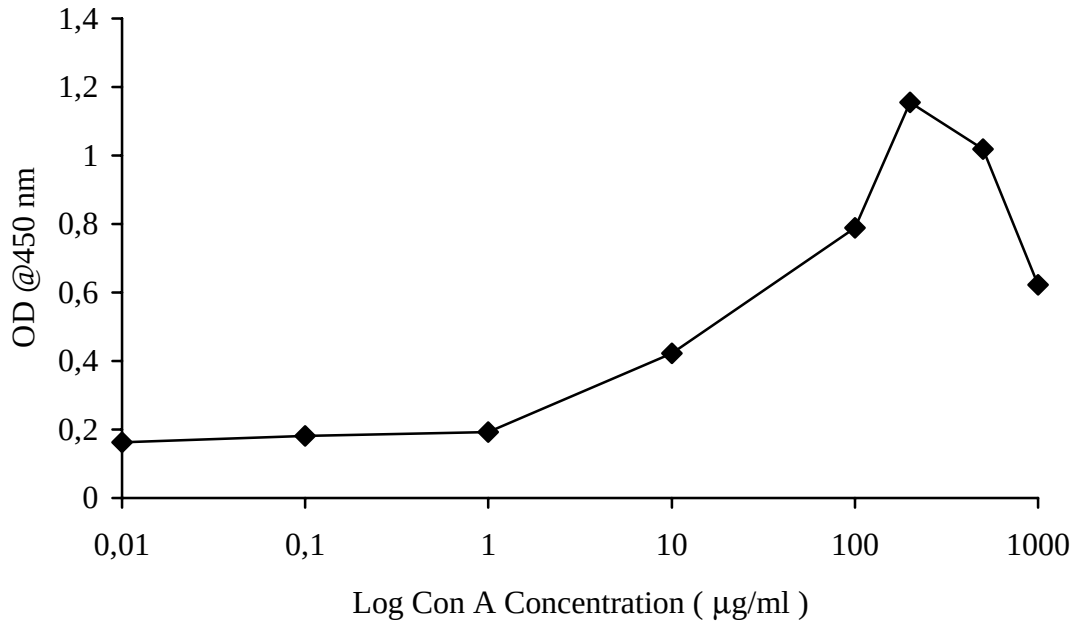


Figure 4.10. Stimulation of PBMC with Con A.

PBMC (2×10^5 cells/well in 96-well plate) were cultured in the presence different concentrations of Con A between 0.01 and 1000 μg/ml. Proliferation of cells were determined by Biotrak Cell Proliferation ELISA kit. Optical densities at 450 nm directly represent the proliferation of PBMC. At 200 μl/ml concentration maximum cell proliferation was detected. Data are the average of duplicate samples from the same experiment.

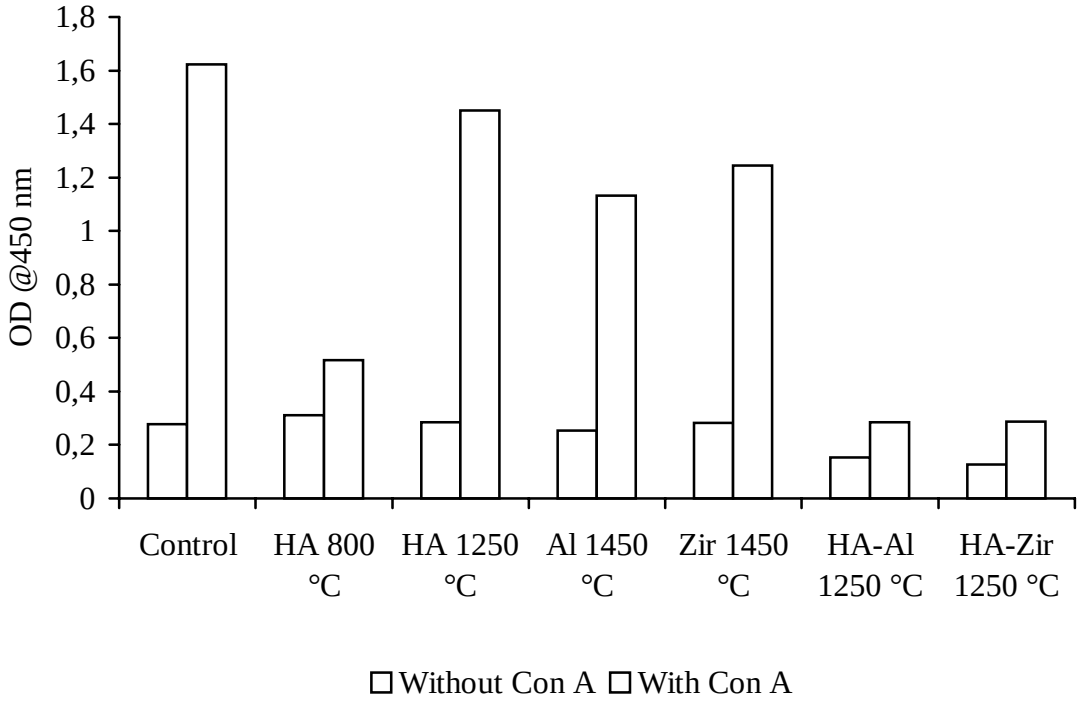


Figure 4.11. Effects of bioceramics on proliferation of PBMC. PBMC (8×10^4 cells/well) were cultured with ceramic pellets (5 mm in diameter) for 72 hours. BrdU was added 24 hours before the end of incubation period. The incorporation of BrdU in proliferating cells was determined with peroxidase-labelled anti-BrdU antibodies. Optical densities at 450 nm were determined after addition of TMB substrate. OD values directly represent the proliferation of PBMC. Con A stimulates the cell proliferation. Effects of ceramic pellets in the presence and absence of Con A (200 μg/ml) were compared. Data are the average of duplicate samples. Test was repeated with the similar results.

4. 4. Effects of Biomaterials on The Activation of PBMC

The activation of PBMC was examined by using MTT assay. For this purpose, ceramic pellets (5 mm in diameter) at different sintering temperatures between 800 °C and 1450 °C and their extracts in PBS (pH 7.4) were used. PBMC were incubated with the samples at optimum cell culture conditions for 24, 48 and 72 hours. MTT in active cells is converted to insoluble formazan crystals that are dissolved in DMSO and the absorbance of formed purple solutions directly represents the cellular activity. After 24 hours, in HA 800 °C, HA 900 °C and HA 1250 °C pellets 24%, 13% and 5% lower absorbance values than the control culture were obtained. On the contrary, in HA 1100 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C pellets 11%, 21% and 47% increase in the absorbance values were recorded. After 48 hours and 72 hours, all tested ceramic samples showed lower response than the control cultures (Figure 4.12).

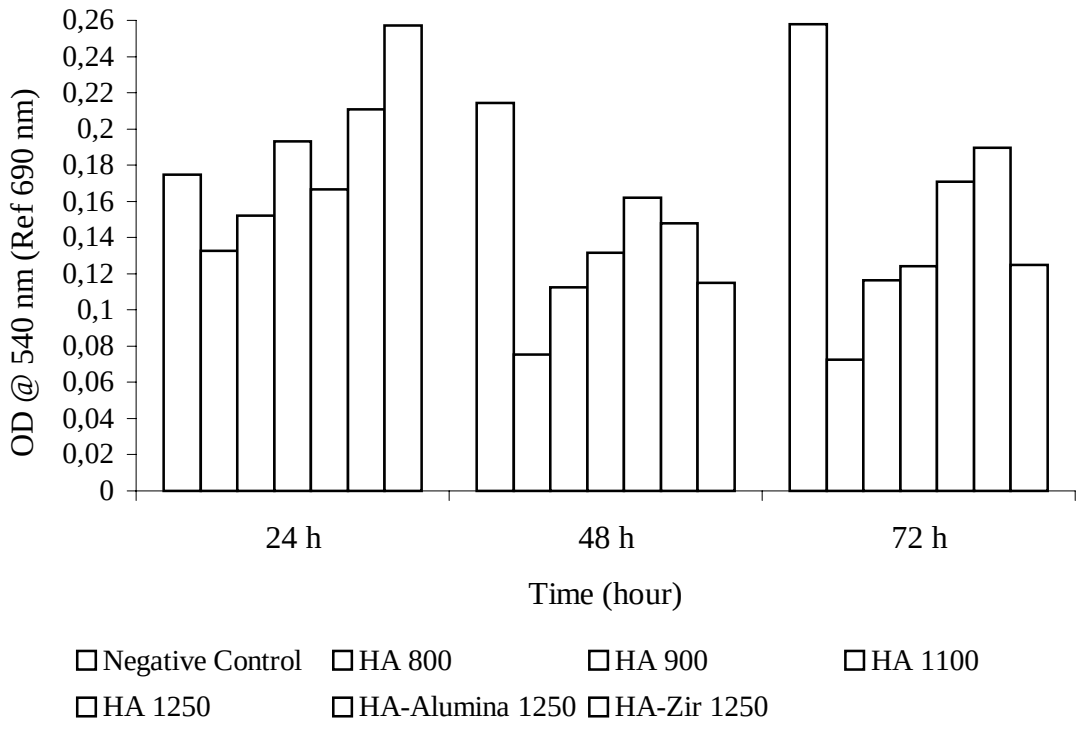


Figure 4.12. Activation of PBMC in the presence on Hydroxyapatite samples. PBMC (1×10^5 cells/well in 96-well plates) were cultured in the presence of HA 800 °C, HA 900 °C, HA 1100 °C, HA 1250 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C pellets (5 mm in diameter) at 37 °C with 5% CO_2 for 24, 48 and 72 hours.

Cell suspensions were highlighted and transferred into blank wells. 3 hours before the end of incubation periods, MTT (5 mg/ml) was added into wells at 1:10 ratio with the culture medium. Formazan crystals were solubilized with DMSO after precipitation of cells by centrifugation at 1800 rpm for 10 minutes. Optical densities were read at 540 nm (reference wavelength 690 nm). Data are the average of duplicate samples from the same experiment.

Extracts of bioceramics at different concentrations and at different time points were tested to examine their effects on the activation of PBMC. In HA 800 °C extracts, after 24 hours, the cells were activated at all concentrations. However, the activation of cells decreased by increasing concentrations of the extracts. After 48 and 72 hours, the absorbance values were lower than the control cultures without any biomaterials. In HA 900 °C extracts, like HA 800 °C samples, after 24 hours, an activation of cells were observed with respect to the control. However, after 48 and 72 hours, the results were lower than the control. After 24 hours incubation of HA 1000 °C and HA 1250 °C extracts with the cells, an activation of cells were obtained at all concentrations. At 48 and 72 hours time points, the results were almost equal to the controls. In the case of alumina 1450 °C and zirconia 1450 °C extracts, at the 24 hours, higher absorbance values than the control were recorded. After 48 and 72 hours, the results were lower than the controls (Figure 4.13). As a result of these data, the extracts of ceramics activated PBMC in 24 hours and at low concentrations. Especially, at 5 µl and 10 µl of HA 800 °C, HA 900 °C, HA 1250 °C and alumina 1450 °C samples higher absorbance values than the controls were obtained. Prolonged exposure to the extracts may become toxic for cells and cells may start to die. Because MTT assay measures cytotoxic effects of test samples, low absorbance values at 540 nm represent the cytotoxic effects of extracts. Taken together, these results showed that even though there is a fluctuation in the OD levels of MTT assay based on prolonged exposure or concentration of the extracts, the difference among OD levels of samples is relatively low. Therefore, it can be concluded that biomaterials used in this assay are not toxic to PBMC.

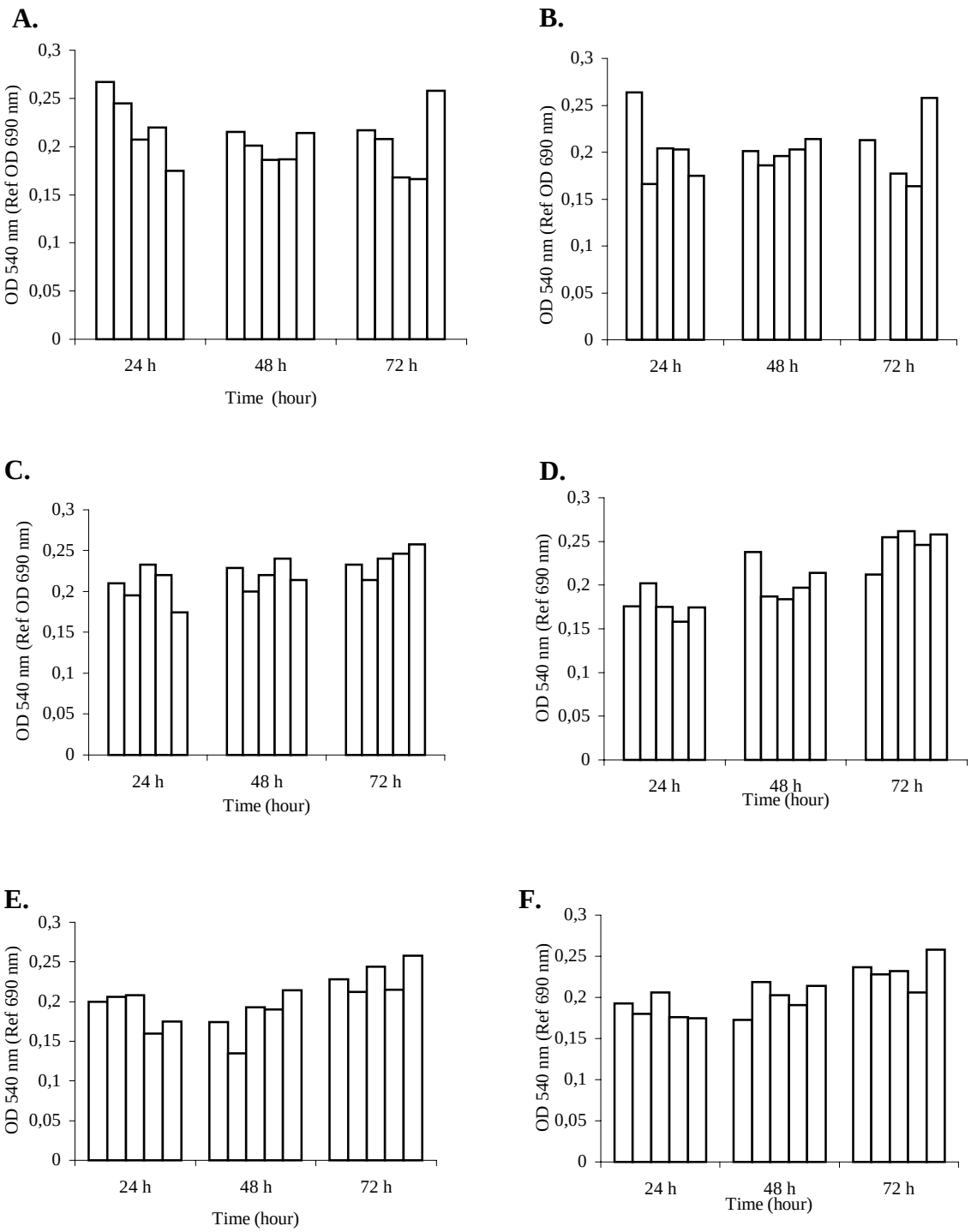


Figure 4.13. Activation of PBMC in the presence of extracts of ceramic materials.

PBMC (1×10^5 cells/well) were incubated with the extracts of HA 800 °C (A), HA 900 °C (B), HA 1000 °C (C), HA 1250 °C (D), Alumina 1450 °C (E) and Zirconia 1450 °C (F) with concentrations of 5 µl, 10 µl, 20 µl and 40 µl at 37 °C with 5% CO_2 for 24, 48 and 72 hours (Columns in the graphs represent concentrations of extracts and the last one is the control culture without any extract of biomaterials, respectively). 3 hours before the end of incubation periods, MTT (5 mg/ml) was added into wells at 1:10 ratio with the culture medium. Formazan crystals were solubilized with DMSO after precipitation of cells by centrifugation at 1800 rpm for 10 minutes. Optical densities were read at 540 nm (reference wavelength 690 nm). Data adapted from one set of experiment.

Cytotoxicity tests are recommended for all biomaterials. These tests allow for a rapid evaluation and to obtain quantitative and comparable data with less expensive results than animal testing. In addition, due to sensitivity of the tests, they allow for discarding toxic materials prior to animal testing. Experimental studies showed good correlation between *in vitro* and *in vivo* cytotoxicity tests. The cells *in vitro* are more sensitive to toxic materials and substances than *in vivo* tissues. Therefore, a material toxic *in vitro* may result not actually toxic for the tissues *in vivo*, even in long-lasting assays [108].

4. 5. Dissolution Properties of Biomaterials

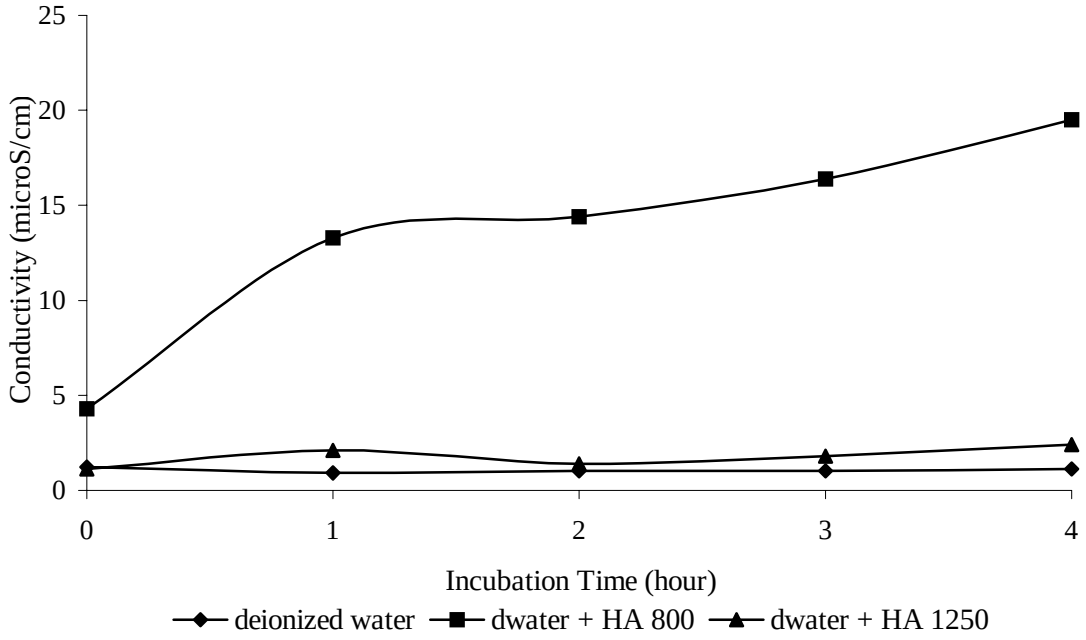
There are two main classes of ceramics used as biomaterials: Structural and resorbable (or soluble). Alumina and zirconia are structural ceramics. Calcium hydroxyapatite, tricalcium phosphate, octacalcium phosphate are the soluble ceramics. Studies on the biomaterials-cell interactions, HA 800 °C pellets and the extracts showed low responses or undesired results with respect to the controls, other ceramic and metallic samples. The reasons of the negative effects of HA 800 °C samples were examined in this part. For this purpose, HA 800 °C, HA 1250 °C, alumina 1450 °C and zirconia 1450 °C pellets in 15 mm in diameter were incubated in the culture medium (RPMI-1640 containing 15% FBS and 50 µg/ml gentamycin sulfate) with and without PBMC and in deionized water for 4 hours, 72 hours and 10 days periods. A sample

solution was collected at the end of time points and it was diluted with sterile deionized water. The conductivity and pH of dilutions were measured.

In the first 4 hours, the data were taken at 1-hour intervals. Dilutions were carried out and measurements were taken immediately. Figure 4.14-A represents ionic alteration in deionized water. In 4 hours period, HA 800 °C pellets led to an increase in the conductivity. In HA 1250 °C pellets almost same results with deionized water were obtained. Also, this test was carried out for long period of time. In 10 days period, an increase in the conductivity of HA 800 °C pellets in deionized water were found. However, HA 1250 °C, zirconia 1450 °C pellets and in deionized water did not change the conductivity up to 10 days (Figure 4.14-B).

Effects of ceramic materials on conductivity were examined in the cell culture medium with and without cells. PBMC may act on ceramic samples to degrade them by phagocytosis. These events can change the ionic environment. In 4 hours period, there was a fluctuation in the data, but there was no sharp increase or decrease in the conductivity values of all samples (Figure 4.15-A). In 72 hours period, the culture medium without and with the cells containing HA 800 °C pellets showed an increase in the conductivity values with time (Figure 4.15-B). In HA 1250 °C pellets, there was some change in the conductivity at different time points, whereas almost no change in HA 1250 °C pellets was obtained in the conductivity at 72 hours. RPMI-1640 medium and the culture medium with the cells slightly increased the conductivity. The reason of that might be the production of waste products by cells or depletion of some minerals or ions in medium with time. HA 800 °C pellets have porous structure and they are not dense as much as HA 1250 °C pellets. Due to density of HA 1250 °C, alumina 1450 °C and zirconia 1450 °C pellets, the release of particles or ions were less than HA 800 °C pellets.

A.



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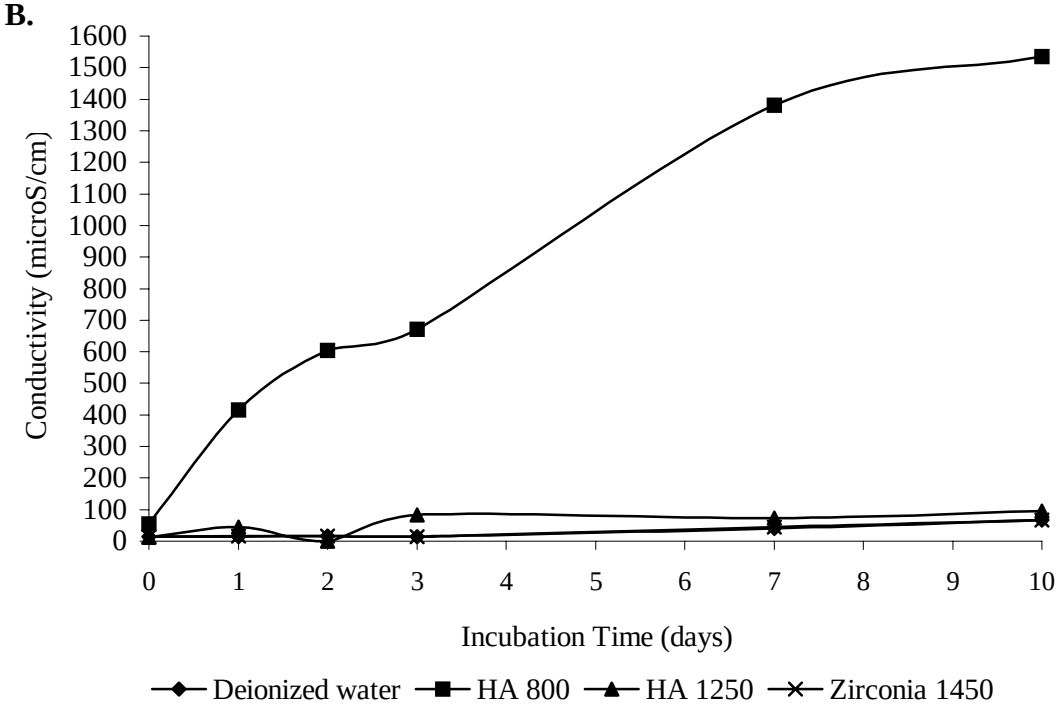


Figure 4.14. Conductivity measurement of Hydroxyapatite pellets in deionized water. The pellets were incubated in deionized water at 37 °C with 5% CO_2 for 4 hours. The samples were diluted with the factor of 25 (A). Ceramic pellets were incubated for 10 days. The measurements were multiplied with the dilution factors (B). Data represent the values from one set of experiment.

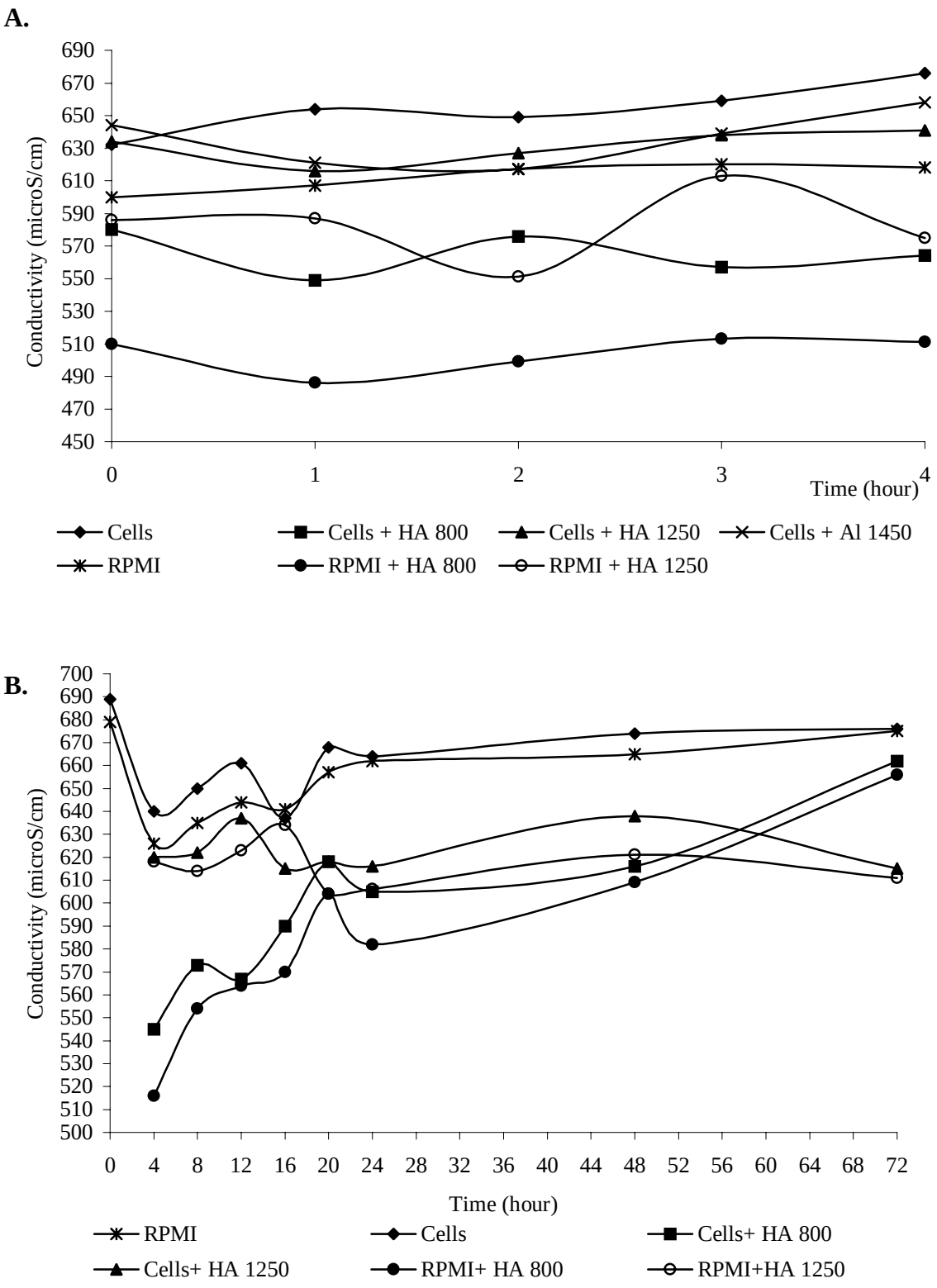
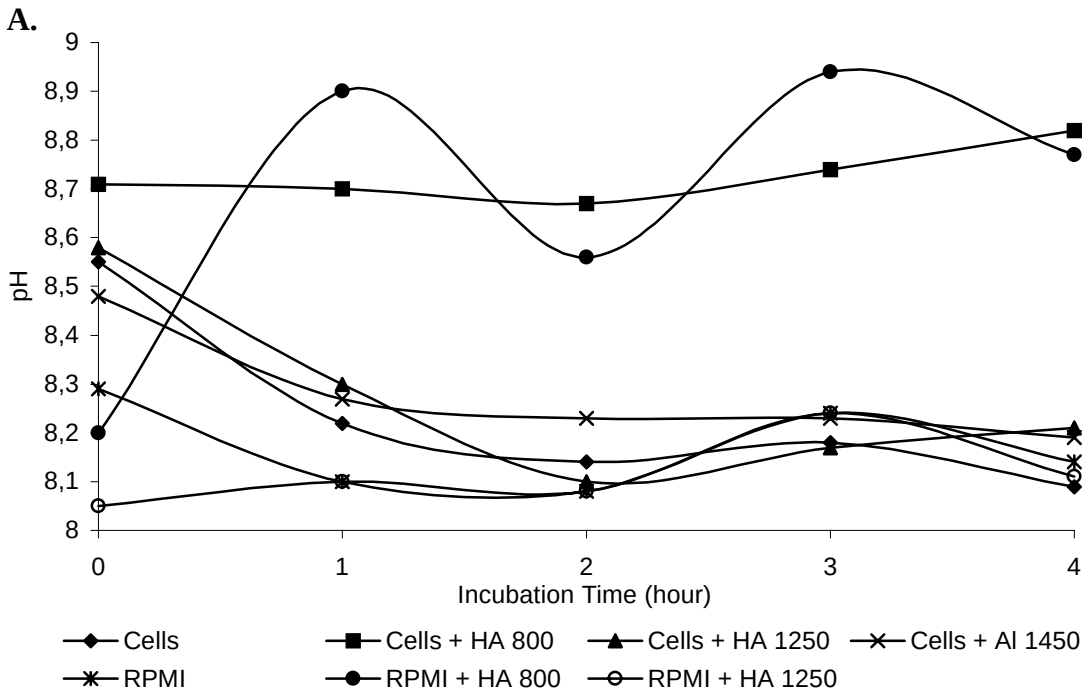


Figure 4.15. Conductivity changes of ceramic samples in the culture medium.

HA 800 °C and HA 1250 °C and alumina 1450 °C pellets were incubated in RPMI-1640 with and without PBMC (2.5×10^5 cells/well in 24-well plates) at 37 °C with 5% CO_2 for 4 hours (A) and 72 hours (B). 1 ml of sample was diluted with 24 ml deionized water and conductivity was measured by using WTW Inolab Cond Level 2P conductivity meter. Data were obtained from one set of experiment.

In addition to conductivity measurements, pH of diluted solutions was measured. In the data, a fluctuation in pH values was obtained. Especially, due to deionized water, a stable value could not be obtained. At 72 hours period, there was a slight increase from 8.5 to 8.8 in the pH of dilutions of HA 800 °C in RPMI-1640 and in the culture medium with PBMC. However, in HA 1250 °C samples in RPMI-1640 with and without cells, there was almost no change in pH (7.92 at 4 hours, 8.07 at 72 hours in RPMI-1640 with cells and 8.04 at 4 hours, 8.12 at 72 hours in RPMI-1640 without cells) (Figure 4.16-A and B).



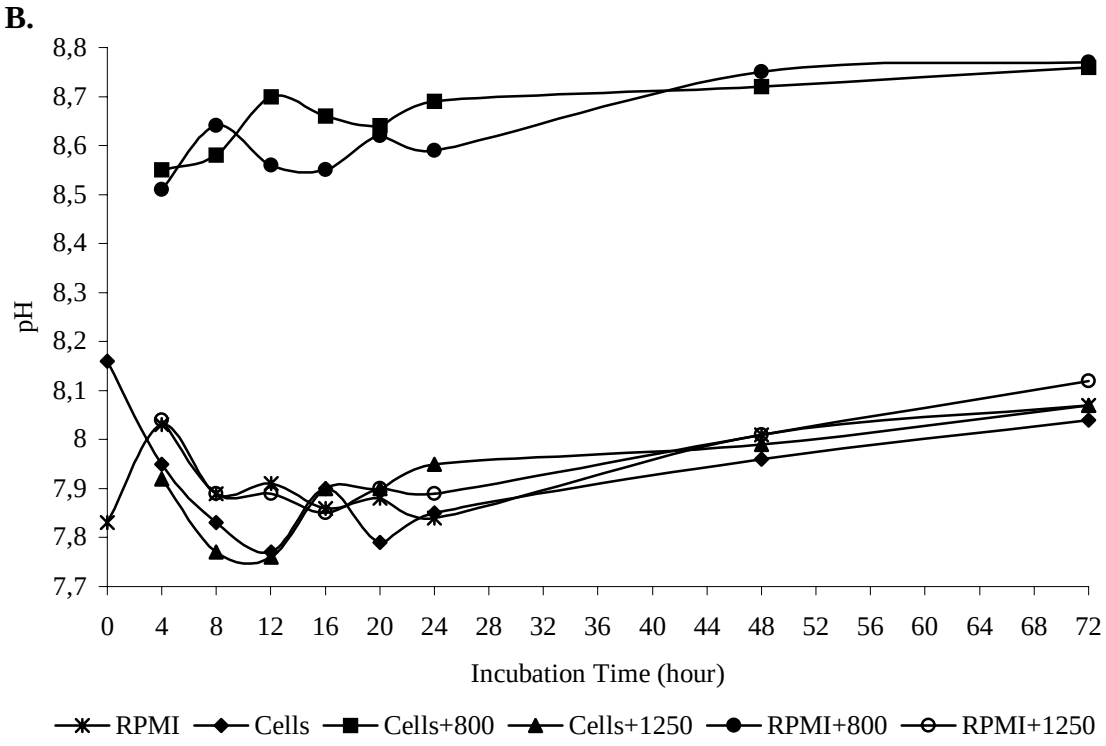


Figure 4.16. pH variance of Hydroxyapatite samples in the culture medium with and without PBMC. Hydroxyapatite pellets in 15 mm diameter were incubated in RPMI-1640 medium with and without cells (2.5×10^5 cells/well in 24-well plate) at 37 °C with 5% CO_2 for 4 hours (A) and 72 hours (B). 1 ml of sample was diluted with 24 ml deionized water. The pH of samples was measured by using Hanna Instruments HI 9321 microprocessor pH meter. Data were obtained from one set of experiment.

pH measurements were also taken in long period of time. In 10 day, the pH of dilutions containing HA 800 °C and HA 1250 °C pellets in deionized water were determined as in Figure 4.17. In HA 800 °C pellets, an increase was observed in 3 days. However, after 3 days, pH values became almost stationary. Due to evaporation of water at 37 °C in long period of incubation time, high dilutions were carried out to measure both conductivity and pH. The reason of slight decrease after 3 days in HA 800 °C pellets might be due to these dilutions. In zirconia 1450 °C samples, there was no change in the pH value. In other words, it was same as in HA 1250 °C pellets, an increase in the pH values was observed. But, with time pH decreased and closed to

beginning pH (at time 0). In short, there was no change in HA 1250 °C pellets at the end of 10 days (Figure 4.17).

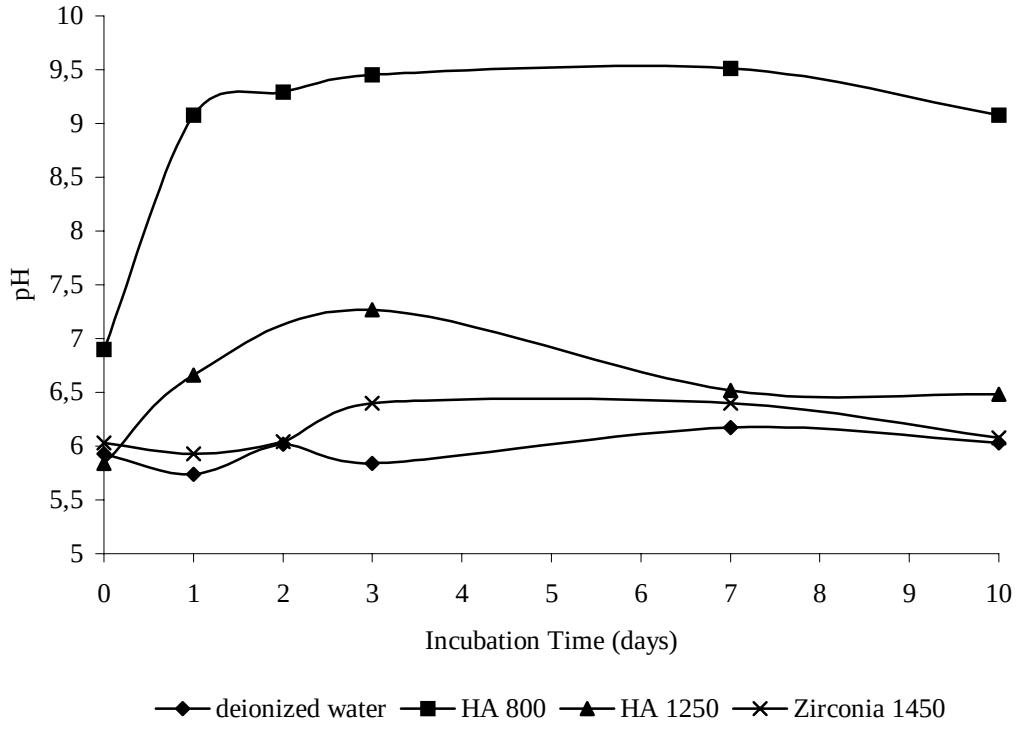


Figure 4.17. pH measurement of Hydroxyapatite pellets in deionized water. HA 800 °C, HA 1250 °C and zirconia 1450 °C pellets were incubated in deionized water at 37 °C for 10 days. Every day 2 ml of samples were diluted with 22 ml deionized water. The pH values were measured by using Hanna Instruments HI 9321 microprocessor pH meter. Data were obtained from one set of experiment.

In the literature, Suzuki *et al*. reported that biocompatible ceramics made of β–tricalcium phosphate (TCP) were found to be actively changing their surface properties in the tissue culture medium [113]. The results showed that the surface structure of TCPs was dynamically varied by not only dissolving Ca and PO_4, but also adsorbing various components dissolved in the culture medium (Eagle's minimum essential medium (MEM) and 10% FBS/MEM). The concentrations of dissolved Ca and PO_4 in distilled water showed that the TCP ceramic powder was highly soluble in distilled water. The conductivity of TCPs at the end of 6 days decreased to the values lower than that for the first day by immersing in 10% FBS/MEM. This situation explains the removal of some solutes such as proteins and minerals, from the solution by adsorbing onto TCPs in 10% FBS/MEM. In addition, pH of the control solutions of MEM and 10% FBS/MEM increased gradually day by day. Because the medium contains $NaHCO_3$, increases in pH of the control solutions seem to be caused by removal of CO_2. Similarly, the decreases in dissolved Ca in MEM and 10% FBS/MEM were considered to be resulted from binding of Ca with carbonate. Moreover, the precipitation of calcium carbonate and calcium phosphate were promoted under mild alkaline conditions [113]. In order to elucidate the dissolution properties of our tested ceramics in the culture medium with and without cells, zeta potentials of the samples, the amounts of released Ca and PO_4 to the environment and the adsorption of proteins or other minerals and vitamins from the culture medium have to be examined in detail.

4. 6. Mutagenic Potential of Biomaterials

Mutagenic effects of biomaterials were examined by applying AMES test. In this test the extracts of test samples in PBS (pH 7.4) were used. 100 µl extract was mixed with 100 µl bacterial culture. In this test, *Salmonella typhimurium* TA 100 strain was used. The number of revertant colonies with background lawn were counted after 48 hours incubation at 37 °C and compared with the positive control (sodium azide) and the negative control without any extracts. Data shown in Table 4.1 are average of replicate samples. The number of revertant colonies in the positive control was 1880. However, in the bacteria incubated with the extracts of all biomaterials, the number of revertant colonies was around that of the negative control. Based on these data, no mutagenic effects of extracts of biomaterials were detected (Figure 4.18).

Table 4.1. The number of revertant colonies of *Salmonella typhimurium* TA100

Biomaterials	TA 100 (n=2)	Biomaterials	TA 100 (n=2)
HA 800 °C	79.5	Zirconia 1450 °C	105
HA 900 °C	116	HA-Alumina 1250 °C	92.5
HA 1000 °C	127	HA-Zirconia 1250 °C	125.5
HA 1250 °C	112.5	Stainless steel (316L)	112.5
HA 1400 °C	110	Ti Alloy (Ti-6Al-4V)	105
Alumina 1000 °C	110	Sodium Azide (positive control)	1880
Alumina 1450 °C	104.5	Neg. Control (only bacteria)	104
Zirconia 1000 °C	84.5	Neg. Control without Histidine	39.5

AMES test with *Salmonella typhimurim* TA100 strain was carried out in the presence of extracts of biomaterials. 100 µl extracts were mixed with 100 µl bacterium culture and poured onto glucose plates. The inoculated plates were incubated at 37 °C for 48 hours. The revertant colonies with background lawn were counted. The mutagenic potentials of extracts of biomaterials were compared with the positive control (sodium azide) and the negative control (only bacteria). Data are the average of duplicate plates from the same set of experiment.

Figure 4.18. Mutagenic potential of biomaterials

4. 7. Adhesion of Pathogenic Bacteria to Biomaterial Surfaces

Formation of a biofilm layer on the surface of biomaterials was detected as described in Materials and Methods Section. For this purpose, *S. aureus*, *E.coli* that are the main causative agents of osteomyelitis were used. Moreover, especially in dental implants Viridans species (*S. mutants* and *S. mitis*) and in pacemaker implants KNS-centred infections are mostly observed. Therefore, these bacteria species were used to determine their adhesion to the surface of ceramic, metallic and polymeric materials. In order to observe bacteria on the surface, pellets were heated on hot plate to fix bacteria. After that, bacteria were stained with methylene blue (Figures 4.19, 4.20, 4.21, 4.22, 4.23, 4.25, 4.26 and 4.27) or acid fuchsin dyes (Figures 4.24 and 4.28). The surfaces of biomaterials were observed by a reflected light microscope and photographs were taken by using Olympus semiautomatic exposure photomicrography system and Kodak gold ultra 400/27° 35 mm film. The photographs directly represent the data. Surface roughness is an important parameter for bacterial adhesion. Especially smooth surfaces are not suitable for attachment of bacteria. Cracks, clefts and rough surface help bacteria to colonize on the surface as seen in Figures 4.19-4.28. Adhesion of pathogenic bacteria onto surface helps the formation of biomaterial-centred infections in the body. These conditions are undesired in the implant applications. These infections result in costly removal of implant material from body also affecting the patient health.

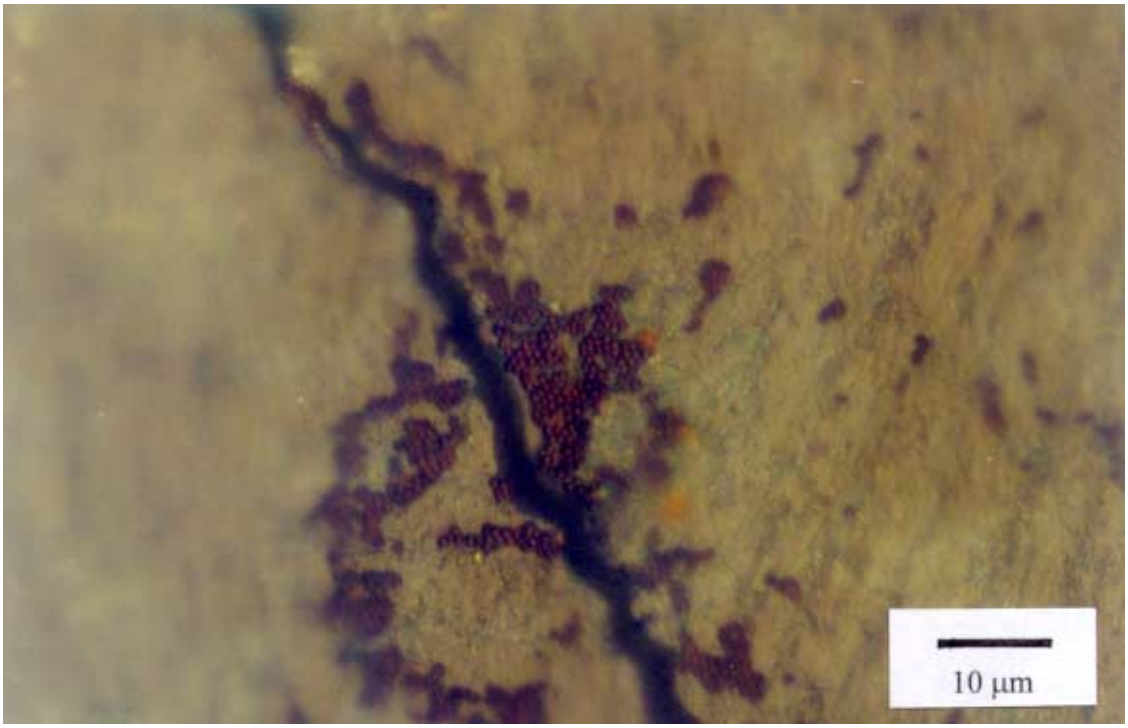


Figure 4.19. *S. aureus* on the surface of HA 800 °C pellet.

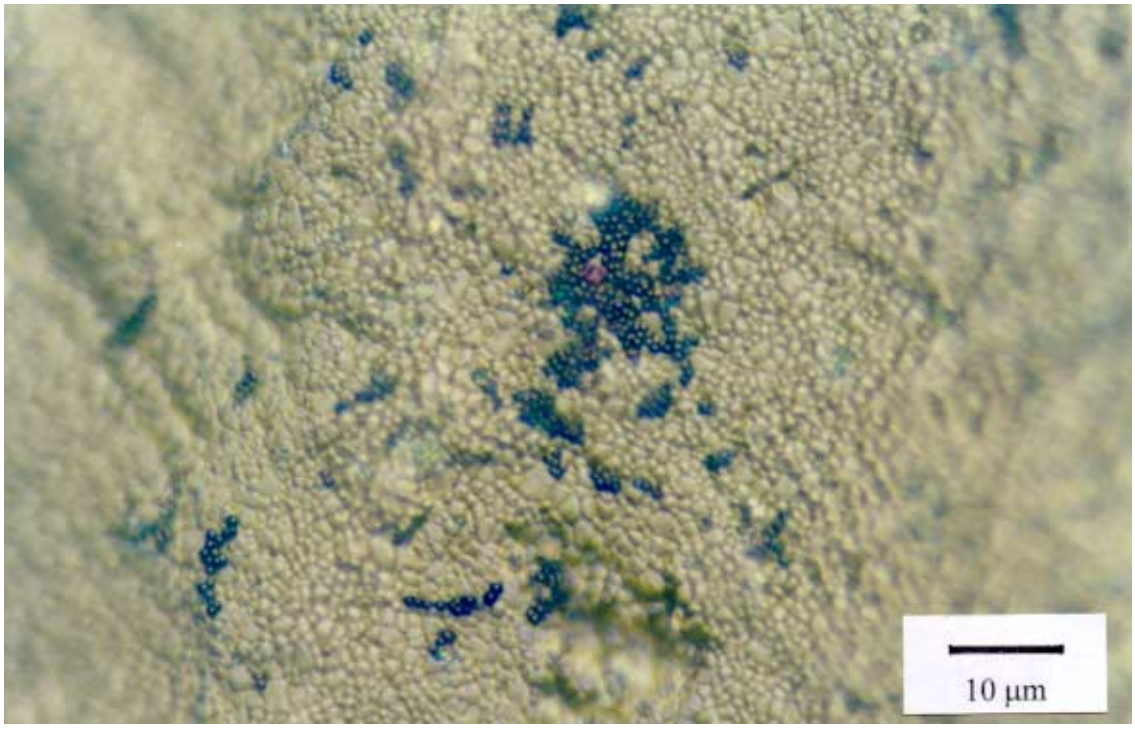


Figure 4.20. *S. aureus* on the surface of HA 1250 °C pellet.

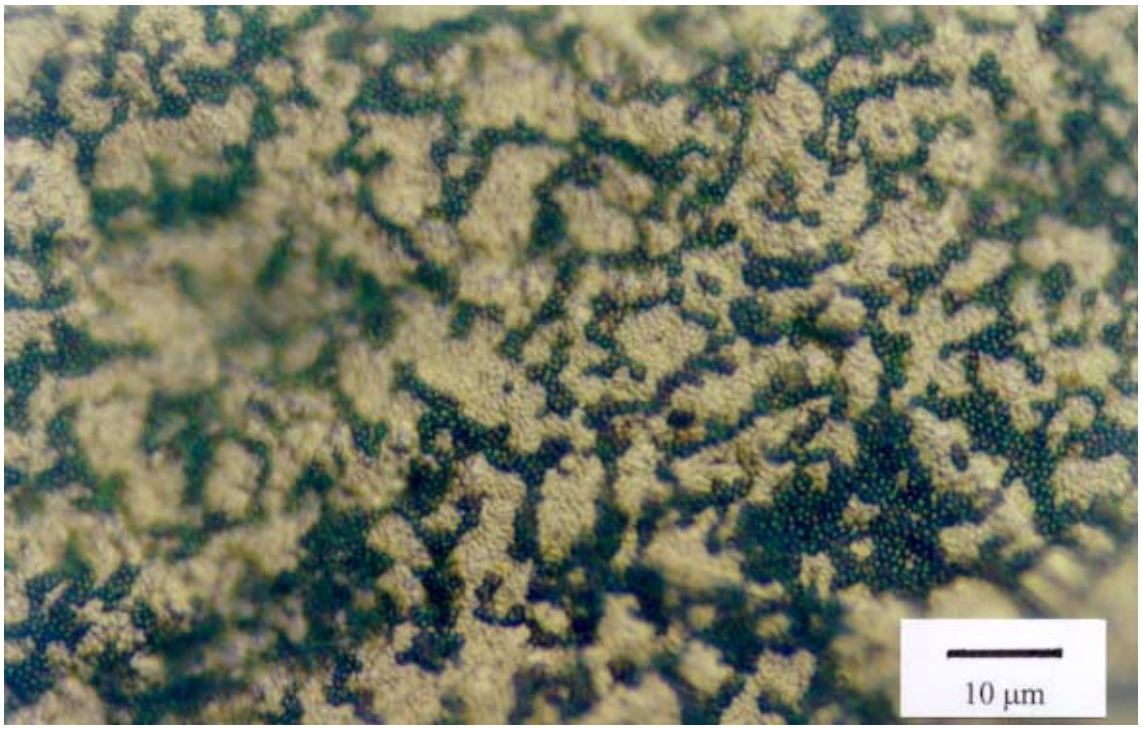


Figure 4.21. *S. aureus* on the surface of Zirconia 1450 °C pellet.

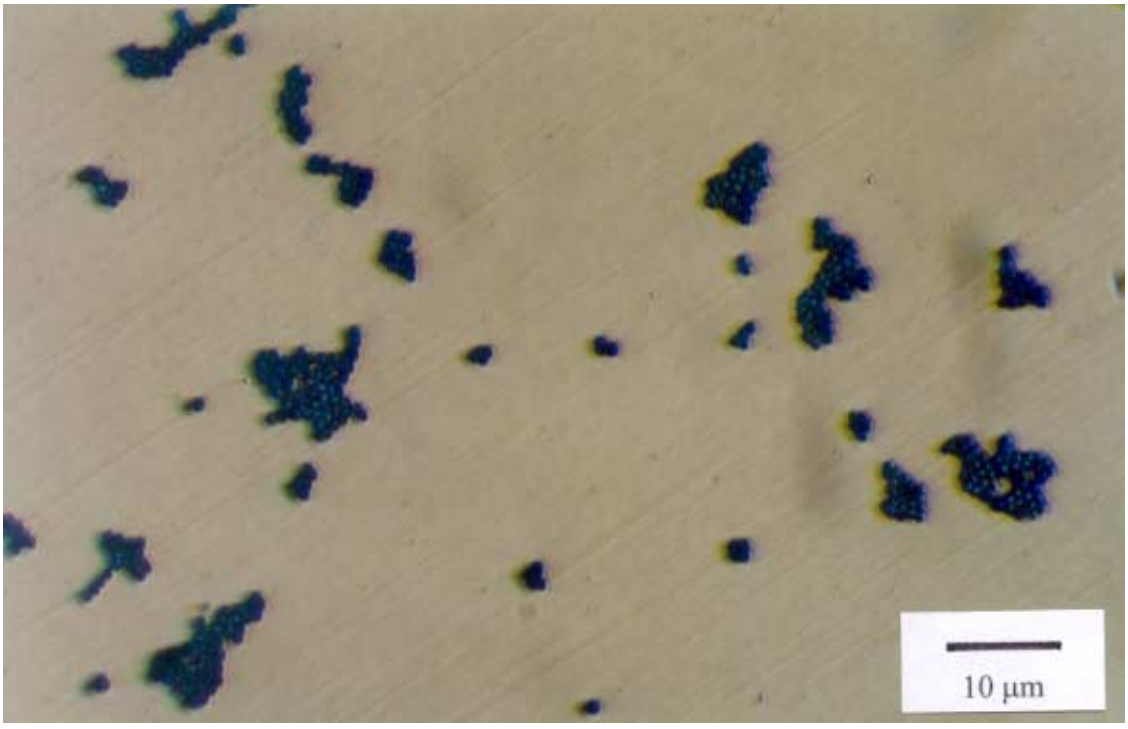


Figure 4.22. *S. aureus* on the surface of polished stainless steel (316L).

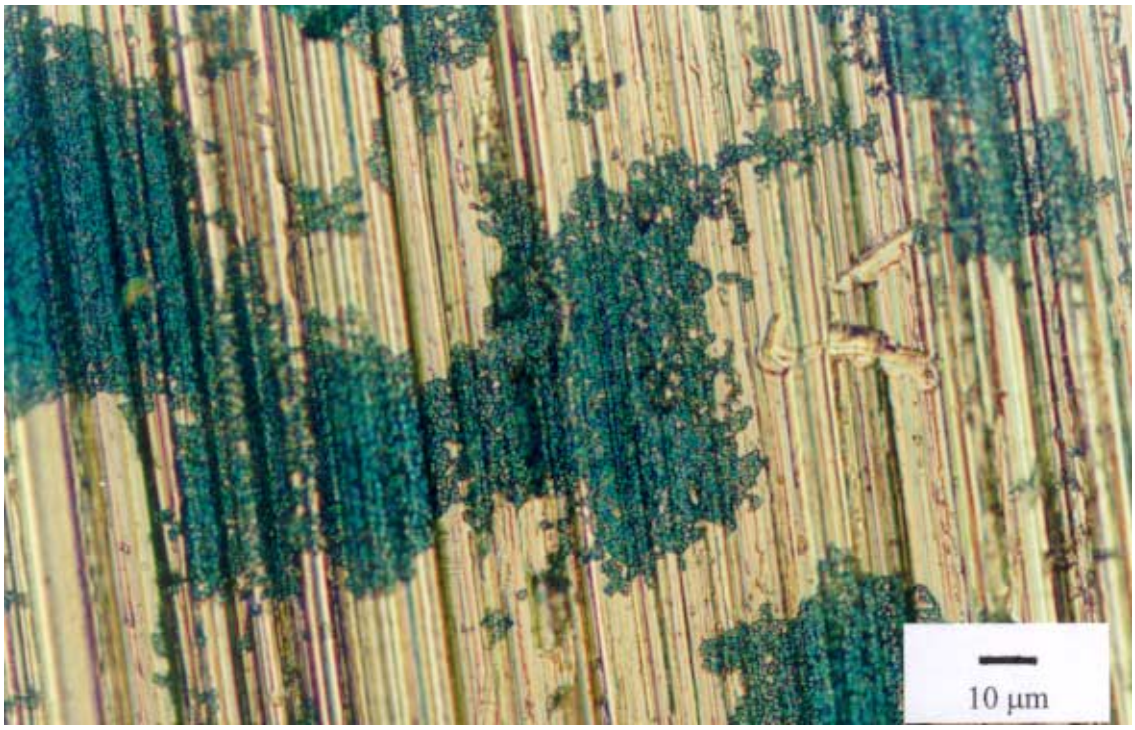


Figure 4.23. *S. aureus* on the surface of stainless steel (316L).

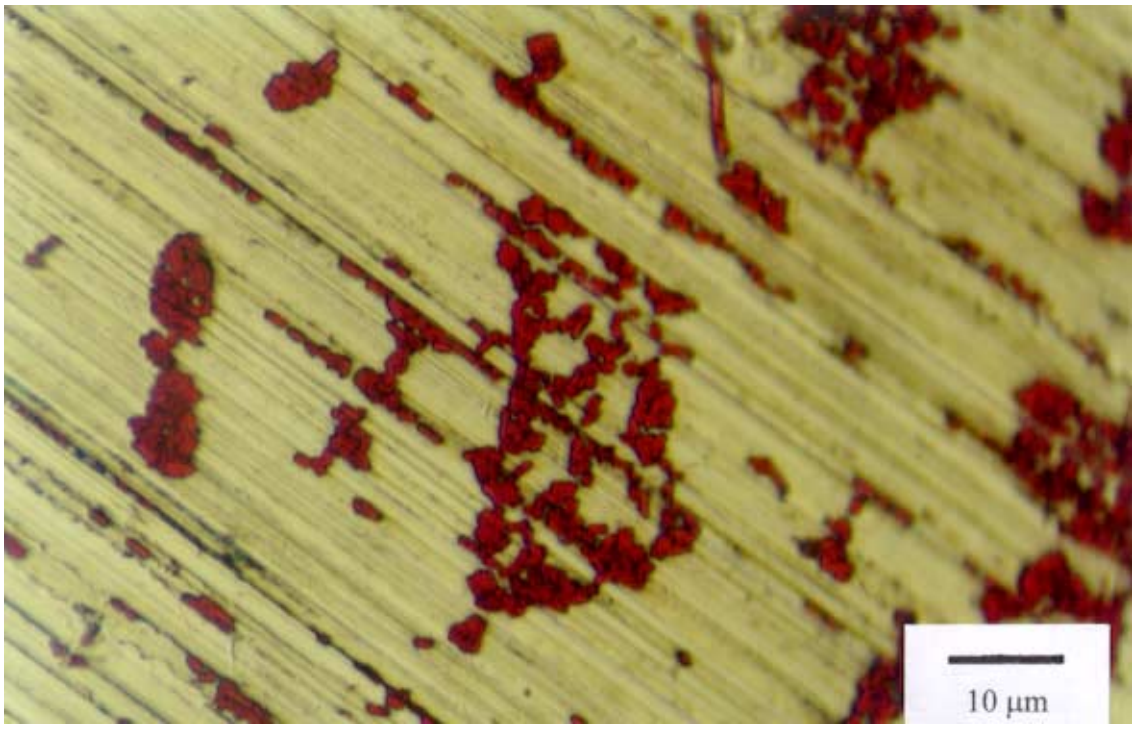


Figure 4.24. *E. coli* on the surface of stainless steel (316L).

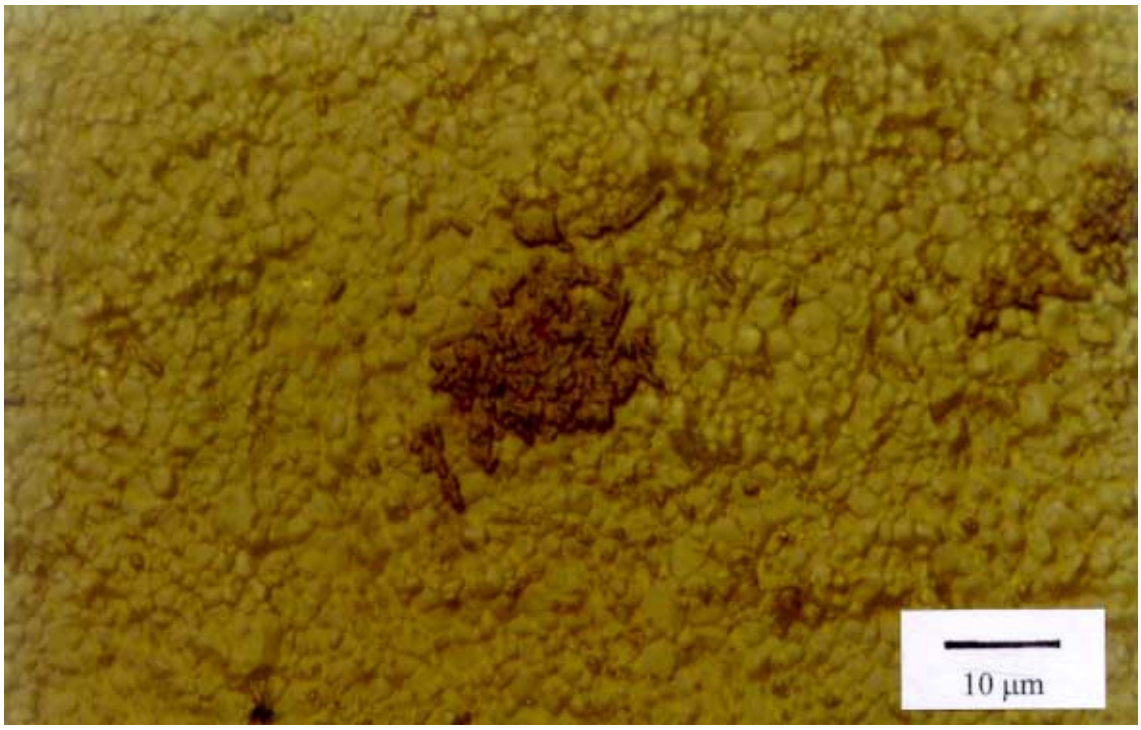


Figure 4.25. *E. coli* on the surface of HA 1250 °C pellet.

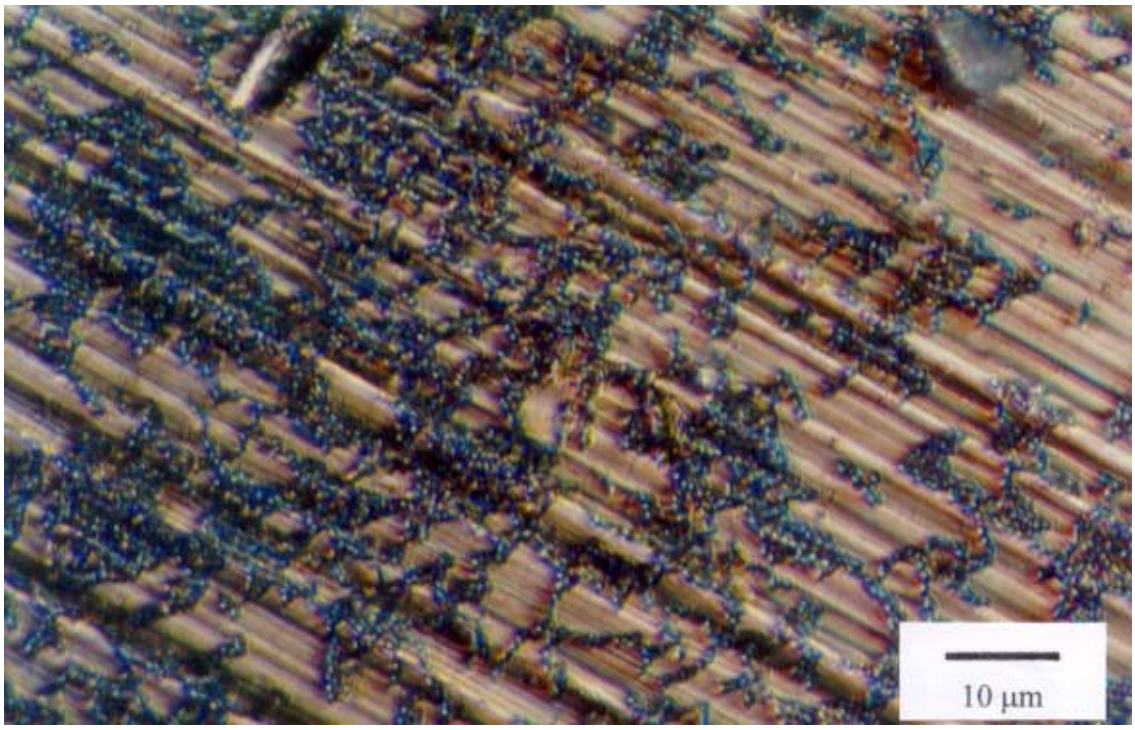


Figure 4.26. Viridans spp. (*S. mitis*) on the surface of stainless steel (316L).

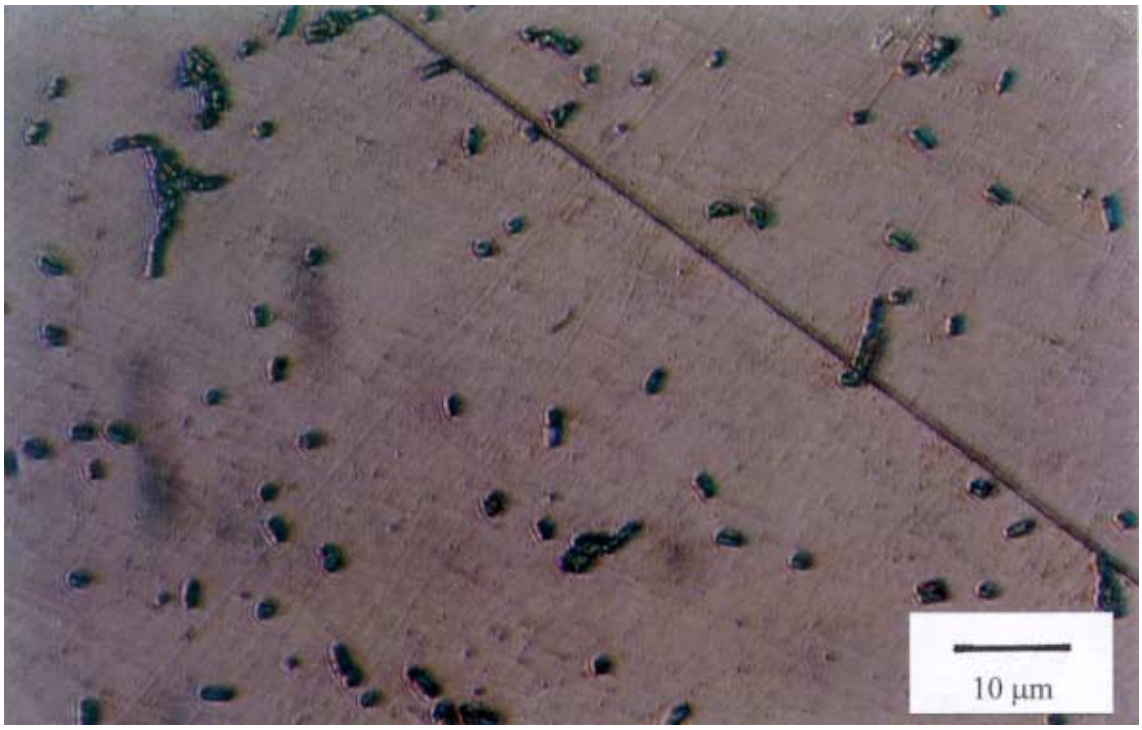


Figure 4.27. KNS on the surface of polished stainless steel (316L).

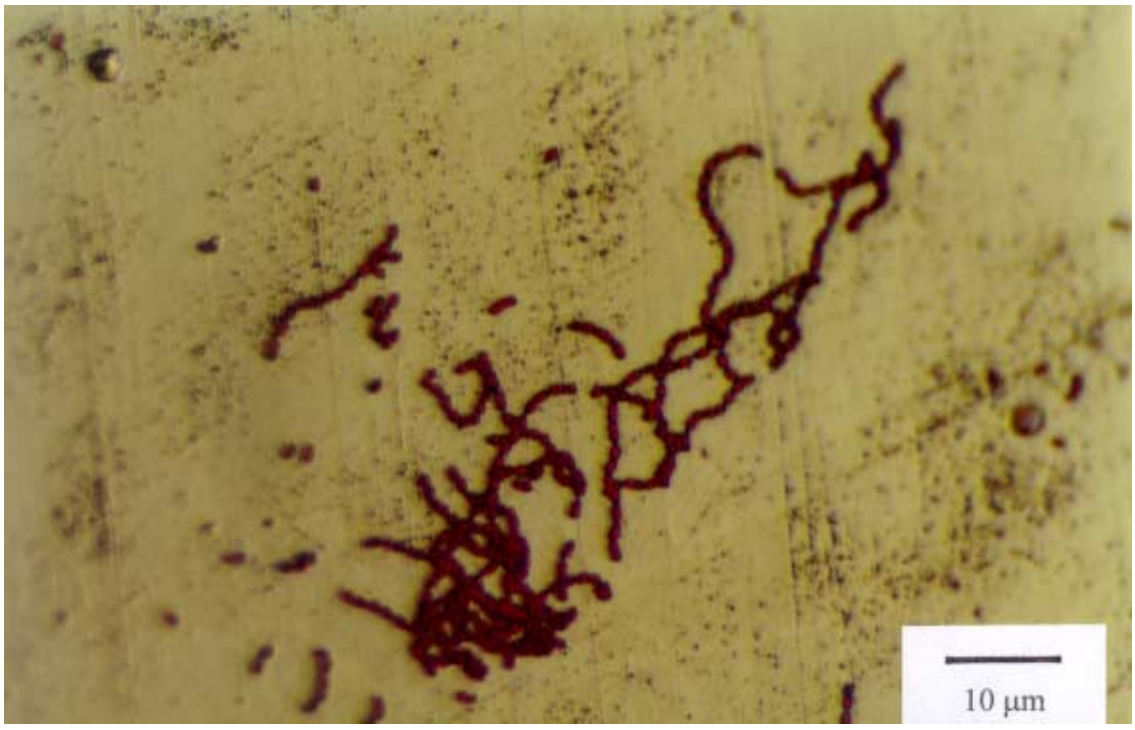


Figure 4.28. *Streptococcus mutans* on the surface of polished stainless steel (316L).

4. 8. Anti-bacterial Effects of Biomaterials

Anti-bacterial effects of biomaterials were investigated by using the agar diffusion method. Gram-negative bacterial strains (*E. coli*, *P. aeruginosa*, *K. pneumonia*, *Proteus spp.*) and gram-positive bacterial strains (*S. aureus* and *S. pyogenes*) were inoculated on Mueller-Hinton agar plates. The plates were incubated at 37 °C with 5% CO_2, humidified incubator. Zone diameters around test samples and the positive controls (antibiotic discs) were measured daily. Meropenem (10 μg) discs for *E. coli*, *P. aeruginosa*, *K. pneumonia*, *Proteus spp.* and *S. pyogenes* and vancomycin (30 μg) discs for *S. aureus* were used. Any inhibition zones around HA, alumina and zirconia powders, HA 800 °C, HA 1000 °C, HA 1250 °C, Alumina 1450 °C, Zirconia 1450 °C, titanium alloy, stainless steel and cirulene (ultra high molecular weight polyethylene) discs were not observed. The comparison between inhibition zones of antibiotic discs and test samples were shown in Figures 4.29 and Figure 4.30. In this test, the test sample should be dissolve in the agar medium to inhibit bacterial growth. However, none of the test samples was soluble in the agar medium. Most possibly the solid forms resulted in these results. As a result of the data, tested biomaterials had no anti-bacterial effect on various pathogenic bacteria.

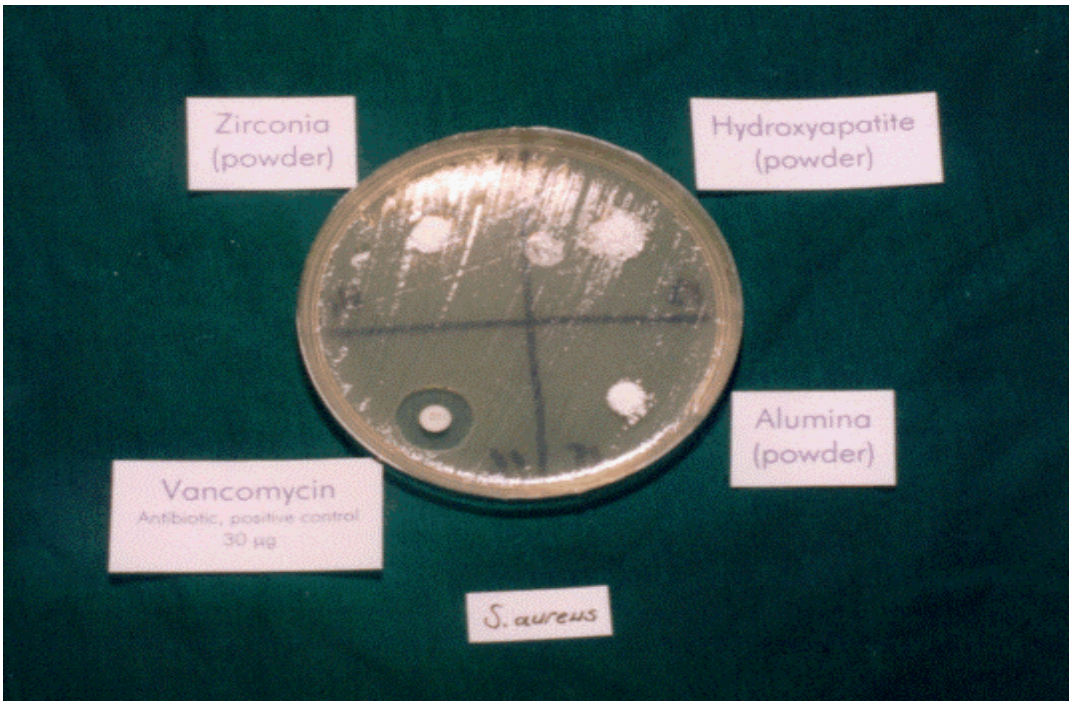


Figure 4.29. Inhibition zone around vancomycin disc and the test materials on *S. aureus* plate after 24 hours incubation.

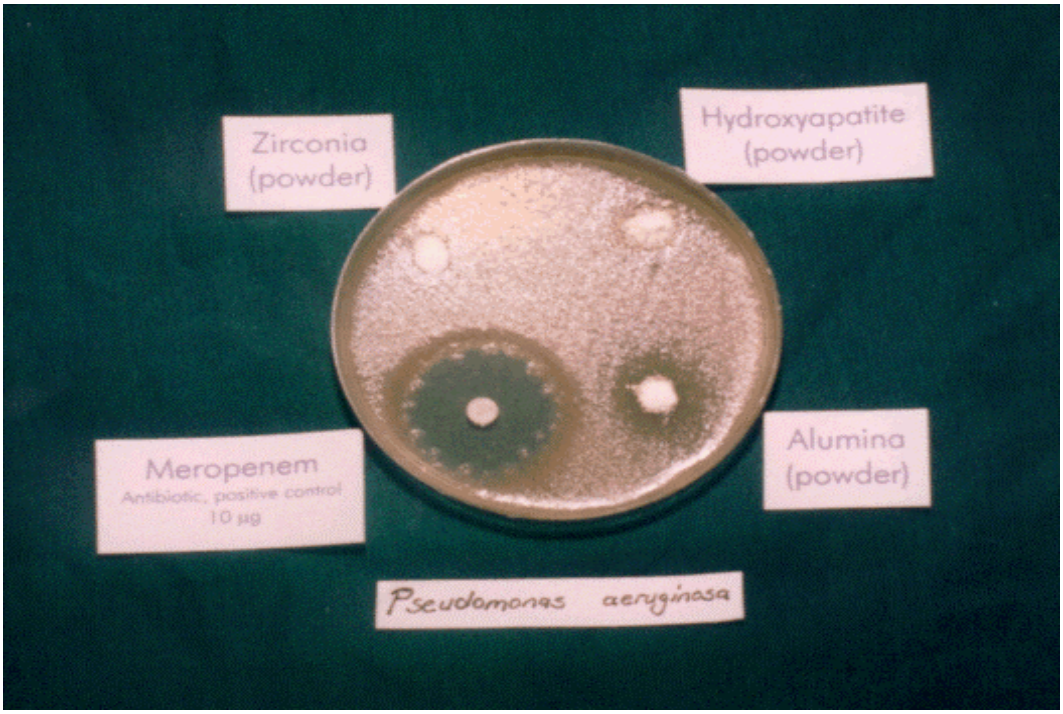


Figure 4.30. Inhibition zone around meropenem disc and the test materials on *Pseudomonas aeruginosa* plate after 24 hours incubation.

Chapter 5

CONCLUSIONS AND FUTURE EXPERIMENTS

5. 1. Conclusions

In summary, ceramic materials (hydroxyapatite, alumina and zirconia), their extracts in PBS, ceramic-ceramic composites (HA-Alumina and HA-Zirconia), metallic materials (stainless steel and titanium alloy) and polymeric material (high molecular weight polyethylene (cirulene)) with different chemical and surface properties were examined to determine their effects on the viability, proliferation and activation of PBMC and on the secretion of inflammatory cytokines (IL-1α and IL-6) from PBMC. Furthermore, the mutagenic potential of extracts of biomaterials, anti-bacterial effects and adhesion of pathogenic bacteria to the surface of biomaterials were investigated. Finally, to elucidate the effects of biomaterials in the cell culture, the dissolution properties of ceramic samples in the culture medium and in deionized water were investigated on the basis of pH and conductivity measurements.

In conclusion, the sintering temperature of HA pellets showed an effect on the cell viability. HA pellets sintered at 800 °C, 900 °C, 1000 °C resulted in a decrease in the viability of PBMC, but HA 1100 °C and HA 1250 °C pellets had no effects on the viability compared to the control culture. However, BSA-coated HA 800 °C pellets increased the cell viability with respect to uncoated HA 800 °C pellets. Especially polished metallic samples and other metallic and polymeric samples showed high percent cell viabilities at 24 and 48 hours. However, after 72 hours, a decline in the cell viabilities was determined most probably due to released ions and particles to the environment. A negative correlation between increasing extract concentration and the cell viability was observed for all ceramic samples especially after 48 and 72 hours treatments. As a result, except for HA 800 °C samples, all samples led to appropriate percent cell viabilities at all incubation periods with respect to the control cultures.

The secretion of inflammatory cytokines from PBMC in the presence of biomaterials was examined by using ELISA kits. In the presence of LPS, a decrease in IL-1α secretion was observed in HA 800 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C pellets. BSA-coated of HA 800 °C pellets did not change IL-1α secretion in the presence of LPS. However, in the absence of LPS, BSA-coating caused an increase in IL-1α secretion in HA 800 °C pellets. Unlike HA 800 °C, BSA-coating of HA 1250 °C, alumina 1450 °C and zirconia 1450 °C showed higher levels of IL-1α secretion in the presence of LPS. Stainless steel, titanium alloy and cirulene pellets resulted in low levels of IL-1α secretion. Because IL-1α secretion is an indication of inflammation, low level of IL-1α secretion in the presence of biomaterials is favorable condition. Therefore, metallic biomaterials and cirulene seems to be desired biomaterials. In the case of IL-6, BSA-coated HA 800 °C pellets increased IL-6 secretion compared to uncoated pellets. In metallic samples, low IL-6 levels were obtained with and without LPS indicating that the metallic samples and cirulene are more biocompatible than BSA-coated ceramics.

HA 800 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C samples had inhibitory effect on the proliferation of PBMC. Especially in the presence of Con A, HA 800 °C, HA-Alumina 1250 °C and HA-Zirconia 1250 °C samples led to significant decreases in the proliferation of cells with respect to other ceramic samples and the control culture. Therefore, these samples are not biocompatible on the parameter of cell proliferation. The inhibition of proliferation of PBMC in the presence of a mitogen by biomaterials may cause immune suppression and this may lead to be the body susceptible to infections.

The results of MTT assay represent both activation of PBMC and cytotoxic potentials of test samples on PBMC. As in the case of cell viabilities, HA 800 °C and HA 900 °C samples showed the lowest cell activation at all incubation periods. Moreover, other ceramic samples showed lower cell activation after 48 and 72 hours treatments compared to the control cultures. In addition, the extracts of ceramics activated PBMC in 24 hours and at low concentrations. Especially, at 5 µl and 10 µl of HA 800 °C, HA 900 °C, HA 1250 °C and alumina 1450 °C samples higher absorbance values than the controls were obtained. Prolonged exposure to the extracts may become

toxic for cells and cells may start to die. Although there was a fluctuation in the OD levels of MTT assay based on prolonged exposure or concentration of the extracts, the difference among OD levels of samples was relatively low. Therefore, it can be concluded that the extracts of biomaterials used in this assay are not toxic to PBMC, but the pellets sintered at low temperatures may be toxic to PBMC due to released products in the dissolution process.

The dissolution properties of ceramic samples were determined in deionized water and in the culture medium with and without PBMC. In deionized water, HA 800 °C pellets showed a significant increase in the conductivity. However, in HA 1250 °C, zirconia 1450 °C and in deionized water itself, there was almost no change up to 10 days period. In the culture medium with and without PBMC, HA 800 °C pellets led to an increase in the conductivity. In other samples, there were no significant alterations in the conductivity during overall incubation periods. Whereas, in the pH values of samples in the culture medium with and without the cells and in deionized water, there were fluctuations. Only HA 800 °C samples in the culture medium with and without the cells resulted in a slight increase in pH of solutions in 4 and 72 hours periods. Only HA 800 °C pellets caused an increase in the pH of deionized water up to 10 days. As a result of these measurements, some ion transfers take place both from materials to medium and from medium to materials. The released ions increased the conductivity and pH of solutions that may be the reasons of death of cells in the presence of HA 800 °C pellets.

According to results of AMES reverse mutation test, none of the extracts of biomaterials in PBS had mutagenic effects on *Salmonella typhimurium* TA100 strain when they were compared to the mutagenic material (sodium azide) and the negative controls (only bacteria).

None of the tested ceramic powders, ceramic pellets, metallic and polymeric materials has anti-bacterial effects on both gram-negative strains (*E. coli, P. aeruginosa, K. pneumonia, Proteus spp.*) and gram-positive strains (*S. aureus* and *S. pyogenes*). Furthermore, in adhesion of pathogenic bacteria to the surface of biomaterials, it was found that surface properties, especially roughness and characteristics of materials are important for attachment, adhesion and colonization of bacteria on the surface of biomaterials.

5. 2. Future Experiments

1. Determination the conductivity, pH alterations and zeta potentials of BSA-coated ceramic samples in dH_2O, the culture medium with and without cells may be useful to elucidate the positive effect of BSA adsorption onto ceramic pellets.
2. Adsorption of different proteins or biologically active molecules onto ceramic, metallic and polymeric samples may be carried out to investigate the effects of these coating materials on different cell types and adhesion of pathogenic bacteria to the surface of materials.
3. Films from organic and inorganic sources may be produced and they may be loaded with antibiotics and coated to the surface of biomaterials to prevent bacterial attachment and adhesion.
4. New ceramic-ceramic, ceramic-metallic, ceramic-polymeric composite materials may be produced from biocompatible materials with desired chemical, physical and mechanical properties.

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APPENDIX A

PROCEDURES OF TESTS

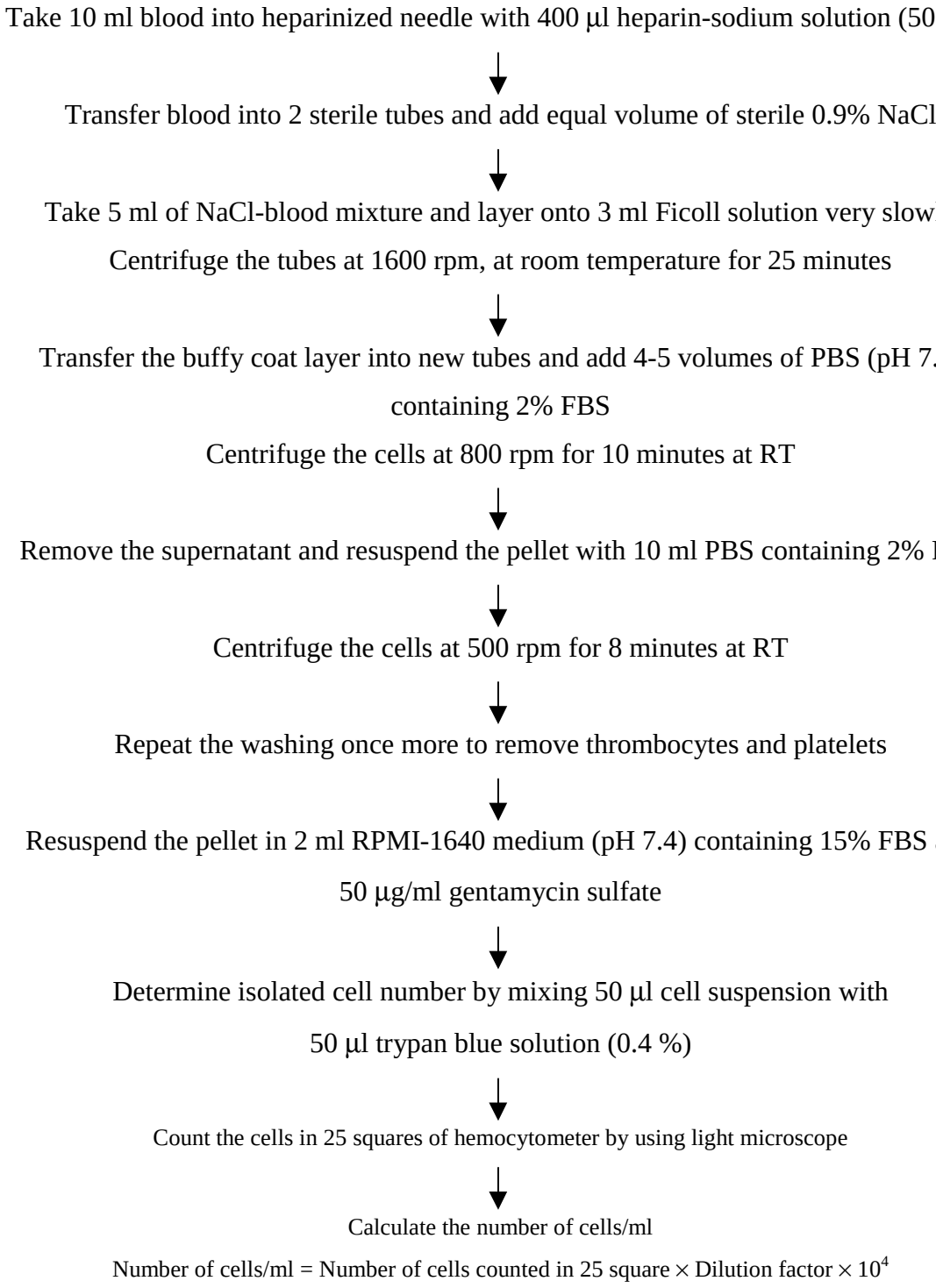

Figure A1. Isolation of PBMC procedure

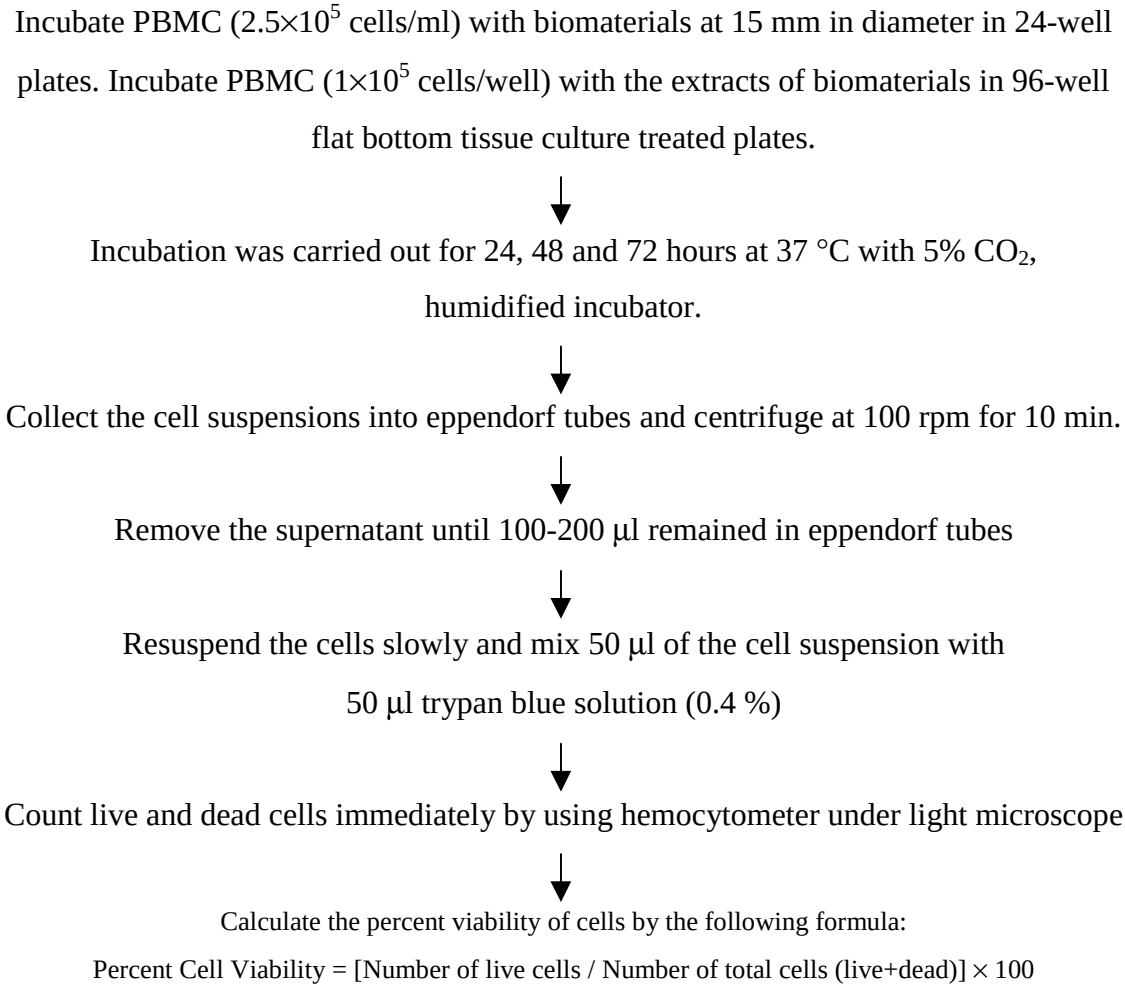

Figure A2. Trypan blue exclusion method

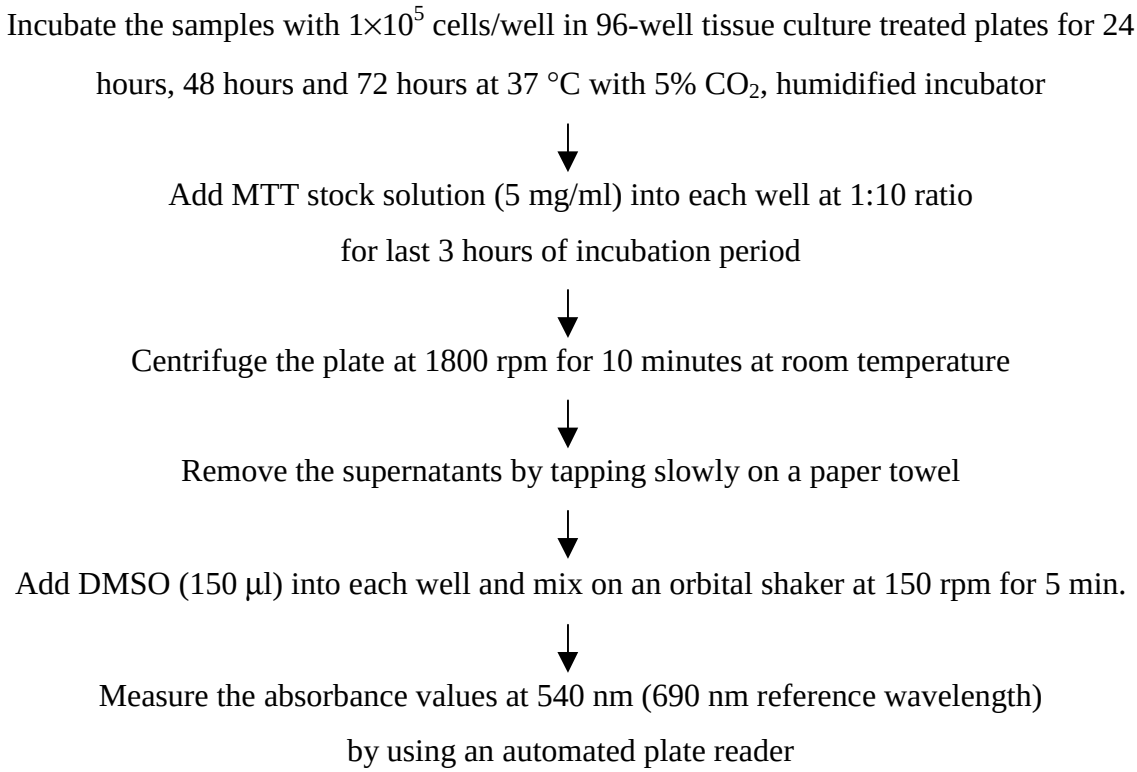

Figure A3. MTT assay

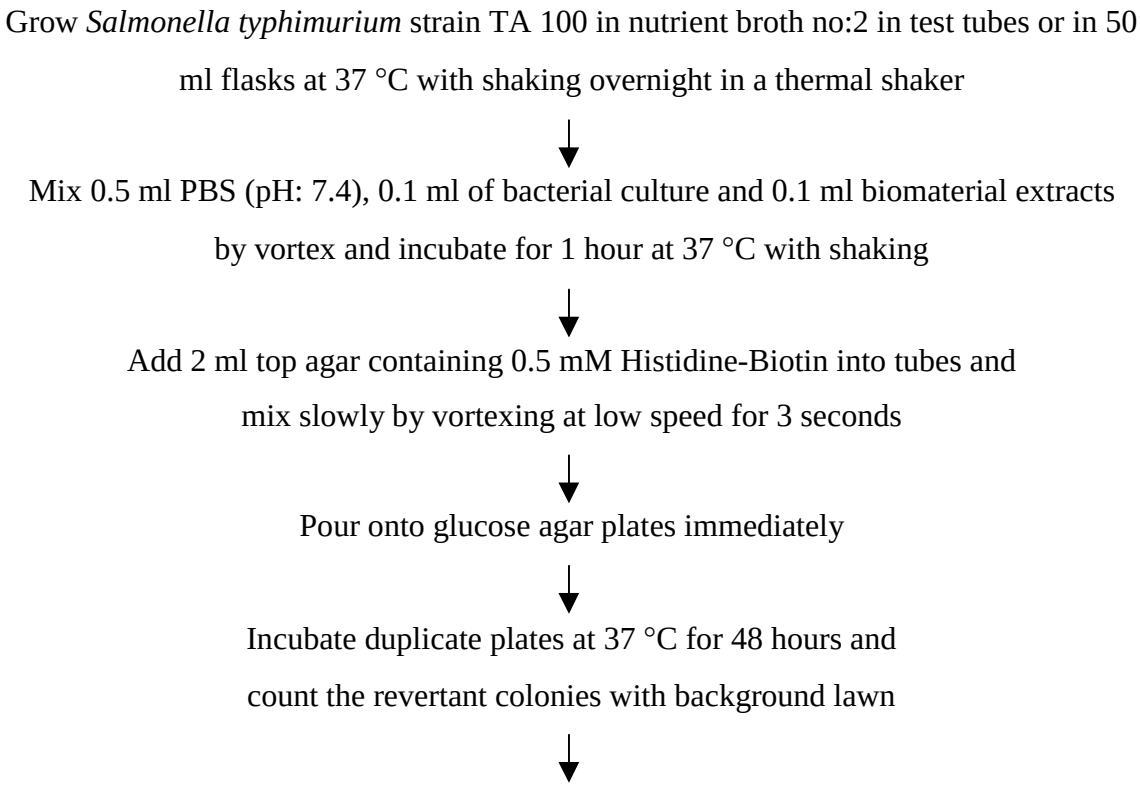

Figure A4. AMES test

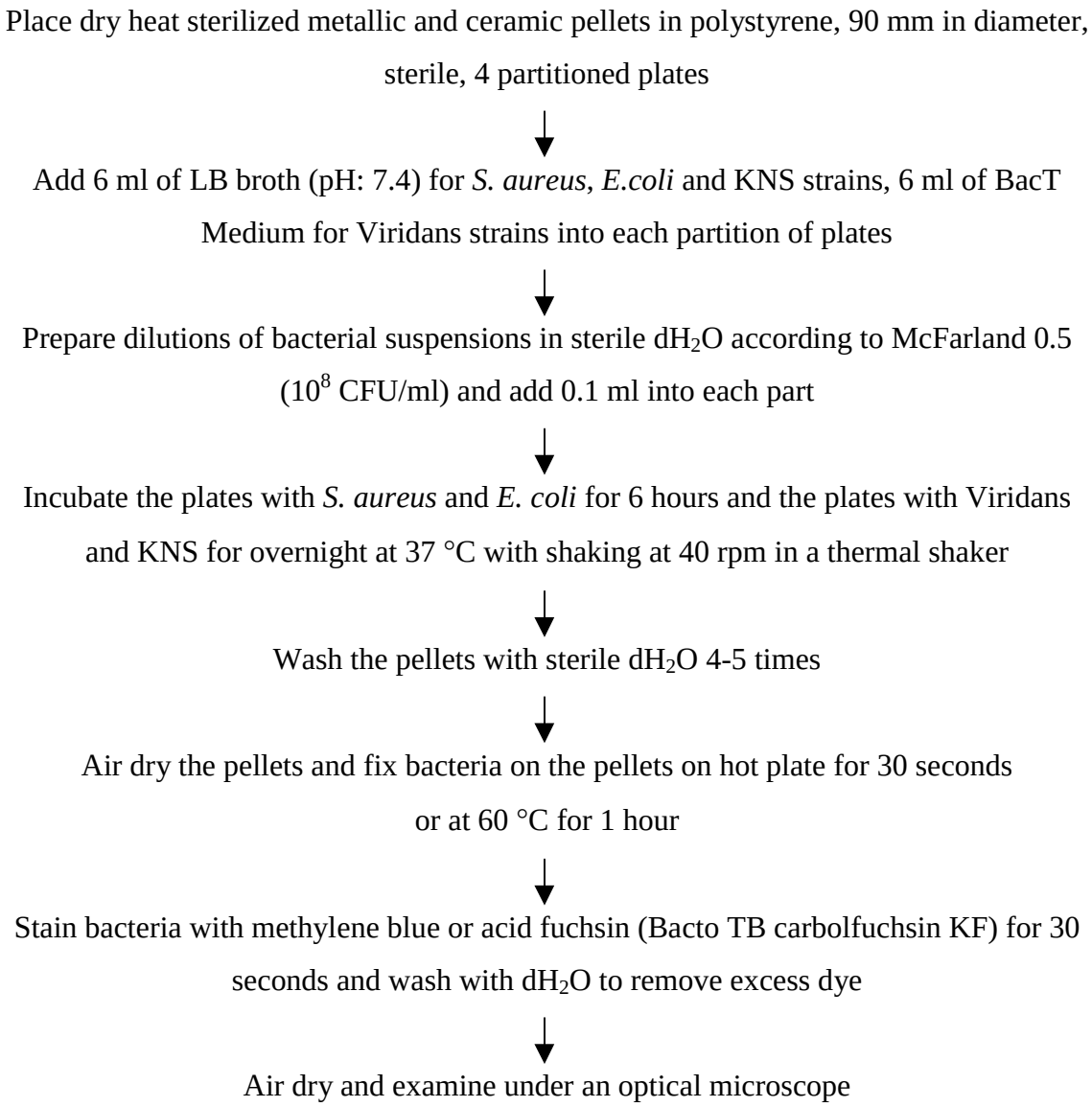

Figure A5. Determination the adhesion of bacteria to biomaterial surfaces

APPENDIX B

TABLES OF DATA

Table B1. Effects of ceramic biomaterials on the viability of PBMC

Percent Viability of PBMC			
Test Sample	**24 hours**	**48 hours**	**72 hours**
Control (Without Biomaterial)	96.12	94.06	85.43
HA 800 °C	72.62	28.22	22.13
HA 1250 °C	96.22	94.63	91.00
Alumina 1450 °C	94.90	93.86	87.33
Zirconia 1450 °C	96.15	96.77	93.14

HA 800 °C, HA 1250 °C, alumina 1450 °C and zirconia 1450 °C ceramic samples (15 mm in diameter) were incubated with 2.5×10^5 cells/well at 37 °C with 5% CO_2 for 24, 48 and 72 hours. Live and dead cells were counted after staining with trypan blue (0.4%) and percent cell viabilities were calculated. Data are the average of duplicate samples from separate experiments and from different donors.

Table B2. Effects of different sintering temperature of ceramic pellets on the viability of PBMC

Percent Viability of PBMC			
Test Sample	**24 hours**	**48 hours**	**72 hours**
Control	94.12	92.12	86.36
HA 800 °C	57.25	29.90	32.23
HA 900 °C	54.52	35.63	30.95
HA 1000 °C	66.81	58.92	28.26
HA 1100 °C	94.12	89.10	83.94
HA 1250 °C	98.00	90.76	82.58
Alumina 1000 °C	84.81	48.51	ND
Alumina 1450 °C	ND	84.85	ND

PBMC (2×10^5 cells/well) were incubated with ceramic samples with different sintering temperatures (15 mm in diameter) at 37 °C with 5% CO_2 for 24, 48 and 72 hours. The percent cell viabilities were determined by trypan blue exclusion method. Data are the average of duplicate samples from separate experiments. ND: not determined.

Table B3. Effects of ceramic extracts on the viability of PBMC

Percent Cell Viability					
		HA 800°C	HA 1250 °C	Al 1450°C	Zir 1450°C
24 hours	5 µl extract	93.80	98.49	86.33	94.39
	10 µl extract	97.98	93.86	91.40	91.67
	20 µl extract	96.64	96.67	93.18	90.62
	40 µl extract	92.59	90.72	92.31	93.10
	Control (Without Extract)				92.70
48 hours	5 µl extract	92.86	90.00	92.78	93.10
	10 µl extract	92.91	91.34	90.00	81.82
	20 µl extract	83.82	95.04	82.05	87.50
	40 µl extract	85.98	90.84	90.35	84.21
	Control (Without Extract)				90.48
72 hours	5 µl extract	88.54	91.84	88.04	85.38
	10 µl extract	78.81	90.42	83.71	84.18
	20 µl extract	84.68	91.27	89.10	88.51
	40 µl extract	68.96	86.81	90.77	88.96
	Control (Without Extract)				91.65

PBMC (1×10^5 cells/well) were incubated with different concentrations of ceramic extracts in PBS at 37 °C with 5% CO_2 for 24, 48 and 72 hours periods. Percent cell viabilities were determined by trypan blue exclusion method. Data are the average of duplicate samples from separate experiments.

Table B4. The viability of PBMC after incubating

with protein adsorbed ceramic pellets

Percent Viability of PBMC			
Test Sample	**24 hours**	**48 hours**	**72 hours**
Control (Without Biomaterial)	97.92	95.73	88.24
HA 800 °C	82.14	57.14	35.90
HA 1250 °C	97.03	90.53	87.74
Alumina 1450 °C	94.87	89.60	89.55
Zirconia 1450 °C	98.98	94.74	85.71

Bovine Serum Albumin (BSA) adsorbed ceramic pellets (15 mm in diameter) were incubated in 10 ml protein solution with the concentration of 20 mg/ml BSA in sodium phosphate buffer (pH 7.3) at 37 °C with shaking at 110 rpm for 3 hours. The pellets were incubated with 1 ml PBMC (2.5×10^5 cells/well) at 37 °C with 5% CO_2 for 24, 48 and 72 hours. The viabilities of cells were determined trypan blue exclusion method. Data for protein adsorbed ceramic samples represent results of one experiment.

Table B5. Effects of metallic and polymeric samples on the viability of PBMC

Percent Cell Viabilities			
Test Sample	**24 hours**	**48 hours**	**72 hours**
Control (Without Biomaterial)	94.40	89.98	90.18
Stainless Steel (316L)	94.64	90.36	70.48
Polished Stainless Steel	100	100	85.53
Titanium Alloy (Ti-6Al-4V)	96.12	87.70	80.98
Polished Titanium Alloy	95.24	87.23	86.21
Cirulene (ultra high molecular weight PE)	95.30	89.91	75.52

Metallic biomaterials stainless steel (316L), titanium alloy (Ti-6Al-4V) and their polished forms with 1200 grid SiC, 6 micron diamond solution, 1 and 0.5 micron alumina suspension and polymeric material cirulene were examined with PBMC. 2×10^5 cells/well in 24-well tissue culture treated plates incubated with 10 mm sample discs at 37 °C with 5% CO_2 for 24, 48 and 72 hours. Percent cell viabilities were determined by using trypan blue exclusion method. Data are the average of duplicate samples from separate experiments.

Table B6. Effects of biomaterials on the secretion of IL-1α from PBMC with and without LPS stimulation

Biomaterial	OD value @450 nm without LPS	OD value @450 nm with LPS
Control	0.072	0.818
HA 800 °C	0.074	0.160
HA 1250 °C	0.078	0.751
Zirconia 1450 °C	0.078	0.785
Alumina 1450 °C	0.074	0.662
HA-Alumina 1250 °C	0.073	0.255
HA-Zirconia 1250 °C	0.077	0.420

Table B7. Effects of metallic, polymeric and protein adsorbed ceramic samples on the secretion of IL-1α from PBMC with and without LPS stimulation

Biomaterial	OD value @450 nm without LPS	OD value @450 nm with LPS
Control (Without Biomaterial)	0,057	0,484
HA 800 °C with prt adsorption	0,151	0,337
HA 1250 °C with prt adsorption	0,097	0,520
Alumina 1450 w/prt adsorption	0,090	0,524
Zirconia 1450 w/prt adsorption	0,101	0,584
Stainless steel (316L)	0,064	0,340
Titanium alloy (Ti-6Al-4V)	0,066	0,438
Cirulene (UHMWPE)	0,074	0,374

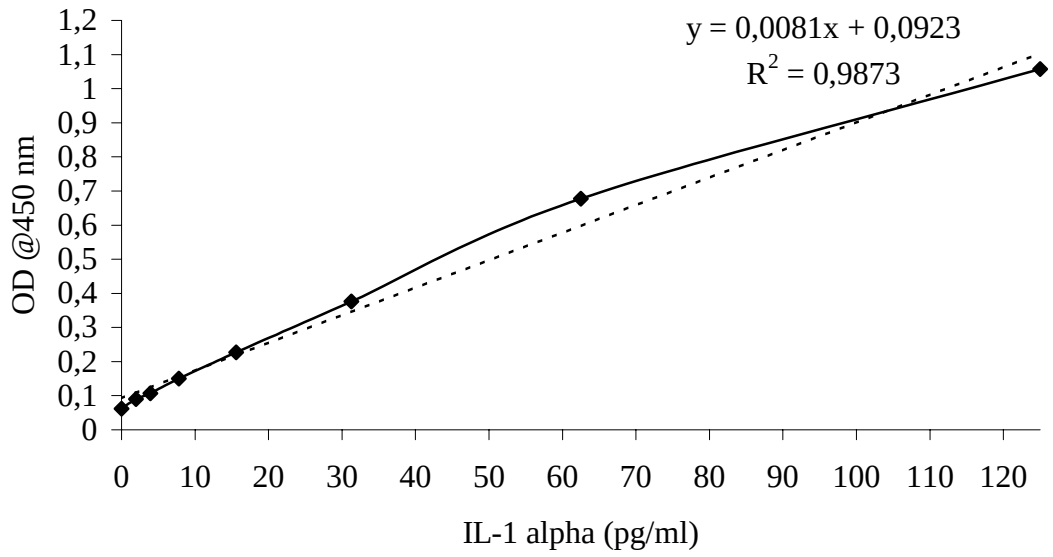

Figure B1. Calibration curve of IL-1α.

IL-1α calibration curve was obtained by using serial dilution of standard IL-1α, which is supplied by the manufacturer. 0, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5 and 125 pg/ml IL-1α were used. Optical densities of standards were determined as test samples described by the manufacturer. Data are the average of triplicate standard samples from two experiments.

Table B8. The amount of IL-1α secretions from PBMC in the presence of biomaterials

Biomaterial	IL-1α (pg/ml) (in the presence of LPS)
Control (Without Biomaterial)	89.59
HA 800 °C	8.36
HA 1250 °C	81.32
Alumina 1450 °C	70.33
Zirconia 1450 °C	85.52
HA-Alumina 1250 °C	20.09
HA-Zirconia 1250 °C	40.46
Control (Without Biomaterial)	48.36
HA 800 °C with protein	30.21
HA 1250 °C with protein	52.80
Alumina 1450 °C with protein	53.30
Zirconia 1450 °C with protein	60.70
Stainless steel	30.58
Titanium alloy	42.68
Cirulene	34.78

Data were calculated on the basis of calibration curve of IL-1α (Figure B1).

Table B9. Effects of biomaterials on the secretion of IL-6 from PBMC

Biomaterial	OD @ 450 nm without LPS
Control	1.029
HA 800 °C	0.371
HA 1250 °C	1.042
Zirconia 1450 °C	1.171
Alumina 1450 °C	1.066
HA-Alumina 1250 °C	0.876
HA-Zirconia 1250 °C	0.780

Table B10. Effects of protein coated ceramic, metallic and polymeric samples on IL-6 secretion from PBMC

Biomaterial	OD @450 nm	OD @450 nm with LPS
Control (Without Biomaterial)	0.392	2.600
HA 800 °C with prt coating	2.939	1.918
HA 1250 °C with prt coating	Out	2.678
Alumina 1450 °C with prt coating	2.928	2.776
Zirconia 1450 °C with prt coating	Out	2.702
Stainless Steel (316L)	0.310	2.279
Titanium Alloy (Ti-6Al-4V)	0.453	2.361
DLC coated AISI 52100	0.956	N.D.
Cirulene (UHMWPE)	0.360	2.368

Data are the average of duplicate samples from the same experiments. Samples containing LPS were diluted 4 times before ELISA experiment. Out represents out of range of automated plate reader at 450 nm. ND: not determined.

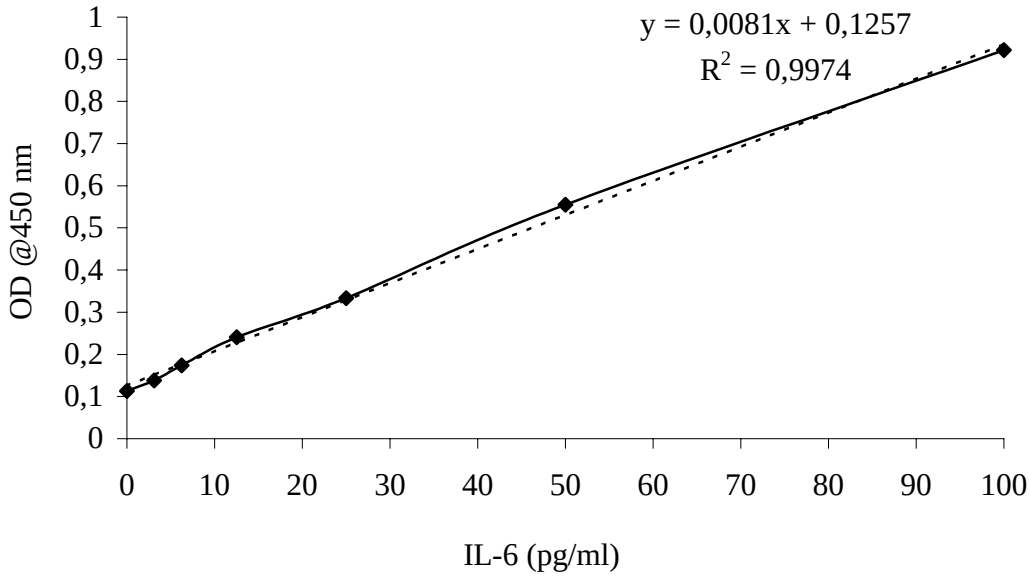

Figure B2. Calibration curve of IL-6

IL-6 calibration curve was obtained by using serial dilution of standard IL-6, which is supplied by the manufacturer. 0, 3.12, 6.25, 12.5, 25, 50 and 100 pg/ml IL-6 were used. Optical densities of standards were determined as test samples described by the manufacturer. Data are the average of triplicate standard samples from two experiments.

Table B11. The amount of IL-6 secretion from PBMC in the presence of biomaterials

Biomaterial	**IL-6 (pg/ml)**	**IL-6 (pg/ml) (in the presence of LPS)**
Control (Without Biomaterial)	111.52	-
HA 800 °C	30.28	-
HA 1250 °C	113.12	-
Zirconia 1450 °C	129.05	-
Alumina 1450 °C	116.09	-
HA-Alumina 1250 °C	92.63	-
HA-Zirconia 1250 °C	80.78	-
Control (Without Biomaterial)	32.88	1221.88
HA 800 °C with prt coating	347.32	885.07
HA 1250 °C with prt coating	-	1260.40
Alumina 1450 °C with prt coating	345.96	1308.79
Zirconia 1450 °C with prt coating	-	1272.25
Stainless Steel (316L)	22.75	1063.36
Titanium Alloy (Ti-6Al-4V)	40.41	1103.85
DLC coated AISI 52100	102.51	-
Cirulene (UHMWPE)	28.92	1107.31

Data were calculated on the basis of calibration curve of IL-6 (Figure B2).

Table B12. Proliferation of PBMC with and without Con A stimulation after treatment with bioceramics

Biomaterial	OD @ 450 nm (PBMC without Con A)	OD @ 450 nm (PBMC with Con A)
Control (Without Biomaterial)	0.278	1.624
HA 800 °C	0.310	0.518
HA 1250 °C	0.284	1.450
Alumina 1450 °C	0.253	1.131
Zirconia 1450 °C	0.283	1.244
HA-Alumina 1250 °C	0,154	0,286
HA-Zirconia 1250 °C	0,127	0,287

Proliferation of PBMC in the presence of ceramic pellets in 5 mm diameter was determined by using Biotrak Cell Proliferation ELISA kit. Con A (200 μg/ml) was used as a mitogen. PBMC (8×10^4 cells/well) were cultured at 37 °C with 5% CO_2 for 72 hours. BrdU was added 24 hours before the end of incubation period. The incorporation of BrdU in proliferating cells was determined with peroxidase-labelled anti-BrdU antibodies. Optical densities at 450 nm were determined after addition of TMB substrate. OD values directly represent the proliferation of cells. Effects of ceramic pellets in the presence and absence of Con A were compared. Data are the average of duplicate samples. Test repeated three times with the cells of different donors.

APPENDIX C

MEDIUM AND SOLUTION FORMULAS

Mammalian cell culture medium and solutions:

0.9 % Sodium Chloride: Dissolve 9 g NaCl in distilled or deionized water to a total volume of 1 liter. Autoclave and store at +4 °C.

Phosphate-buffered saline (PBS), pH 7.4: Dissolve 0.34 g KH_2PO_4, 1.58 g K_2HPO_4 and 8.0 g NaCl in distilled or deionized water, adjust pH to 7.4 and bring to a total volume 1 liter. Autoclave and store at +4 °C.

Heparin solution: Prepare 50 I.U./ml stock solution with sterile 0.9% NaCl. Take 400 µl (50 I.U./ml stock) into 10 ml needle to obtain 2 I.U./ml heparin in blood. Store heparin solution at +4 °C.

Growth medium: Add 45 ml FBS (15%) and gentamycin sulfate with final concentration 50 µg/ml to 300 ml RPMI-1640. Keep growth medium and gentamycin sulfate at 4 °C. Prepare gentamycin sulfate stock solution with sterile ultrapure deionized water. Store FBS at –20 °C and bring to 37 °C all growth medium constituents before use.

Lipopolysaccharide from *E.coli* 055:B5 (LPS): Dissolve 1 mg LPS in 2 ml sterile PBS for 0.5 mg/ml stock. A final concentration of 10 µg/ml was used in cell culture. Store at +4 °C.

Protein adsorption solutions:

Phosphate buffer, pH 7.3: Dissolve 0.69 g $NaH_2PO_4.H_2O$ in 250 ml dH_2O and 0.7098 g Na_2HPO_4 in 250 ml dH_2O separately. Take 23 ml $NaH_2PO_4.H_2O$ and 77 ml Na_2HPO_4 and adjust pH to 7.3 and complete to 200 ml with dH_2O to obtain 0.01 M buffer. Autoclave and store at +4 °C.

Protein solution: Add appropriate amount of BSA with aseptic conditions to yield a final concentration of 20 mg/ml into sterile phosphate buffer (pH 7.3). Put a sterile magnet to mix protein. Store at +4 °C.

Cell proliferation solutions :

Concanavalin A (Con A) solution: Dissolve 5 mg Con A in 2.5 ml sterile 0.9% NaCl to obtain 2 mg/ml stock solution. Store at –20 °C.

Bacterial culture medium and solutions:

Dissolve 12.5 g of LB broth and 20 g LB agar in dH_2O. Adjust the pH of the media to 7.4 with 1 M NaOH or 1M HCl. Complete the volume to 500 ml with dH_2O and autoclave at 121 °C for 15 minutes.

Dissolve 10.5 g of Mueller-Hinton broth (Oxoid CM 405) and 19 g Mueller-Hinton agar (Oxoid CM 337) in dH_2O. Adjust the pH of the media to 7.2-7.4 with 1 M NaOH or 1M HCl. Complete the volume to 500 ml with dH_2O. Bring to boil Mueller-Hinton agar to dissolve completely and autoclave at 121 °C for 15 minutes.

Dissolve 12.5 g of Nutrient Broth No:2 (Oxoid CM 67) in dH_2O. Adjust the pH of the media to 7.4 with 1 M NaOH or 1M HCl. Complete the volume to 500 ml with dH_2O and autoclave at 121 °C for 15 minutes.

Mutagenicity assay solutions:

Vogel-Bonner medium E (50X) (per liter): 670 ml Warm dH_2O (45 °C)
10 g $MgSO_4 \cdot 7\ H_2O$
100 g Citric acid monohydrate
500 g K_2HPO_4 (anhydrous)
175 g $NaHNH_4\ PO_4 \cdot 4\ H_2O$

Add salts in the order indicated above to warm water in a beaker or flask placed on a hot plate with stirring. Dissolve completely each salt before adding the next. Adjust the volume 1 liter. Distribute into 2 1-liter flasks and autoclave at 121 °C for 20 minutes.

0.5 mM Histidine/Biotin solution
(per 250 ml): 30.9 mg d-Biotin (F.W. 247.3)
24 mg L-Histidine·HCl (F.W. 191.7)
250 ml dH_2O

Dissolve the biotin by heating the water to the boiling point. Add histidine and mix well. Autoclave or sterilize by filtration through a 0.22-μm membrane filter. Store in a glass bottle at +4 °C. (Add 10 ml His/Biotin solution to 100 ml of top agar)

Top agar (per liter): 6 g Agar
5 g NaCl
1000 ml dH_2O

Mix thoroughly and transfer 100-ml aliquots. Autoclave for 20 minutes and cool the top agar. Store at room temperature. Melt the top agar before using and bring to 45 °C before using mutagenicity assay.

Minimal glucose plates (per liter): 15 g Agar
930 ml dH_2O
20 ml 50X VB salts
50 ml 40% Glucose

Add 15 g agar to 930 ml dH_2O and autoclave for 20 minutes using slow exhaust. When agar solution has cooled slightly, add 20 ml sterile 50X VB salts and 50 ml 40% glucose. A magnetic bar was added to the flask before autoclaving. Mix well all the ingredients. Pour 25 ml into each petri plate. Store plates at +4 °C.

40% Glucose: Dissolve 40 g glucose in dH_2O by mixing and heating on a hot plate. Autoclave and store at room temperature.

Turbidity standard for inoculum preparations:

$BaSO_4$ turbidity standard equivalent to McFarland 0.5 is used. 0.5 ml 0.048 mol/l $BaCl_2$ (1.175 % w/v $BaCl_2 \cdot H_2O$) is added to 99.5 ml 0.18 mol/l (0.36 N) H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The absorbance of prepared solution should be 0.08-0.10 at 625 nm for McFarland 0.5 standard solution. Standard solution is stored in the dark at room temperature after 4- to 6-ml aliquots in tubes, which are tightly sealed.

MTT stock solution:

Dissolve MTT dye at a concentration of 5 mg/ml in sterile phosphate-buffered saline (PBS) and filter through a 0.22 μm filter to remove any formazan crystals. Aliquot and store MTT stock solution at –20 °C in the dark.