Whole Blood and Vessel Interactions to Biomaterials; leukocyte filtration and angiogenesis stimulation effect of calcium phosphate biomaterials

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All that I am, or hope to be, I owe to my angel mother and a real man called father.

Dedicated to my lovely parents, my sisters, and brother, who have been there for me day
and night.

Table of Contents

Abstract

Background

When a material is implanted in the body, invariably, the first fluid it comes into contact with is blood. While whole blood mostly consists of red blood cells, leucocytes also account for 1% of the blood, making them substantially less numerous than the red blood cells. Although leukocytes are a small cell population in blood, they are the most crucial blood components to protect the body against foreign substances and infectious factors. Little is known about their interaction with biomaterials beyond initial attachment following activation. Similarly, while there are thousands of reports examining biomaterials and endothelial cells, there are barely 100 investigating the interaction of the tunica adventitia of blood vessels with biomaterials, even though it is more probable that an implanted material will contact the exterior of the blood vessel system than its interior lumen. In this study, the interaction of blood and blood vessels with calcium phosphate biomaterial, as the most common material used in bone repair and tissue engineering, was investigated. This project's main focus was on the leuko-reduction effect of hydroxyapatite biomaterial, and the attachment and survival of immune cells on this material, besides investigating vein tissue cell attachment and proliferation on cylindrical calcium phosphate biomaterial.

Methods

Blood samples and vein tissues for this study were harvested from healthy male rats. Hydroxyapatite (HAp) interaction with leukocytes in whole blood and its leukoreduction effect were tested after incubating two types of hydroxyapatite (HAp-1 and HAp-2) and cotton with blood samples for 1h, 2h, and 4h. Leukocyte manual counting, differential counting using Giemsa staining, and MTT assay were accomplished to examine the leukocyte number in whole blood after incubation with HAp materials. Live and Dead cell assay and cell detachment were performed to

determine cell viability and identify cells attached to the biomaterials. Blood vessel interaction with calcium-phosphate biomaterial was tested by detecting cells growing on the biomaterial using Live and Dead assay and Scanning Electron Microscope (SEM) imaging.

Results

The number of leukocytes in whole blood after incubation with HAp-1 reduced significantly after 4h by increasing the biomaterial mass ($p<0.05$). Approximately 30% of leukocytes in whole blood attached to HAp-1, which in comparison to HAp-2 showed more leukoreduction effect. As the most common material in leukocyte filters, cotton showed more than 50% of leukoreduction effect from the first hour of incubation. According to the Live and Dead assay, more cells were attached to the biomaterial (HAp-1, HAp-2, cotton, and commercial filter) after 4h rather than 1h. Lymphocytes and monocytes could be identified after cell detachment on hydroxyapatite biomaterial.

Conclusion

According to obtained data from different experiments with blood and vein tissue, hydroxyapatite materials' leukoreduction effect in whole blood is mass-dependent and time-dependent. Increasing the amount of material enhanced their efficiency by increasing the surface area. Leukocytes can stay attached and survive on HAp biomaterials for 5 days, and their identification showed that lymphocytes and monocytes attached more. Besides these, vein tissue can well-attached to cylindrical microporous calcium phosphate biomaterial, and according to SEM and fluorescence imaging, cells were growing through the biomaterial, and extracellular matrix was penetrating the pores.

In conclusion, calcium phosphate biomaterial showed good affinity to leukocytes; furthermore, blood cells and vein tissue cells can be well-attached and survive, proliferate and differentiate.

Résumé

Contexte

Lorsqu'un matériau est implanté dans le corps, invariablement, le premier fluide avec lequel il entre en contact est le sang. Alors que le sang total est principalement constitué de globules rouges, les leucocytes représentent 1% du sang, ce qui les rend nettement moins nombreux que les globules rouges. Bien que les leucocytes soient une petite population de cellules dans le sang, ils sont les composants sanguins les plus essentiels pour protéger le corps contre les substances étrangères et les facteurs infectieux. On sait peu de choses sur leur interaction avec les biomatériaux au-delà de l'attachement initial après l'activation. De même, alors qu'il existe des milliers de rapports examinant les biomatériaux et les cellules endothéliales, il y en a à peine 100 enquêtant sur l'interaction de l'adventice ou tunique externe des vaisseaux sanguins avec les biomatériaux, même s'il est fort probable qu'un matériau implanté entre en contact avec l'extérieur du vaisseau sanguin qu'avec sa lumière. Dans cette étude, l'interaction du sang et des vaisseaux sanguins avec le biomatériau phosphate de calcium, en tant que matériau le plus couramment utilisé dans la réparation osseuse et l'ingénierie tissulaire, a été étudiée. Ce projet se concentrait principalement sur l'effet de leucoréduction du biomatériau d'hydroxyapatite, l'attachement et la survie des cellules immunitaires sur ce matériau, en plus d'étudier la fixation et la prolifération des cellules tissulaires veineuses sur le biomatériau cylindrique de phosphate de calcium.

Méthodes

Les échantillons de sang et de tissu veineux pour cette étude ont été prélevés de rats mâles en bonne santé. L'interaction de l'hydroxyapatite avec les leucocytes dans le sang total et son effet de réduction des leucocytes ont été déterminés après incubation de deux types d'hydroxyapatite (HAp-1 et HAp-2) et de coton avec des échantillons de sang pendant 1h, 2h et 4h. Le comptage manuel

des leucocytes, le comptage différentiel en utilisant la coloration au Giemsa et le test MTT ont été réalisés pour examiner le nombre de leucocytes dans le sang entier après incubation avec des matériaux HAp. Le test Vivant et Mort (¨Live and Dead¨) ainsi que le détachement des cellules ont été effectués pour determiner la viabilité cellulaire et identifier les cellules attachées aux biomatériaux. L'interaction des vaisseaux sanguins avec le biomatériau de calcium-phosphate a été testée en détectant la croissance cellulaire sur le biomatérieau avec le test Vivant et Mort et l'imagerie par ¨Scanning Electron Microscope¨ (SEM).

Résultats

Le nombre de leucocytes dans le sang total après incubation avec HAp-1 a diminué de manière significative après 4h en augmentant la masse du biomatériau ($p < 0,05$). Environ 30% des leucocytes du sang total ont été attachés à l'HAp-1, qui, par rapport à l'HAp-2, a montré un plus grand effet de réduction des leucocytes. Le coton, matériau le plus courant dans les filtres à leucocytes, a montré plus de 50% de réduction des leucocytes dès la première heure d'incubation. Selon le test Mort et Vivant, une plus grande quantité de cellules était attachée au biomatériau après 4h plutôt que 1h. Les lymphocytes et les monocytes ont pu être identifiés après détachement cellulaire sur un biomatériau d'hydroxyapatite.

Conclusion

Selon les données obtenues à partir de différents essais avec du sang et des tissus veineux, l'effet de leucoréduction des matériaux d'hydroxyapatite dans le sang total dépend de la masse et du temps. L'augmentation de la quantité de matériau améliore leur efficacité en augmentant la surface. Les leucocytes peuvent rester attachés aux biomatériaux HAp pendant 5 jours et survivre, et leur identification a montré que les lymphocytes et les monocytes se sont davantage attachés. De plus,

le tissu veineux peut être bien attaché à un biomatériau de phosphate de calcium microporeux cylindrique, et selon la SEM et l'imagerie par fluorescence, les cellules se développaient à travers le biomatériau et la matrice extracellulaire pénétrait les pores.

En conclusion, le biomatériau phosphate de calcium a montré une bonne affinité pour les leucocytes; en outre, les cellules sanguines et les cellules des tissus veineux peuvent être bien fixées et peuvent survivre, proliférer et se différencier.

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Author Contributions and Statements of Originality

The work described here was performed by the author and is original. This is the first report that describes the whole blood and vessel interactions to biomaterials, focusing on the leukocyte filtration and angiogenesis stimulation effect of calcium phosphate biomaterials.

Part I Leuko-reduction Effect of Hydroxyapatite

I. Chapter 1- Introduction

1.1Rationale

When a material is implanted in the body, invariably, the first fluid it comes into contact with is blood. While mostly consisting of red blood cells, leucocytes account for 1% of the blood volume. Little is known about their interaction with biomaterials beyond initial attachment following activation. Similarly, while there are thousands of reports examining biomaterials and endothelial cells, there are barely 100 investigating the interaction of the tunica adventitia of blood vessels with biomaterials, even though it is more probable that an implanted material will contact the exterior of the blood vessel than its interior lumen. This thesis explores whole blood interactions and explanted vessels with calcium phosphate materials commonly used to repair bone defects to characterize these overlooked early-stage material-cell interactions.

It was discovered in the 1950s that leukocytes present in the blood led to side effects in transfusion recipients. Since then, allogeneic leukocytes in various blood components have affected recipients with some adverse responses. According to the studies, there was a relationship between leukocyte numbers in blood and side effects controlled by reducing the residual leukocytes (1). There are different methods for removing leukocytes from whole blood, including centrifugation, washing, freezing, buffy coat removal, and filtration (2-4). The different efficiencies of each procedure make them suitable for different aims. For blood products, sterility is essential, and leukocyte removal before transfusion by filters is sterile, fast, and efficient.

There are several diseases and surgical cases for which blood transfusion should be done after a leukocyte reduction filtering process, such as thalassemia major (5, 6), sickle cell anemia (7, 8), leukemia (9, 10), transfusion-transmitted, and cytomegalovirus infection (10, 11), patients awaiting organ transplantation (12), prevention of platelet refractoriness in thrombocytopenia, multiple or chronically transfused patients (13).

Reperfusion injury is one of the critical factors that can happen following ischemic injury, and according to recent studies, leukocyte reduction filters can control it (14-16).

The ischemic injury occurs when the blood supply to an area of tissue is restricted, and the shortage of oxygen and nutrition causes damage to the tissue area. The ischemic injury occurs in a wide

range of diseases such as myocardial infarction, stroke, and thrombotic disorders. Ischemia affects 1.3 million people annually in the US. Heart disease is the second most prevalent cause of death in Canada. Ischemic disorders of the heart are the most common form of heart disease, the main reason for shortened lifespan (17), and the second leading cause of disability-adjusted life years lost (the number of years lost due to ill-health, disability, or early death) time (18, 19). For patients with acute coronary syndrome and myocardial infarction (MI), preventing and limiting the ischemic damaged area is vital to lower the chance of heart failure, and prevention of reperfusion effects is an essential step for that (20-22).

Ischemic injury also occurs in cardiac surgery when blood vessels are cross-clamped and in organ transplantation (23). A key player in reperfusion injury is leukocyte attachment to capillary walls and neutrophil-mediated necrosis; hence leukocyte filtration can reduce ischemic size in the damaged area (14, 24, 25). Although leukoreduction filters in blood banks have been used for decades, using leukocyte filters during surgery is a relatively new approach. In the initial design of the blood filter, compacted cotton wool was used as the filtering material. The first generation of leukoreduction filters used cellulose material with 98% of leukocyte removal. Their limitations included complement C3 activation and filtration time dependent on the blood flow speed through the filter (26). After years of study and enhancement of leukocyte filter design, current modern filters have been developed as composite filters where synthetic nonwoven microfiber materials are used in their design. In these filters, filter materials are found in the form of compressed cotton wool fibers, polyester, and sometimes polyurethane. The new generation of filters combines a high filtration rate with speed of flow (16).

1.2 Scope of the Project

Since altering leukocyte numbers in tissues by using biomaterials had a profound effect on its healing, as is clearly demonstrated in the transplantation literature, this project investigated whether bioceramics might behave similarly to fibrous leukocyte filters and might support their survival and expansion.

1.3 Project Contributions to Healthcare

The proposed project is primarily concerned with studying the leukocyte filtration effect of calcium phosphates compared to cotton fibers, a material used initially to remove leukocytes from blood, and a commercial leukocyte filter. Based on the vast range of diseases and surgical situations favoring the use of leukoreduction filters and the low number of studies on cell survival and attachment on the filters, this study can lead to a new high-efficiency generation of leukocyte filters.

II. Chapter 2- Overview of Leukocyte Filtration

2.1 Leukocytes

Leukocytes or White Blood Cells (WBC) are immune system cells that protect the body against infectious agents, the damage they cause, toxins, and foreign threats. The normal range of leukocyte number is wide, from 4000-11000 cells/µl, in human blood, and a change outside of this normal range indicates diseases as the body's response to infectious and foreign factors (27). White blood cells make up approximately 1% of the total blood volume in a healthy adult, making them substantially less numerous than red blood cells. Hematopoietic cells (HSCs) are pluripotent cells in the bone marrow from which all the cellular elements of blood, including the red blood cells (RBC) that transport oxygen, the platelets that trigger blood clotting in damaged tissues, B cells, T cells, and other circulating WBC, ultimately derived. Figure II.1 shows the origin of blood cells and their differentiation from pluripotent hematopoietic stem cells that give rise to marrow resident lineage cells and the blood cells. Most of the leukocytes develop and mature in the bone marrow (Figure II.1).

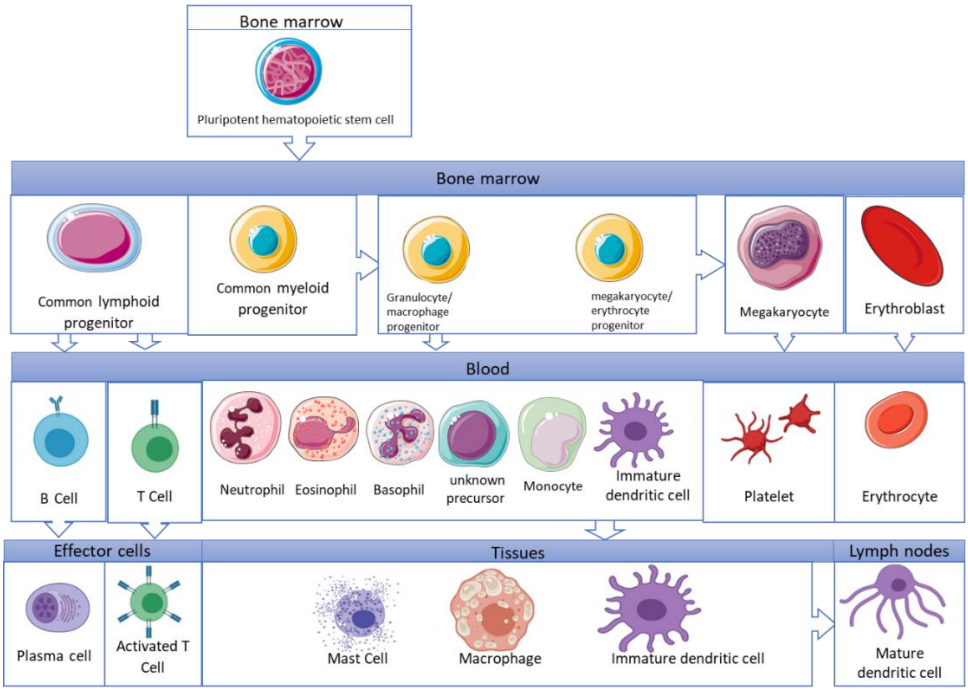


Figure II.1. All the blood's cellular elements, including the immune system cells derived from hematopoietic stem cells in bone marrow (with permission to reproduce picture (Zimmerli, 2005 #151)).

Leukocytes can be distinguished from the other blood cells by their nucleus as red blood cells (RBC) and platelets are nuclei free (28, 29).

According to their origin, structure, and functional characteristics, leukocytes are classified into different types. Leukocytes are commonly identified by their color and morphology using high resolutions light microscopy with the Giemsa stain. This stain differentiates the nuclear and cytoplasmic morphology of the different blood cells. Figure II.2 shows the morphology of the different types of leukocytes. They could be classified based on their structure as granulocytes and agranulocytes or based on their cell line of development as myeloid and lymphoid cells (29).

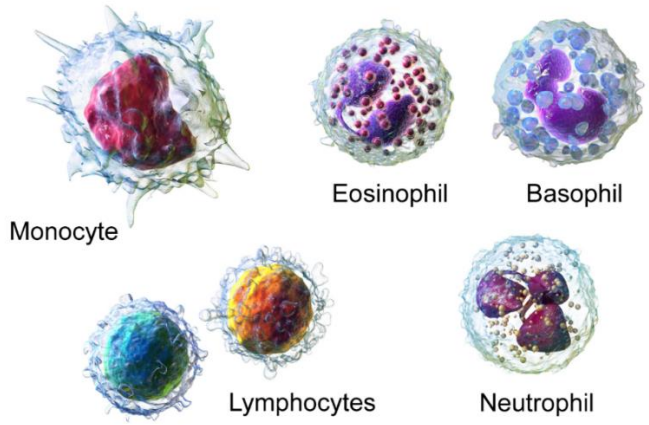


Figure II.2 Schematic picture of white blood cells showing their size morphology and nucleus differences (Blausen.com 2014 #103)

Granulocytes, which are named for the densely staining granules in their cytoplasm, are also called polymorphonuclear (PMN) because of the variety in their nucleus shape, which makes them distinguishable from agranulocytes. Neutrophils, basophils, eosinophils, and mast cells are subtypes of granulocytes. These types of leukocytes are all relatively short-lived, surviving for only a few days. Different approaches have reported the average lifespan of inactivated human neutrophils in the circulation to range between 5 and 135 hours. Upon activation, they migrate into tissues, where they survive for 1–2 days (30). Neutrophils are phagocytic cells and the most numerous and essential cells in innate immune responses (60-65% of the total circulating leukocytes). Eosinophils and basophils are present less than neutrophils in blood flow (0-6%), but like neutrophils, they have granules containing various enzymes and toxic proteins, which are released when these cells are activated by attraction to infectious tissues (29, 31). Mast cell development begins in the bone

marrow, and their maturation happens in peripheral tissues such as skin, intestines, and airway mucosa (29).

Agranulocytes, also called mononuclear leukocytes, are noted by the absence of granules in their cytoplasm. Lymphocytes (B cells and T cells) and monocytes are two subtypes of agranulocytes that make up 35% of blood circulation (27). Monocytes in response to infection differentiate into macrophages and dendritic cells; macrophages remain in the tissue and do phagocytosis, whereas dendritic cells present antigen fragments to lymphocytes. Figure II.3 depicts the different percentage of the leukocyte types in human blood compared to rat blood (32-34). While rodents are often the choice model for studying healing and biomaterial interactions, their immune cell characteristics differ from those of humans. The human immune system is neutrophil rich (~2:1 ratio of neutrophil: lymphocyte), whereas, in rodents, it is lymphocyte rich; 80-90% of immune cells are lymphocytes (35, 36).

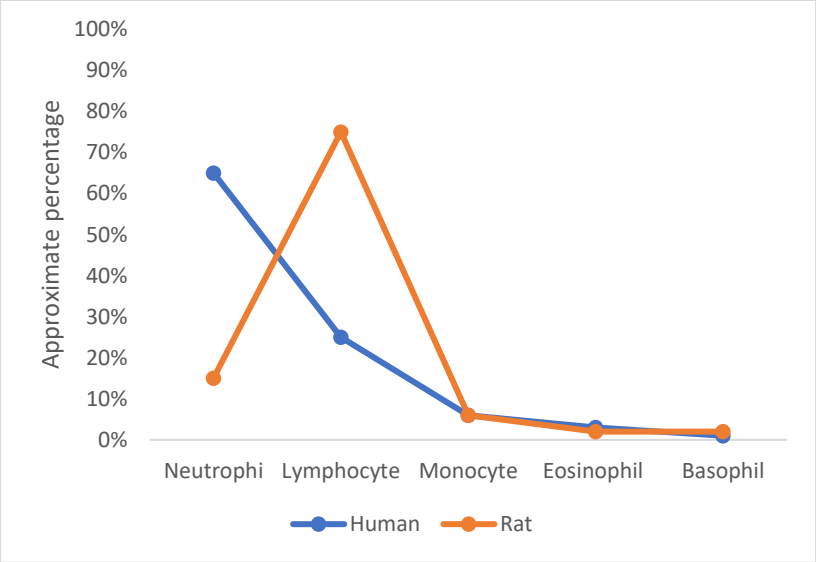


*Figure.*II.3. *Approximate percentage of different types of leukocytes in healthy human blood in comparison to their percentage in healthy rat blood.*

2.2 Reperfusion Injury and Related Factors

Reperfusion injury, also known as Ischemic-Reperfusion Injury (IRI) (37), results from the adverse effect of blood flow restoration in the ischemic site leading to cellular death, tissue dysfunction, and expansion of the damaged area. There are several vital organs that IRI can affect, including the heart, lung, brain, kidney, and muscles. By destroying any of these organs, reperfusion injury affects related organs and can lead to systemic disorders. Several factors participate in extensive tissue damage. Reactive oxygen species (ROS), nitric oxide (NO), endothelins (potent peptide vasoconstrictors produced by the vascular endothelium), cytokines (tumor necrosis factor-alpha (TNF- α,) interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and platelet-activating factor (PAF)) are influential factors that contribute to reperfusion injury (18, 38). Complement system[1] activation following ischemic injury produces chemotactic factors for leukocytes and stimulates neutrophil activation (39). Leukocytes play a vital role in reperfusion injury by different mechanisms. Leukocytes' trafficking and plugging of the capillaries are associated with microvascular dysfunction and IRI. An inflammatory response to molecular changes in the vascular wall after ischemia can be an intensive reaction created by leukocytes in returning blood flow that leads to reperfusion injury (40, 41). Activated leukocytes with or without platelets attach to the vascular wall and by plugging of capillaries disrupt blood flow and cause extension of injury. Between all types of leukocytes, activated neutrophils have a crucial role in IRI. Neutrophils are considered as the primary cells in response to IRI that, just after activation, migrate to the damaged site. There is a positive feedback loop of neutrophil activation and enlistment, which starts after capillary blockage by neutrophils and causes tissue necrosis. Tissue damage stimulates an immune response followed by secretion of pro-inflammatory chemokines by neutrophils (42-44).

In addition to these, endothelial dysfunction plays a critical role in the pathogenesis of reperfusion injury. Studies demonstrated that endothelial dysfunction results from the interaction of neutrophils and other inflammatory cells with endothelium during critical early and later phases of IRI. Adhesion molecules on endothelial cells and neutrophils are the main factor of interaction between these two types of cells. Among all transmembrane adhesion molecules, selectins[2] cause the initial interactions of neutrophil–endothelial cells. Besides neutrophils, monocyte activation in the

[1] Complement is a system of plasma proteins that can be activated directly by pathogens or indirectly by pathogen-bound antibody, leading to a cascade of reactions that occurs on the surface of pathogens and generates active components with various effector functions

[2] Selectins are a family of transmembrane molecules, expressed on the surface of leukocytes, activated endothelial cells and in platelets.

ischemic area leads to infiltration of chemotactic protein and cytokines associated with IRI and extension of the injured site (18, 42, 45, 46).

Controlling and preventing IRI is crucial for the limitation and treatment of the injured area after ischemia. However, there are many different mechanisms associated with reperfusion injury in different organs; leukocytes play a central role, and filtering them can help IRI prevention or limitation (18, 20).

2.3 Leukocyte Filters

A Fleming first introduced filtration to remove leukocytes from blood in 1928, and a cotton column was the first material used for small quantities of blood (47). Standardized leukocyte filters emerged, and evolution was simultaneous with commercial blood collection containers available in blood banks (48). It was in the 1960s for the first time when the reperfusion effect on open-heart bypass procedures was reported to be controlled by filters that led to the design of a different type of leukocyte filters with 40μm woven polyester mesh, which became a worldwide standard (49, 50). Different leukocyte filters were designed and developed to overcome the limitation of blood volume and particle size they can filter. The initial studies on leukoreduction filters focused on disorders associated with blood transfusion in blood banks. Other disorders were then suggested that could be controlled by leukocyte filterings, such as preventing alloantibody-mediated refractoriness to platelet transfusion support or colorectal surgery (51). The field of these studies has broadened and includes a wide range of diseases and surgical situations in different organs that can be improved in terms of morbidity and mortality using leukocyte filters.

From the initial years of using leukocyte filters, different types of filters with different materials were developed. In 1972, P.Diepenhorst reported an improvement in leukocyte filtration with 95% efficiency by making blood cell suspensions in saline and using filters with tightly packed cotton wool columns (52).

There are three categories of filtration, including surface filtration, cake filtration, and depth filtration, and each may have some limitations. During surface filtration, particle size is essential, and those that are bigger than filter size cannot pass, but because of the aggregation of big particles on the surface, surface filtration is just suitable for a low concentration of particles. In cake filtration, filtered particles make a porous layer on the surface, and filtering will be done as long as the porous

flow is possible. During depth filtration, because of the filter's specific porous structure, filtration of particles occurs throughout the filter, not just on the surface, and retention of particles is not restricted in the surface (4). Filtration of leukocytes from whole blood is more like depth filtration, but it can be a combination of all three mechanisms.

Adhesion of cells to the material is essential, especially when the diameters of cells are significantly smaller than the filter's pore size. Adhesion helps retain cells in the filter pores without mechanical forces such as gravity and hydrodynamic pressure. The main application of in-depth filtering is removing particles with a size significantly smaller than pore size, and adhesion can overcome this problem. There are several characteristics of filter material that can affect the filtration efficacy and cell adhesion. Chemical properties, electrical charge, morphology, and microstructure are the most influential factors in the filtration process. As a result of deformability differences, leukocytes have difficulty passing pores with a diameter of less than 5µm, whereas red blood cells will easily pass 3µm pores (16, 53). Several investigators have tried to relate the extent of cell adhesion to the substrate surface's chemical composition, but very few specific studies using leukocytes are available. Physical and biological factors contribute to the leuko-reduction effect of filters; however, the precise mechanism is not exact.

2.4 Hydroxyapatite (HAp)

Hydroxyapatite ($Ca_5(PO_4)_3(OH)$) belongs to a broad group of calcium minerals based around the phosphate $(PO_4)^{3-}$ group. The chemistry of HAp is complex and with many possible ionic exchanges (54, 55).

Hydroxyapatite is a bioceramic that has been used in bone repair and osseous defects studies for decades because of its similar chemical structure to the main inorganic component of bone. Its biocompatibility, bioactivity, and hydrodynamic stability in the body make it a suitable biomaterial for a wide range of repair and tissue engineering aims (56). Bone defects, bone grafting, and prosthesis revision surgery are the situations in that HAp has been used as filling material besides being used as a coating material on biomedical implants and drug delivery applications (57-59).

In recent years, biomaterial scientists attracted more interest in the preparation of HAp bioceramics with porous morphology. The mechanical interlocking of porous HAp coatings reduces the motion between implant and host tissue, leading to faster healing processes. Pore size and shape have a

critical effect on the functionality of these bioceramics. The potential of vascular restoration and bone tissue penetration throughout the repair site made the porous HAp (60) attractive material to be developed to fill load-bearing and non-load-bearing bone injuries. Porous HAp materials have found a wide range of use in biomedical applications (61-63). In addition to being used as a hard tissue scaffold, porous HAp is reported to be used for cell loading, drug-releasing factor, and chromatography analysis (63, 64). Besides their mechanical, chemical, and thermal resistances, porous ceramic biomaterials' high surface area makes them suitable for cells to adhere to and grow.

2.5 Limitations of Commercial Filters

Even though current leukocyte filters are efficient, making more optimized filters for different usages is desirable. Filters that can produce RBC concentrates entirely free of leukocytes have not yet been developed but may be beneficial for the various clinical situations discussed previously. The filter capacity is one of the characteristics that should be optimized. Increased leukocyte removal capacity may reduce filter size and a higher yield of purified RBCs, desirable for clinical and logistical purposes. Overall, leukocyte filtration is still a relatively expensive method to prepare leukocyte-poor blood compared with other differential centrifugation and washing methods. Reduction of the processing time is advantageous for the rapid provision of leukocyte-poor blood and directly applying filtration at the bedside. The development of new filters would benefit from a better understanding of the mechanisms causing leukocyte depletion. So far, these mechanisms have not been studied systematically, although several investigators have suggested that leukocyte filtration is managed by screen and adhesion (53, 60, 65, 66). However, the quantitative contribution of each of these factors has never been rigorously studied. Leukocyte survival and their proliferation are the other factors that need to be further investigated.

III. Chapter 3- Leukocyte-Biomaterial Interaction and Leukocyte Filters; Review of the Literature

3.1 Leukocyte-Biomaterial Interaction

When implanted in vivo, all biomaterials stimulate cellular and tissue responses, including inflammatory responses and foreign body reactions.

The surgical procedure of implanting a biomaterial for bone repair or replacement heightens the biomaterial's chance to contact blood. Whole blood components have a different reaction to biomaterials based on their biological structures. Blood/biomaterial interactions begin simultaneously after implanting, with protein adsorption to the biomaterial surface. In 2005 Cameron J. Wilson and his colleagues (67) studied adsorbed proteins during cell interaction with implanted bone tissue engineering and orthopedic disorders materials, and Robert A. Latour (68) did a related study on protein-surface interaction on biomaterials for tissue engineering. In addition to protein-biomaterial interaction, platelet- biomaterial interactions have been widely studied. Platelet activation effect on hemocompatibility (69), surface adhesion (70), and surface design of implanted materials for cardiovascular and orthopedic disorders are investigated (71, 72).

While material interactions with other blood components are extensively studied, little is known about the induction of leukocyte activation by materials. Foreign body inflammatory responses mediated by implanted medical devices are critical to their integration and sensitivity to infection.

Leukocyte activation induced by biomaterials has been investigated in several studies that examined the accumulation of inflammatory products after a particular time (73, 74).

Adhesion of polymorphonuclear (PMN) cells and monocytes on modified surface of biomaterials using titanium was studied in 2001, and according to their data, the thickness of coated material has a different effect on adhesion of different leukocyte cell types (75). Induction of leukocyte activation by implanted materials was studied, focusing on the structural effect of biomaterial in 2012 by Xiaojian Chang and Maud Gorbet (76).

A study involving ceramic biomaterials showed PMNs had been activated in the absence of serum by oxygen burst of $CaHPO_4$ powder (77), and differential response of human blood leukocytes to calcium polyphosphate biomaterials was examined by Noah Fine and his colleagues in 2019. In a

recent study, calcium phosphate-based biomaterials interaction with PMNs and monocytes was analyzed, and they showed that these cells are highly activated after exposure to some types of calcium-phosphate biomaterials with specific structures (78).

3.2 Leukocyte Removal Applications and Modifications

Considering the reverse effect of leukocytes in special situations such as blood transfusion and reperfusion injure, the necessity of leukocyte removal from whole blood and the injured site has led to several studies on the effect of leukocyte removal filters. After cardiac surgery, activated leukocytes cause a generalized inflammatory response. Removing leukocytes is suggested in several studies to minimize or even prevent postoperative tissue injury (79-81). Although leukocyte reduction using filtration is common in blood banks (60, 82), the application during surgery is a relatively novel approach.

The first time in 1991a leukocyte-removing filter developed for use in the cardiopulmonary bypass (CPB) circuit was created by Pall Biomedical Products Company (East Hills, NY). Their filter was made of a 40μm rated polyester screen with a surface modified by polycarbonate (83). During a study in 1989, researchers showed that neutrophil depletion reduces myocardial infarct size after 90 minutes of ischemia by limiting reperfusion injury (14). Several studies approved the effect of leukoreduction on limiting ischemic site expansion (16, 37, 84).

Surface modification of materials in leuko-reduction filters enhances the efficiency of filtering. Polymethoxyethylacry used as a coating material on leukocyte filter resulted in a significant reduction of the systemic inflammatory response's incidence modulation and its side effects in post-cardiopulmonary bypass atrial fibrillation (85).

Eun Jin Kim and his colleagues modified melt-blown poly(butylene terephthalate) nonwoven (PBT-NW) using hydroxyapatite (HAp) for removal of leukocytes from whole blood, and they could remove 98.5% of the leukocytes and recovered 99.5% of the erythrocytes using this modification (86). Hydroxyapatite is used as a coating material on nonwoven fabrics for enhancement of cell differentiation, and according to obtained results, HAp affected the enhancement of lineage-specific differentiation to myocardial-like cells (87).

In this study, hydroxyapatite's leukoreduction effect will be investigated besides the survival and differentiation ability of attached cells to this type of bioceramic.

IV. Chapter 4- Hydroxyapatite Leukocyte Filtration Effect; Leukocyte Attachment and Survival on Hydroxyapatite

4.1 Hypothesis and Objectives

Hypothesis:

Our preliminary data support the hypothesis that hydroxyapatite (a material widely used to repair healing bone) has a good affinity with leukocytes. Leukocyte affinity of hydroxyapatite was examined by detecting cell attachment to biomaterials and leukocyte number reduction in whole blood samples in interaction with hydroxyapatite (88). We hypothesized that different material properties such as surface area, porosity, ion release will not only affect immune cells attachment but will also influence their survival and differentiation.

Objectives/Specific Aims:

This research project's main objective was to quantify leucocyte attachment to different hydroxyapatite forms and assess its reproducibility and kinetics to verify hydroxyapatite's filtration effect by monitoring leukocyte numbers using manual cell count and colorimetric methods to measure leukocyte reduction in whole blood after introducing to biomaterials. Then find cells attached to biomaterials using fluorescent microscopy. This project's first step was to study leukocyte dynamics (changes in number and viability) in whole blood under different conditions with biomaterials. WBC count was initially performed manually using peripheral rat blood with a hemocytometer. Different time points, materials, and incubation conditions were tested.

4.2 Introduction

Calcium phosphate-based biomaterials are extensively used in bone repair and tissue engineering study, and surgical applications. Bone implant biomaterials are used to repair or enhance injured hard tissue healing by replacement or tissue regeneration. The similarity of calcium-phosphate biomaterials to the prominent inorganic bone and teeth tissue components makes them suitable for bone replacement studies (88-90). The body's reaction following material implanting incorporates several processes such as blood–material interactions, acute and chronic inflammatory response, foreign body reaction, and fibrous capsule development. Blood is the first fluid in the body that will contact implanted material. Surgical implantation of bone repair materials initiates a series of responses in the body, starting with blood–biomaterial contact and leukocyte responses (91, 92).

Leukocyte presence in the injured site could lead to reverse responses. Ischemia (restriction of blood supply to tissues, causing a shortage of oxygen needed for cellular metabolism) has been identified as one of the factors inflicting enormous damage reported in several orthopedic and clinical cardiac disorders (93-95). One of the side effects of ischemia is reperfusion injury, also called Ischemia-Reperfusion Injury (37). Reperfusion injury is defined as the paradoxical phenomena that cause increased cellular damage following the restoration of blood flow to previously ischaemic tissues. An inflammatory response occurs when tissues are injured, and the damaged cells release chemotactic factors such as cytokines, interleukins, histamine, and prostaglandins. The swelling will be one of the consequences of these chemicals that cause blood vessels to leak fluid into the tissues.

Moreover, the blockage of vessels and capillaries' wall by leukocytes in the returned blood is one of the other factors related to reperfusion injury. The accumulation of activated leukocytes by activation of the complement system and the release of inflammatory mediators in the pulmonary circulation plays an essential role in myocardial damage associated with the cardiopulmonary bypass (CPB) due to surgical reperfusion injury. Leukocyte filtration from whole blood is commonly used in CPB to avoid ischemic damage created by leukocytes in myocardia. Although leukocyte filtration has been applied in various other surgical and non-surgical conditions such as cardiac surgery, reduced postoperative infections, and blood transfusion cases, factors inducing leukocyte attachment in the filters have not been investigated adequately. Physical and biological characteristics such as pore size and shape of filtering materials or cellular adhesive factors are

suggested as the most effective leukocyte removal elements, but the exact mechanism of leukocyte attachment is unknown. On the other hand, different types of WBCs in the whole blood have different lifespans, and there is no significant documented study examining how attachment to materials affects leukocyte survival on filtering biomaterials.

In this study leuko-reduction effect of calcium-phosphate bioceramics (as the most common material in bone repair studies) is investigated in the interaction of whole blood with hydroxyapatite. Leukocyte attachment, survival, and proliferation are compared in different materials.

We will examine changes in the whole blood leukocyte population interacting with biomaterials *in vitro* by exposing them to hydroxyapatites besides detecting cells attached to the materials.

V. Chapter 5. Methods and materials

Cell viability and leukocyte cell count were determined using the standard MTT assay (Sigma-Aldrich) and manual cell counting with Trypan blue staining (Sigma-Aldrich). For observation of leukocyte attachment on biomaterials and more precise cell viability assessment, Live & Dead assay (LIVE/DEAD®, ThermoFisher) was conducted after incubation of whole blood with biomaterials after different time points using fluorescence microscopy. Leukocytes attached to biomaterials were cultured using DMEM medium with 10% FBS (Gibco) to test their survival after attachment to biomaterials.

5.1 WBC Counting:

5.1.1 Blood Withdrawal:

Three 6-10 weeks old healthy Wistar male rats' peripheral blood was used in each experiment. In the experiments where a small amount of blood was needed, lateral saphenous vein blood collection protocol (SOP #403) was used, and for high volume, blood collection was done using the intracardiac puncture procedure.

Intracardiac puncture blood collection is a terminal procedure and can only be done under anesthesia or less than a minute after euthanasia. The procedure of blood collection according to the protocol, including the following steps (96):

A syringe between 3cc and 10cc with a 20G 1½" needle was prepared.

The rat was placed in the dorsal position (Figure V.1).

The xiphoid process between the last two ribs at the tip of the sternum was touched.

The needle tip was inserted between the left side of the xiphoid process and the last rib.

The thoracic cavity was penetrated slowly while directing the needle toward the heart at an angle of approximately 40-45 degrees.

Note: The heart is slightly in the left of the midline.

When a small quantity of blood flows into the needle's hub, we stabilize the needle and continue to pull back on the plunger slowly. The blood should flow into the syringe at a steady rate.

Note: If the blood flow stops, we change the needle's angle slightly, rotate it, or make minimal movements to alter the needle placement.

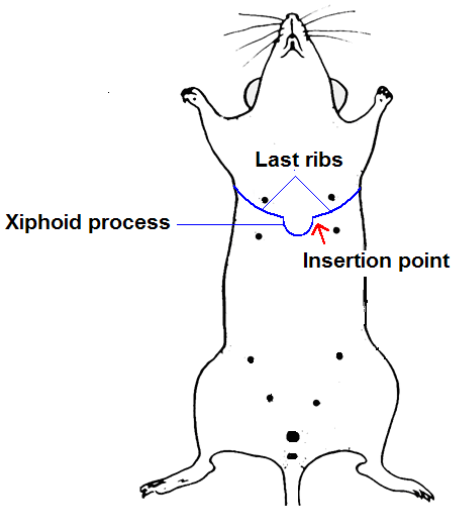


Figure V.1 Animal position and finding insertion point for intracardiac puncture blood collection.

Collected blood was transferred to the laboratory in heparinized tubes (10 IU/ml) (97) on ice (4°C).

5.1.2 WBC Manual Counting

For each biomaterial and control group, three samples were prepared at any time point from each rat's blood.

Studied materials in this project:

- Hydroxyapatite type 1 (HAp-1)
- Hydroxyapatite type 2 (HAp-2)
- Cotton (as common material in commercial leukocyte filters)
- Commercial filter[3] material
- Control group: blood sample without any biomaterial.

Calcium phosphate microspheres made by emulsion processing were kindly supplied by Dr. Uwe Gbureck, University of Wurzburg.

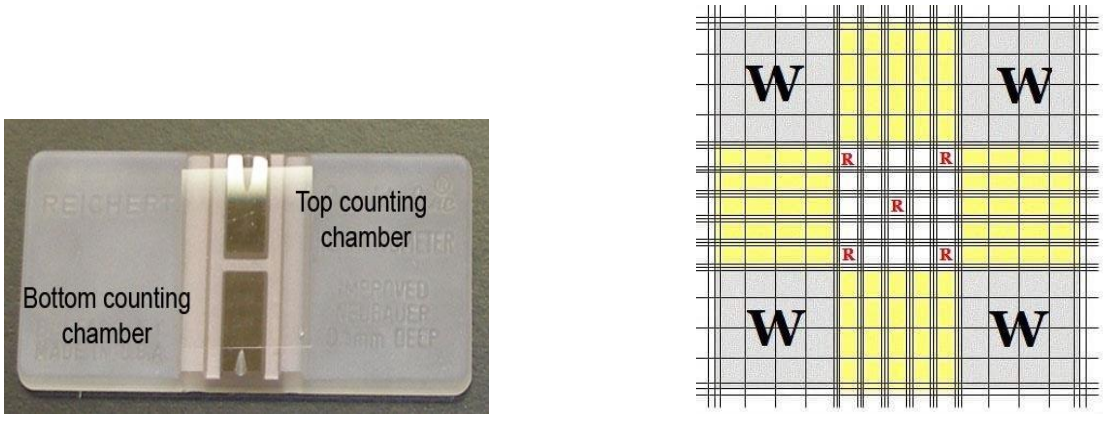
[3] Salvaged Blood Filters, for the filtration of up to one unit of washed or unwashed intraoperative or postoperative salvaged blood with 90% of leukocyte filtration efficiency.

Hydroxyapatites and cotton were added to 250 µl of the blood samples and incubated for one hour, two hours, and four hours at room temperature. White blood cell manual counting was done with different amounts of biomaterial (5, 10, and 25 beads of HAp ~ 3mg, 6mg, and 15mg). The Control group was in the same condition as the test groups. After 1h, 2h, and 4h incubation, materials were removed from blood samples. Whole blood samples were diluted in a 1:20 or 1:50 ratio with RBC lysis and incubated for 5 minutes at room temperature. The RBC lysis, which facilitates manual counting by lysis of RBCs, was prepared according to Table V-1(98).

Table V-1 RBC lysis solution; Ammonium chloride lyse (10X concentration)

NH_4Cl	8.02g
$NaHCO_3$	0.84g
EDTA	0.37g
Reach 100ml with Millipore water. Store at 4°C for up to 6 months	
Working solution Dilute 10ml 10X concentrate with 90 ml Millipore water. Refrigerate until use	

The diluted blood suspension was loaded in a hemocytometer chamber (Figure V.2) after being stained with Trypan blue, and all the cells in four large squares were counted.



A B

Figure V.2 . A) Hemocytometer and B) its counting area for manual counting of WBC detected by W.

Leukocyte number in each animal's sample was counted three times, and the mean number of counted cells was considered for statistics calculations. In each group of samples, the fresh blood

WBC count is considered as control. Leukocyte counts in individual animals are different and fall in the normal range of 4000-11000 leukocytes/µl (99).

5.2 Giemsa Staining

Giemsa staining was accomplished to determine the differential cell count change after incubation of blood samples with biomaterial in comparison to the control group. 25 beads of HAp-1(~ 15mg) and the same weight of cotton were added to 250 µl of whole blood samples, and after 1h and 4h incubation at room temperature, Giemsa staining was done for blood smears prepared on slides. Blood sample with no material was considered as the control group. Like any Romanowsky stains, Giemsa is a buffered thiazine-eosinate solution for interaction with acidophil or basophil blood cells' tendency. Azure and methylene blue, a basic dye, binds to the acid nucleus producing blue-purple color. Eosin is an acidic dye attracted by the cytoplasmic granules, which are alkaline-producing and cytoplasmic staining will vary from blue to light pink (100).

In this study, Giemsa and May Grünwald solution (Sigma-Aldrich®) were used according to the company procedure after preparation of blood smears on slides and fixing them in methanol 5 minutes for each sample;

Giemsa Stain was diluted 1:20 with deionized water or water buffered at pH 7.2 to achieve a bluer coloration.

- Slides were inserted in May-Grünwald Stain for 5 minutes.
- Slides were transferred to PBS 1X (pH 7.2) for 15 min.
- Slides were placed in the diluted Giemsa stain for 15-20 minutes.
- Slides were washed using deionized water very gently.
- After air-drying of the slides, the microscopy was done.

5.2.1 WBC Differential Manual Counting

After staining blood sample slides, different leukocytes were identified based on their morphology and nucleus staining. Lymphocytes, neutrophils, and monocytes are the most common leukocyte types that were attempted to be identified in this study. Their changes after incubation of blood with different materials were monitored to be compared to the control group. By counting 100 leukocytes

in a monolayer of Giemsa stained slide during the differential count, general categories of leukocytes could be identified, and their number presented as a percentage.

5.3 MTT Assay

After incubation with materials, cell viability and leukocyte cell amount remaining in the blood sample were determined using the standard MTT assay. MTT assay reflects the mitochondria's capacity in living cells to convert MTT to purple formazan crystals that can be solubilized with dimethylsulphoxide (DMSO) isopropanol.

In this study, 15mg of HAp-1, HAp-2, and Cotton were added into 250µl whole blood samples and were incubated at room temperature for 4 hours. After 4h of incubation, materials were removed, leukocytes were isolated using RBC lysis and centrifugation technique. Isolated leukocytes were seeded on 96 well cell culture plates after suspension in 200µl of cell culture media, and MTT tested their viability. Leukocyte numbers in each sample were calculated using the MTT standard curve.

Standard curve: Leukocytes in whole blood from the fresh sample was harvested using the centrifuging method and counted using Trypan blue and a hemocytometer. Cells were then serially diluted in a cell culture plate and incubated for 4h with MTT reagent at 37°C. After incubation, cells were treated with MTT solvent for 15 minutes at room temperature. Absorbance was measured at 570 nm.

In addition to the estimation of cell number, MTT assay was accomplished for checking the color change of hydroxyapatites, which were incubated in blood samples for 4h after they were removed from blood samples. They were washed with RBC lysis buffer in order to remove RBCs attached to their surface. A control group of biomaterials was incubated in PBS (instead of blood), and MTT was done using the same condition as the test groups.

5.4 Leukocyte Culture:

The attached leukocytes on biomaterials were cultured in cell culture conditions (37 °C, CO_2 5%). Five HAps exposed to whole blood samples at room temperature for 4h were seeded in 24well cell culture plates. DMEM was added and incubated in the cell culture condition for 7 days and monitored every day.

5.5 Live and Dead Assay:

The cell viability of attached cells on the materials was assessed using a LIVE/DEAD® Viability/Cytotoxicity Kit to observe leukocyte attachment on biomaterials. The two-color assay discriminates vital from dead cells by simultaneously staining with green fluorescent (494–517 nm) calcein-acetoxymethyl (calcein-AM) to indicate intracellular esterase activity and red fluorescent (528–617 nm) ethidium homodimer-1 to predict the loss of plasma membrane integrity(101). Biomaterials (HAp-1, HAp-2, Cotton, and a commercial filter) were incubated with the whole blood samples for 1h and 4h at room temperature. After the mentioned time pints, materials were removed from blood samples and were washed three times with PBS 1X to remove RBCs on their surface. The Live & dead assay was performed as recommended by the manufacturer. A 1:100 dilution of each dye was prepared. Hoechst dye (blue fluorescent stain specific for DNA (i.e., nuclei of eukaryotic cells)) with a ratio of 2:1000 was added to the staining solution. The staining solution was added to the materials and incubated at room temperature for 15-20 minutes. Imaging was done with a fluorescence microscope (Zeiss 416).

Cell attachment was done with isolated leukocytes as well. Leukocytes were harvested from whole blood using RBC lysis and centrifuging techniques.

Whole blood samples were diluted with RBC lysis 1:20, incubated at room temperature for 10 minutes.

Diluted samples were centrifuged at 2000 RPM for 5 minutes.

The supernatant was discarded.

Cells in the pellet were suspended in PBS 1X and centrifuged at 2000 RPM for 5 minutes.

Cell pellets (harvested leukocytes) were suspended in DMEM cell culture media.

Leukocytes were counted using a hemocytometer.

An equal amount of cell suspension was added to each material (15mg of material) and incubated for 1h and 4h at cell culture conditions.

After incubation times, materials were removed from the cell suspension.

One sample from each material was stained on the same day, and the others were cultured in DMEM at cell culture conditions.

Cultured materials were stained with Live & Dead assay after three days and five days of being cultured.

5.6 Cell Detachment and Identification of Attached Cells

After cells were detected on materials with live& dead assay, for identification of attached cells, they were detached from HAps. Cell detachment was done by trypsinization of cells on biomaterials. Trypsin 0.25% was added as much as it can cover the material's surface (100µl for 25 beads of HAps) and incubated 2-3 minutes in the incubator (37 °C, CO_2 5%). DMEM was used for the deactivation of trypsin, and the cell suspension was centrifuged in small tubes after being watched under an inverted microscope. Cell pellets were suspended in 50µl of plasma, which was isolated from rats' blood by centrifuging.

Cell type detection was done using Giemsa staining according to the above-mentioned protocol in this study.

5.7 Statistical Analysis:

All data were analyzed with Excel. The manual WBC counts were compared using a paired t-test (paired two samples for means). The WBC numbers were counted in three different tubes (for each sample), and the mean numbers were compared to the control group. Results with $p<0.05$ were considered to be significant

VI. Chapter 6. Results

6.1 SEM Imaging

Morphology, particle size, and the surface of the hydroxyapatite materials and cotton materials were investigated with SEM (Inspect F50, FEI Company, Hillsboro, ORA, USA) High voltage 5kv. Figure VI.1 depicts the surface microstructures of the HAp-1(A), HAp-2(B), cotton(C), and a random area of the commercial filter (D). HAp-1's particles have a flattened, rounded shape, and the size is around 1mm in diameter and 300 µm thick. The structure of the material is crystalline with about 1-3µm pore diameter.

According to the FESEM image of HAp-2 surface microstructure (Figure VI.1 B), HAp-2 particles are rounded (500-750 µm in diameter), it has a platey crystal (1µm across) structure, and the porosity mainly consists of the spaces between these crystals.

Figure VI.1 (C) shows the appearance of a random area of cotton fibers that were used in this study. The fibers are slightly flattened with a diameter of 10µm and a length of some hundreds of microns. The fibers are reasonably straight and somewhat aligned in regions; the spacing between fibers is up to 100 microns and typically 20-30 microns.

The Salvaged Blood Filter has a complicated structure with several layers. Figure VI.1 (D) depicts a FESEM image of some random areas. Compared to cotton, the fibers are much more densely packed and are more curled. Higher magnification shows fiber diameter varies from around 5-15 microns, and most fibers touch a neighboring fiber at least every 100 microns.

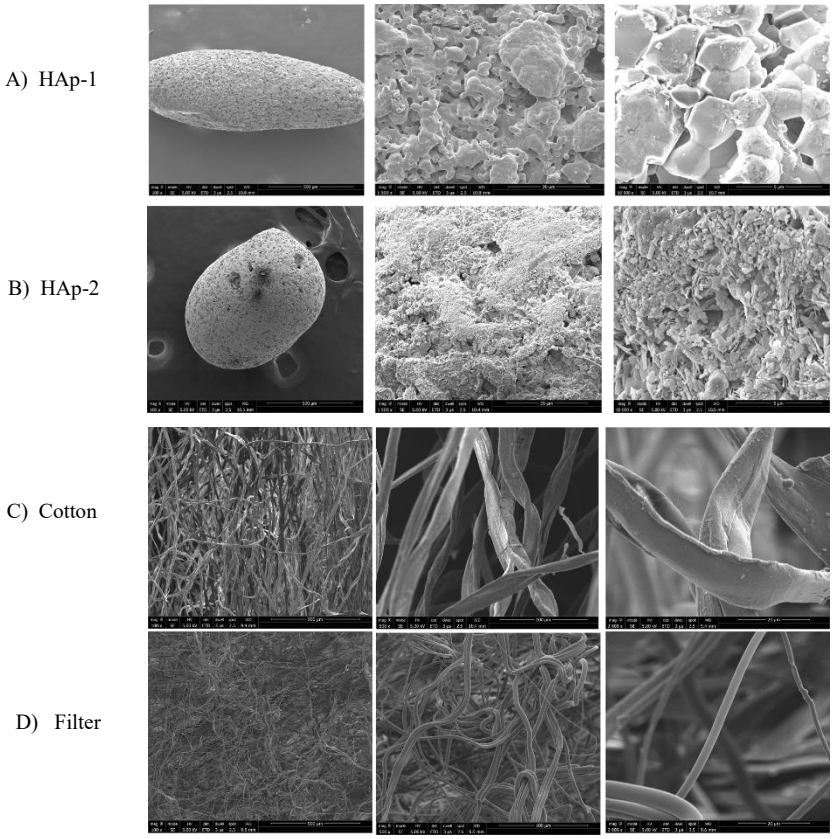


Figure VI.1 FESEM images of different materials' surface structures; (A)the HAp-1 has a flattened, rounded shape with 1mm diameter and 1-3µm pore size. B) FESEM image of HAp-2 microstructure; rounded 500-750 µm in diameter particles with a platey crystal structure. C) FESEM image of a random area of cotton fibers with a diameter of 10µm. D) FESEM image of a random area in Salvaged Blood Filter with various fibers diameter of around 5-15.

6.2 WBC Counting

To find a sufficient amount of hydroxyapatite with leuko-reduction ability, white blood cell counting with a range of 5-25 beads of these materials (3mg-15mg) was performed.

Figure VI.2 compares total WBC/ µl (of blood) counted manually after incubation of 250µl blood samples with five beads of Hydroxyapatite type 1 (~3mg) at time points of one hour, two hours, and four hours at room temperature. The Control group (no biomaterial) allows the change in leukocyte number in blood samples to be determined. Leukocytes were counted manually three times per rat with a hemocytometer, and cell number was calculated with the formula in Eqn 1.

$$\frac{total\ cell\ counted\ \ in\ 4\ squers \times dilution\ \ factor}{Area\ \ counted\ \ (4) \times dept\ h\ (0.1)}\ cell/\mu l = N \times 10^{6} cell \qquad \text{Eqn 1.}$$

The mean number of leukocytes in three rats' blood samples was 10083 (± 1700) cell/ µl of blood.

There was little change with time, and after 4h, cell number had reduced to 8400 (± 1200) cells/ µl blood. There was no significant decrease in leukocyte number after 4h (P> 0.05).

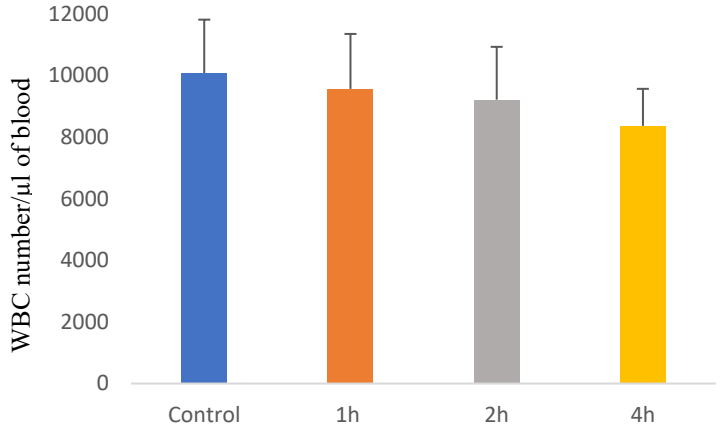


Figure VI.2 The WBC number/ µl of blood after incubating 250µl blood for 1h, 2h and 4h with five beads (~3mg) of HAP-1 in comparison to the control group. The Control group indicates the time zero leukocyte number in the blood samples without biomaterials

In the next experiment, blood samples were incubated with ten beads (~6mg) of HAp-1 for one hour, two hours, and four hours. According to the obtained data, the leukocyte number in blood samples changed after being incubated with the material compared to the control group.

Figure VI.3 depicts the total WBC/ µl (of blood) counted manually after incubation of 250µl blood samples with ten beads of HAP-1 (~6mg) at time points of 1h, 2h 4h room temperature in comparison to control. The Control group indicates the time zero leukocyte number in blood samples without biomaterials.

The mean number of leukocytes in three control samples was 11200 (± 1100) cell/ µl of blood. There was a significant change in leukocyte number after 4h (P<0.05), when the cell number had reduced to 9300 (± 670) cells/ µl blood.

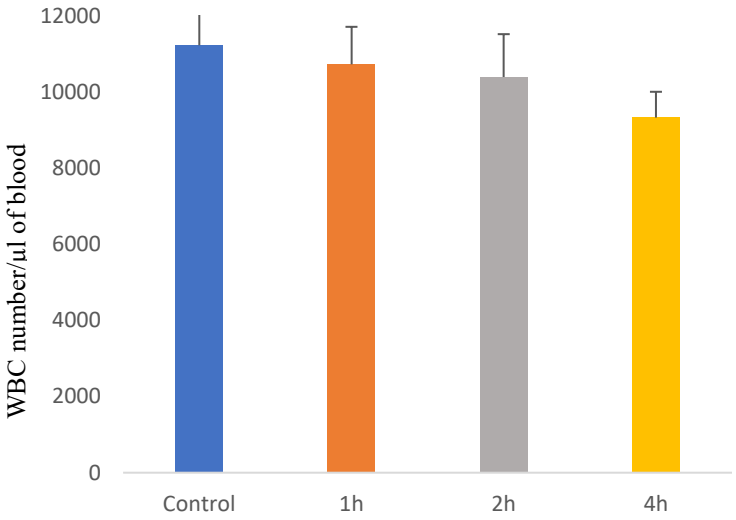


Figure VI.3. The WBC number/ µl of blood after incubating 250µl blood for 1h, 2h and 4h with ten beads (~6mg) of HAP-1 compared to the control group. The Control group indicates the time zero leukocyte number in the blood samples without biomaterials.

By increasing hydroxyapatites to 25 beads (~15mg) in blood samples, the leukocyte numbers that remained in the blood changed more. Figure VI.4 comparing total WBC/ µl (of blood) counted manually after incubation of 250µl blood samples with 25 beads of Hydroxyapatite type 1 (~15mg) at time points of one hour, two hours, and four hours at room temperature. The Control group indicates the time zero leukocyte number in blood samples without biomaterials.

The mean number of leukocytes was 10680 (± 800) cell/ µl of blood. There was a significant change in leukocyte number ($P<0.05$, $P\sim0.01$) after 4h incubation of blood samples with HAp-1and cell number had reduced to 7650 (± 700) cells/ µl blood.

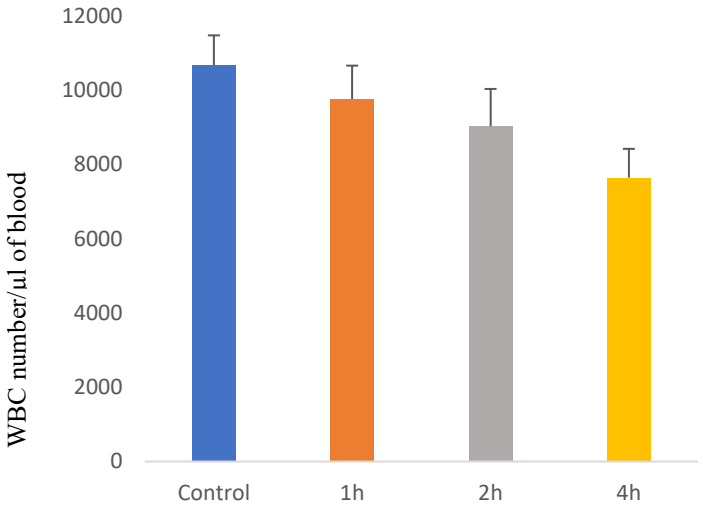


Figure VI.4 . The WBC number/ µl of blood after incubating 250µl blood for 1h, 2h and 4h with 25 beads (~15mg) of HAP-1 compared to the control group. The Control group indicates the time zero leukocyte number in the blood samples without biomaterials

A white blood cell counting experiment was accomplished with 15mg of another type of hydroxyapatite (HAp-2) to test whether its leuko-reduction effect was composition or structurally related. Figure VI.5 shows WBC/µl (of blood) after incubating 250µl blood samples with 15mg of HAp-2. WBC counting was performed manually after 1hour, 2hours, and 4 hours of incubation.

According to the obtained data, The mean number of leukocytes in blood samples was 10183 (± 1300) cell/ µl of blood and is reduced to 7950 (± 1290) cells/ µl. HAp-2 did not show a significant leuko-reduction effect after 4h ($P<0.05$, $P\sim0.04$).

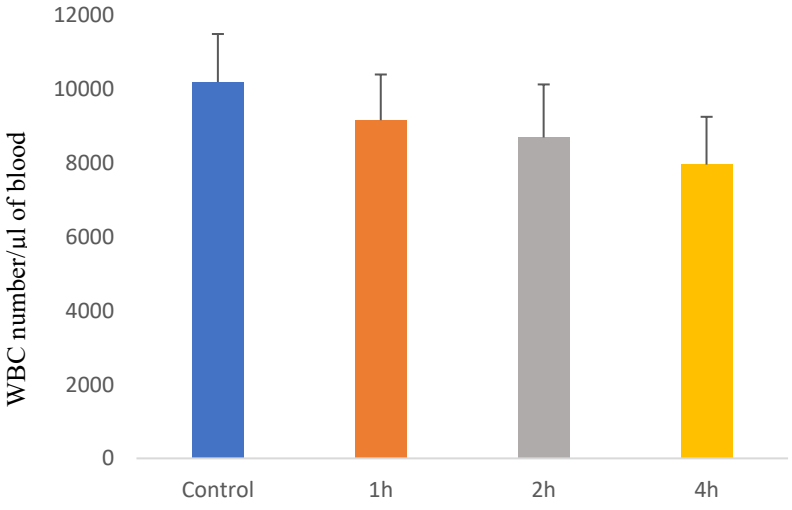


Figure VI.5 The WBC number/µl of blood after incubating 250µl blood for 1h, 2h and 4h with 15mg of HAP-2 in compared to the control group. The Control group indicates the time zero leukocyte number in the blood samples without biomaterials.

Figure VI.6 summarizes the relation between material mass and percentage of cell reduction after incubation of 250µl of blood samples with a range of 3mg to 15mg of HAp-1. As it is depicted, the percentage of cell reduction is increased by raising the material mass. The mean percentage of reduced cells with 15mg of HAp-1 is about 28% (±2%) as the maximum reduction in the examined mass range.

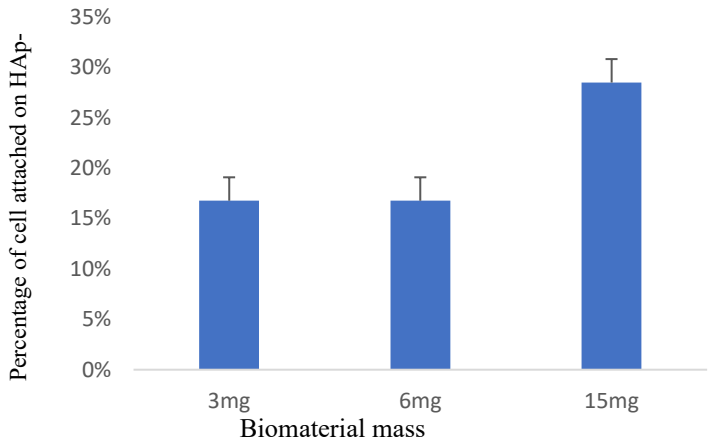


Figure VI.6 the relation between HAp-1 material mass and reduced cell percentage

In the next step, the leuko-reduction effect of HAps was tested in the same condition with commercial filter material and cotton as a commonly used material in commercial filters, and they were compared with the control group. Figure VI.7 demonstrates the total WBC/µl of blood samples after incubation of 250 µl with 15mg of HAp-1, HAp-2, cotton, and a piece of the commercial filter.

Commercial filter reduced WBC number in whole blood after 1h significantly ($P<0.05$, $P\sim0.01$). The mean number of leukocytes was 9616 (± 2417) cell/ µl in control that reduced to 3383 WBC/µl (± 652) after 1hour of incubation with the commercial filter and 3560 WBC/µl (±929) after 1h incubation with cotton ($P<0.05$, $P\sim0.02$). After 4h, the filter removed about 83%, and cotton removed 74% of leukocytes from whole blood. After Cotton, HAp-1 showed the most significant leuko-reduction effect after 4h of incubation by reducing the leukocyte mean number to 5910 WBC/µl (±1500) ($P<0.05$, $P\sim0.018$).

HAp-2 had a less reduction effect on the total number of WBC than other materials. After 4h incubation with HAp-2, the leukocyte means number reduced significantly to 6816 WBC/ µl (±1580) (P>0.05, P~0.04).

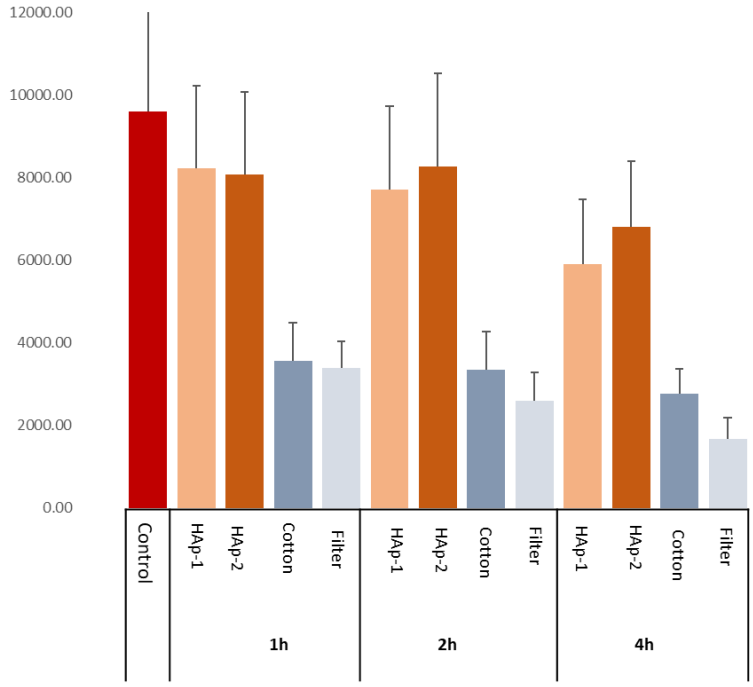


Figure VI.7 The WBC number/µl of blood after incubating 250µl blood for 1h, 2h, and 4h with 15mg of different materials (HAp-1, HAp-2, filter, and cotton) in comparison to the control group. The Control group indicates the time zero leukocyte number in the blood samples without biomaterials. Comercial filter and cotton significantly changed leukocyte numbers after 1h, 2h, and 4h (p<0.05). HAp-1 and HAp-2 after 4h showed a significant reduction in cell numbers (p<0.05).

6.3 WBC Differential Counting (Giemsa Staining)

Differential counting of leukocytes was accomplished manually by counting 100 leukocytes in the smear monolayer using Giemsa staining. The smear is scanned using a pattern that prevents repetitive counting (Figure VI.8), and when the total number of cells observed reaches 100, the percentage of each type can be easily determined as the number of that cell type/100. This method provides relative proportions of WBC generally found in the blood. During a differential count, leukocytes are separated into general categories, including Lymphocytes, Monocytes, and neutrophils as the most common leukocyte types in rat's blood (Figure VI.9).

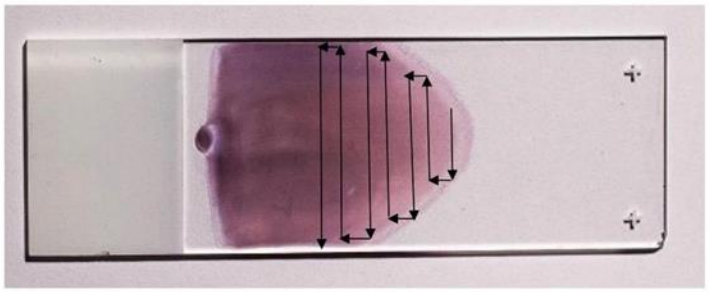


Figure VI.8 path pattern microscopic scan for differential WBC counting.

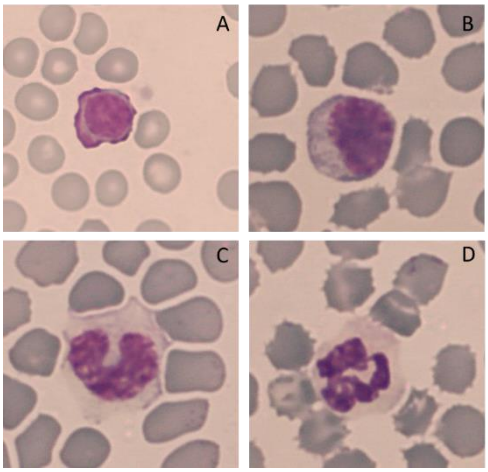


Figure VI.9 : Type of leukocytes in whole blood, A-B: lymphocyte, C: Monocyte, D: Neutrophil. Magnification x100.

Giemsa staining was done to detect each leukocyte type number and their number change after incubating 250µl whole blood with 15mg of materials for 4h.

Counting was accomplished manually using three rats' blood samples. Two observers counted cells (each observer two times) for each rat. Table VI-1 depicts the mean number of most common leukocyte types in rat's blood samples, including lymphocyte, monocyte, and neutrophil. The Control group indicates the time zero leukocyte type number in the blood samples without biomaterials. According to both observers, each cell type's mean number is in the rat's blood's normal range of leukocyte types. In the test groups, there are differences between the two observer counts. The changes are not significant, and we cannot conclude which type is attached more to the materials' surface.

Table VI-1 WBC differential manual counting based on Giemsa staining. Whole blood samples were incubated with HAp-1, HAp-2, and Cotton. Lymphocyte, monocyte, and neutrophils were counted after 1h and 4h. control indicated whole blood without material WBC differential counting.

Observer 1	Mean						STDV					
	Control		HA-1		Cotton		Control		HA-1		Cotton	
	fresh	4h	1h	4h	1h	4h	1h	4h	1h	4h	1h	4h
Monocyte	13.50	16.00	6.17	8.50	11.50	8.17	1.80	5.196	0.76	3.5	4.44	1.44
Neut	15.33	15.00	4.83	5.83	4.83	5.83	1.52	4.58	1.04	2.08	2.56	3.61
Lymphocyte	69.50	68.67	87.83	84.67	82.50	85.83	2.64	9.45	1.89	4.3	3.96	4.36

Observer 2	Mean						STDV					
	Control		HA-1		Cotton		Control		HA-1		Cotton	
	fresh	4h	1h	4h	1h	4h	1h	4h	1h	4h	1h	4h
Monocyte	10.67	12.33	8.00	2.67	14.67	11.00	4.16	4.61	2	1.15	1.52	1.73
Neutrophil	10.67	13.33	14.00	12.67	16.00	13.67	3.05	3.511	6	3.05	5.29	4.50
lymphocyte	76.00	74.33	78.00	83.33	72.67	75.33	4	6.02	5.29	4.16	6.65	3.21

Average	Mean						STDV					
	Control		HA-1		Cotton		Control		HA-1		Cotton	
	fresh	4h	1h	4h	1h	4h	1h	4h	1h	4h	1h	4h
Monocyte	12.08	14.17	7.08	5.58	13.08	9.58	1.65	0.40	0.87	1.65	2.06	0.204
Neutrophil	13.00	14.17	9.42	9.25	10.42	9.75	1.08	0.75	3.50	0.68	1.92	0.63
lymphocyte	72.75	71.50	82.92	84.00	77.58	80.58	0.95	2.42	2.40	0.145	1.90	0.815

6.4 MTT Test

The leukocyte number changes tested using an MTT assay. Obtained data from MTT approved the leukocyte removal after incubating blood samples with 15mg of biomaterials. Leukocyte numbers were calculated based on the standard curve (Figure VI.10). During isolation of leukocytes from blood samples using centrifuging technique, missing some number of cells is unavoidable. Figure VI.11 compare leukocyte number remained in blood samples after incubation with biomaterials. The Control group is considered as the leukocyte number in blood samples with no biomaterial. The mean number of leukocytes in the control groups was about 2.3×10^5cell/well ($\pm$ 0.08 $\times10^5$). According to the data, leukocyte numbers in the samples with cotton showed the highest difference with the control group by decreasing to 1.07 $\times10^5$cell/well ($\pm$ 0.1 $\times10^5$). HAp-1 led to a reduction of leukocytes to 1.5×10^5cell/well ($\pm$ 0.15 $\times10^5$), and HAp-2 reduced leukocyte numbers to 1.8 1.5×10^5cell/well ($\pm$ 0.18 $\times10^5$). MTT assay confirmed the obtained data from manual counting.

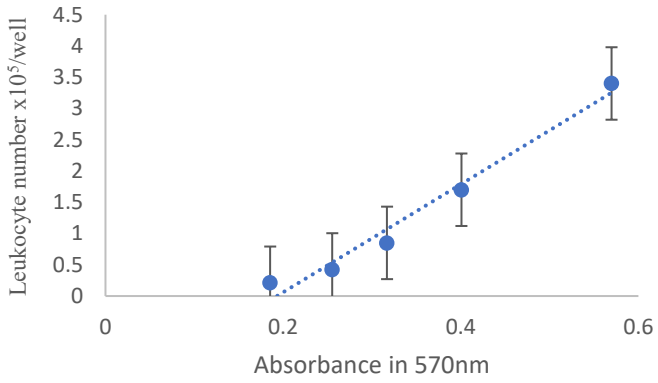


Figure VI.10 The relationship between MTT-formazan absorbance (O.D. 570nm) and viable cell number for the leukocytes harvested from whole blood as determined by the MTT assay (with 5 mg/ mL MTT). Values for MTT-formazan absorbance are expressed as the optical density (O.D.) at 570nm minus the mean background absorbance. The lowest limit of detection is 20,000 cells/well, and the highest is 3.4 × 105 ell/well. The error bars show ± standard deviation about that mean.

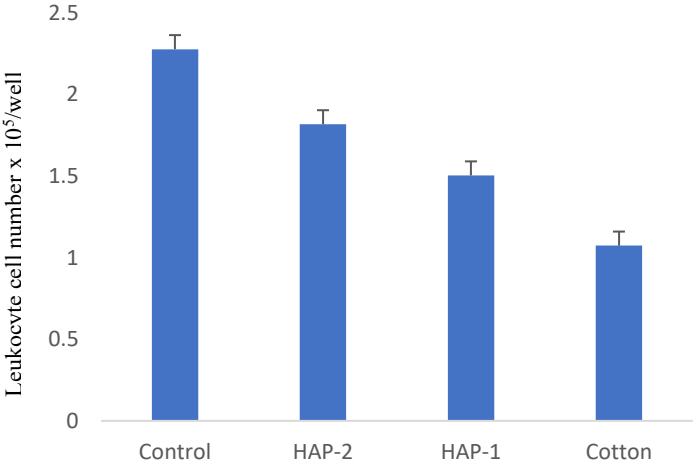


Figure VI.7 Estimation of leukocyte number in blood samples after incubation with biomaterials for 4h according to MTT- formazan absorbance (O.D. 570nm). The blood sample without any biomaterial is considered as control. Indicate significant values

6.5 Leukocyte Culture:

Leukocytes filtered by hydroxyapatites were cultured in cell culture conditions with HAp particles. Figure VI.12 shows the cells grown from five pieces of HAs exposed to a whole blood sample at room temperature for 4h. Biomaterials were seeded in 24well cell culture plates in DMEM medium with 10% FBS in cell culture condition (37 °C, 5% CO_2) were monitored for 5 days. The Control group indicated leukocytes isolated from blood cultured in the same condition without any material.

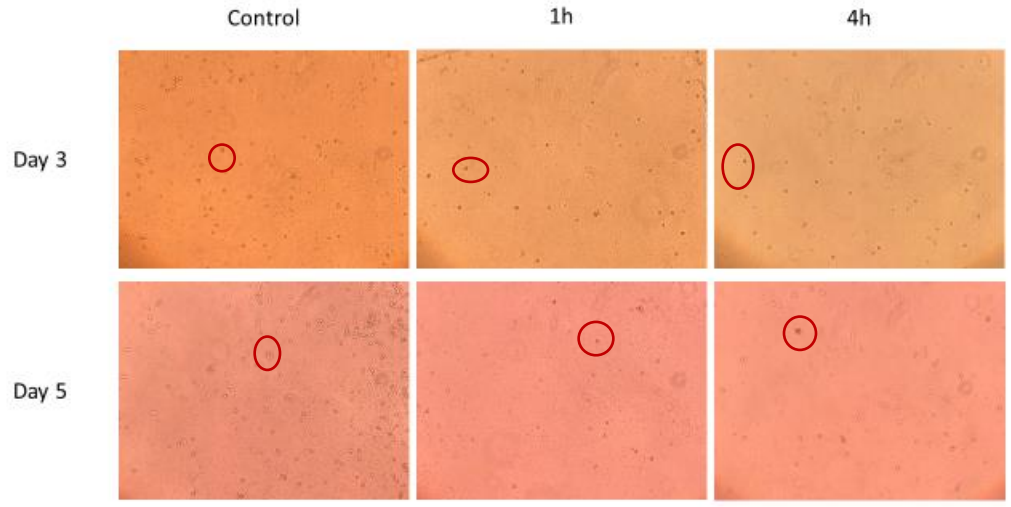


Figure VI.8 leukocyte culture after attachment on biomaterial demonstrated leukocyte filtration by hydroxyapatites

6.6 Cell Detection on Biomaterial Surface and Viability (Live and dead assay):

To detect cells attached to biomaterials' surface, cells were stained by Live & Dead assay. This test was accomplished with both whole blood and isolated leukocytes from the blood. After incubation of 15mg of biomaterials with blood, Live &Dead assay was done after 1h and 4h of incubation. As there were live cells attached to the surface of biomaterials, they were incubated in cell culture conditions with DMED cell culture media for 3 days and 5 days. Cell viability was tested for attached cells after 3days and 5 days. Live & Dead assay was done for cells attached on HAp-1, HAp-2, Cotton, and a commercial filter.

Figure VI.13 depicts cells attached on HAp-1 after 1h and 4h by live & dead staining. According to the pictures, after three days, alive cells stay attached to the biomaterial, but most seem to be detached and dead by day fifth.

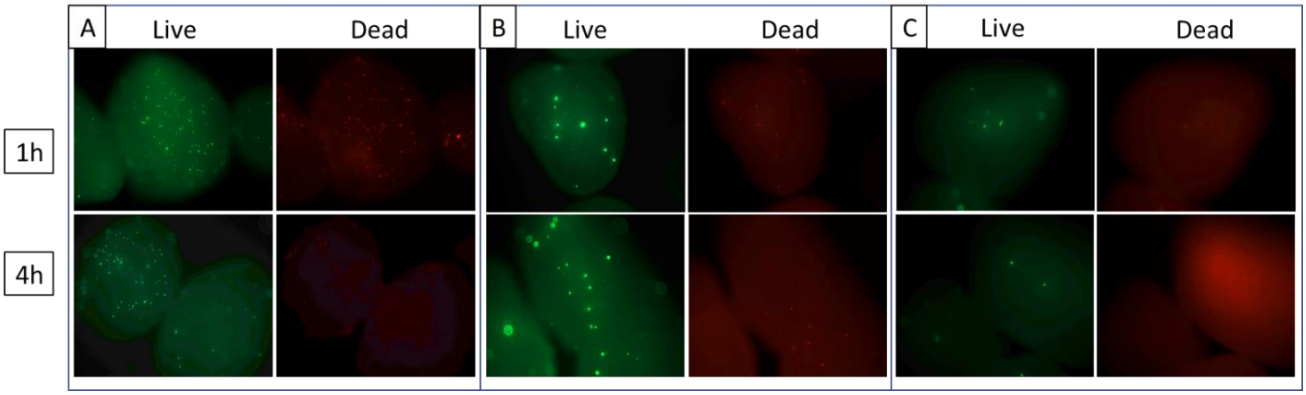


Figure VI.13 Cell staining and viability assay (live/dead) for HA-1. A: Day0, B: 3 days, and C: 5 days. The Green channel depicts live cells, and the red channel depicts compromised/dead cells

Figure VI.14 depicts cells attached on HAp-2 after 1h and 4h by live & dead staining. According to the pictures, after three and five days, a small number of alive cells stay attached to the biomaterial.

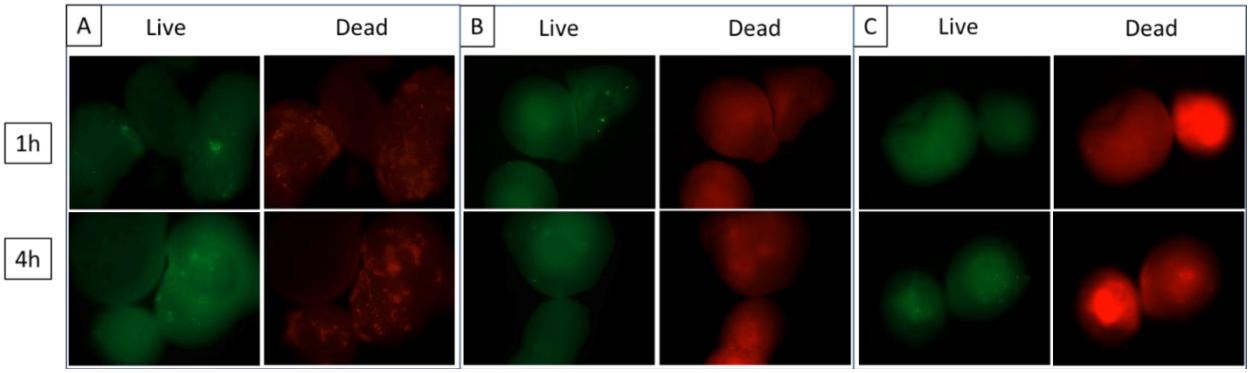


Figure VI.9: Cell staining and viability assay for HA-2 (live/dead) after three days and 5 days. A: Day0, B: 3 days, and C: 5 days. The Green channel depicts live cells, and the red channel depicts compromised/dead cells.

Cell attached to the cotton surface was stained in the same condition with hydroxyapatites. Figure VI.15 compare attached cells on cotton after 1h and 4h. As it is depicted, a small number of cells stay attached to cotton, and after five days, some cells stayed attached alive.

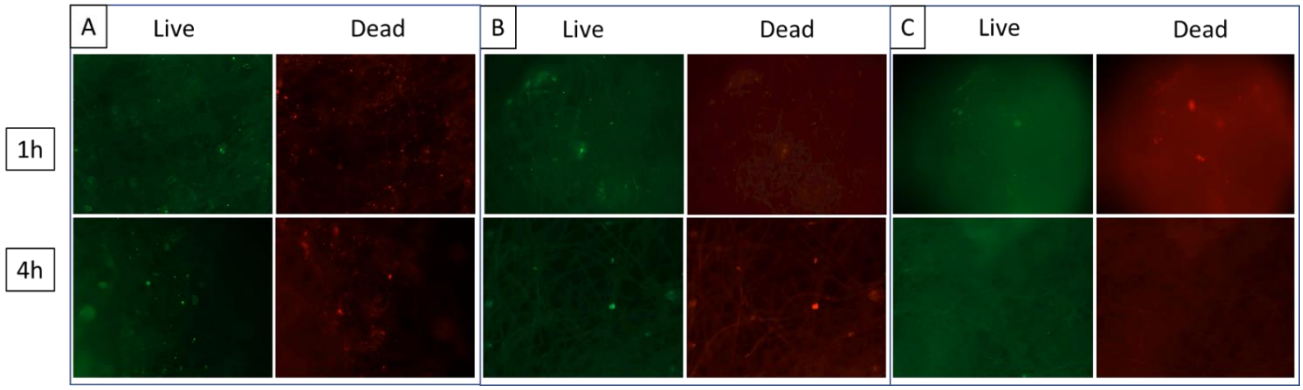


Figure VI.10 Cells stained on cotton and their viability were tested using live/dead assay after 3 days and 5 days. A: Day0, B: 3 days, and C: 5 days. The Green channel depicts live cells, and the red channel depicts compromised/dead cells.

Filtered cells by commercial filter were stained, and their viability on filter material was tested in the same condition with other biomaterials. As is shown in Figure VI.16, attached cells on the filter increased after 4h. Cell viability of attached cells on commercial filter reduced after three days, and on day five, there is no significant number of alive cells stay attached on the filter

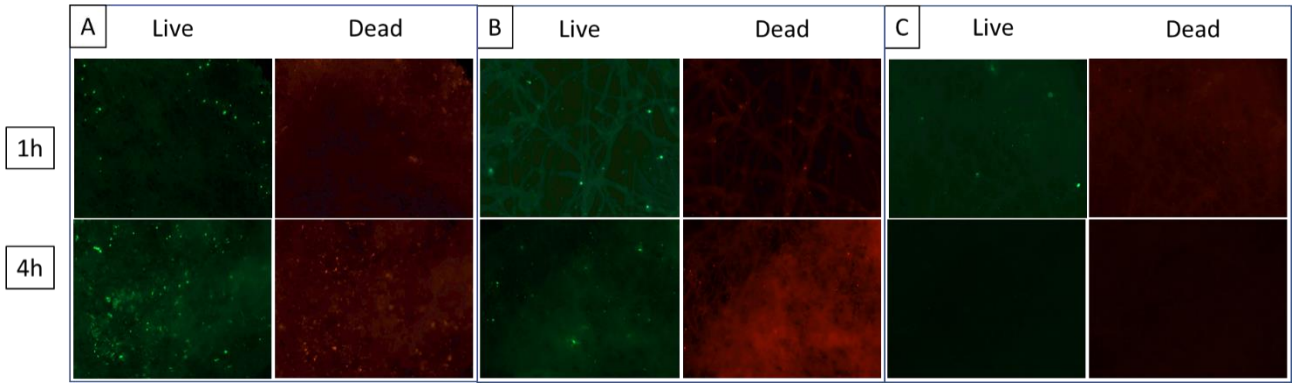


Figure VI.11 Cells stained on Commercial filter and their viability were tested using live/dead assay after 3 days and 5 days. A: Day0, B: 3 days and C: 5 days. Green channel depicts live cells and red channel depicts compromised/dead cells.

6.7 Cell Identification

Cells were detached from filter materials to determine which type of leukocytes tend to be attached to HAp bioceramics. Cells were detached from HAp by the trypsinizing method. Detached cells were diluted with plasma, and Giemsa staining was performed for their smear on the slide. Figure VI.17 shown that there are some monocytes and neutrophils detected from cells attached to HAp.

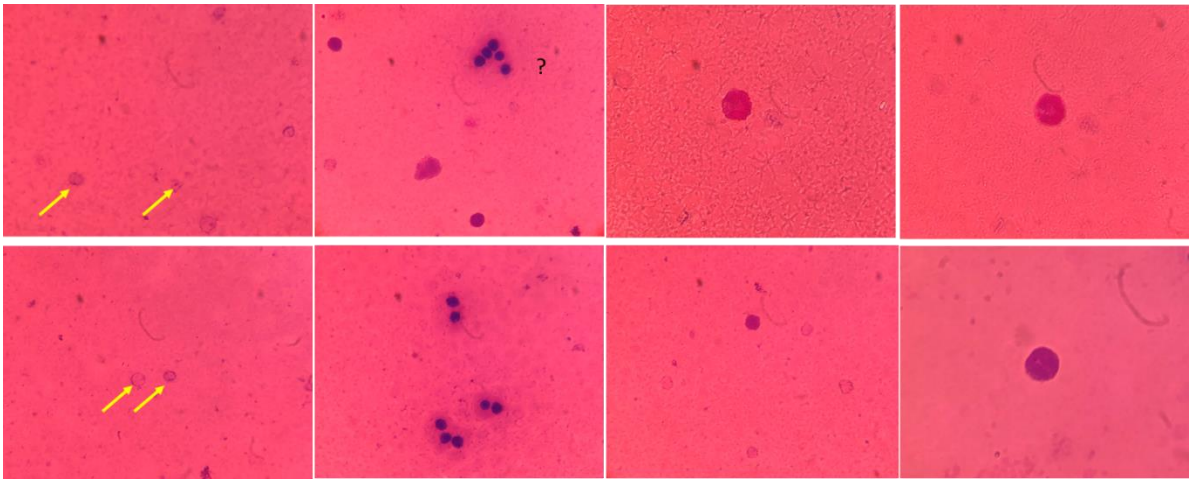


Figure VI.12 Giemsa staining of solitary cells on HAp with trypsin. Most of the cells are dead (yellow indicator), and the other difficulty results in cell identification, but we can see different types of leukocytes: Lymphocytes, Monocytes.

Discussion

This study aimed to investigate whole blood interaction with the biomaterial, focusing on leukocyte attachment to hydroxyapatite and studying its leuko-reduction effect. We hypothesized that leukocytes in whole blood could attach to hydroxyapatites and stay alive.

Studies showed that biomaterial surface characteristics affect the foreign body reaction's modulation in the first couple of weeks following implantation. The biocompatibility of biomaterial could be affected by inflammatory responses to the implant and impact short-term and long-term implantation success (102, 103). Having an in-depth understanding of immune cell behavior in the healing process can lead to smart biomaterial design for tissue engineering. Macrophages as the giant phagocytic immune cells against implanted biomaterials are studies by Sheikh et al. in 2015. They showed that macrophage responses to particulate biomaterials are dependent on the average particle size of the materials (102).

Several physical, chemical, and biological factors affect the attachment of cells on the biomaterial surface and their leukoreduction ability, including the contact surface area, pore size, material surface charge, chemical components of the material, and cell adhesion ability (104, 105).

According to the experiments that were accomplished in this study, hydroxyapatite materials' leuko-reduction effect is mass-dependent and time-dependent. As the contact surface area increases by the rising mass of material, the reduced leukocyte number was more significant in a higher amount of used material.

Based on previous studies on leukocyte filtration, the longer contact time of the leukocytes in the filter increases the filter efficacy and, over time, decreases the efficiency because of surface saturation (25, 106). As blood samples in this study were used in vitro, the average lifespan of leukocytes was considered. Most of the leukocyte types can stay alive for less than 5 hours in the blood sample, but their life span in blood flow and after activation in tissues can be for a couple of weeks, months, and even yeard (memory T cells). Our time limit in this study was 4h that guaranteed leukocytes could stay alive, and after 4h the most significant change in leukocyte number was observed.

In the differential counting, random areas of Giemsa stained blood smear were counted by different observers in a determined pathing pattern. There was no significant change in any type of leukocyte number. As the maximum reduction of leukocytes by HAp was around 30%, it is reasonable that differential cell count doesn't show significant change by Giemsa staining while 70% of leukocytes are present in blood samples.

According to the Live & Dead assay, cells were more attached after 4h, and HAp-1 got more cells than HAp-2. The effect of material surface topography on neutrophil survival in previous studies showed that roughened materials stimulate reactive oxygen species production and neutrophil death more than smooth surfaces (107). SEM imaging of two types of hydroxyapatite demonstrates their surface topology differences that can affect cell survival.

The attached cells were cultured in cell culture conditions for 3 days and 5 days. Live and Dead assays demonstrate that cells stay attached and alive on the surface of biomaterials after 3 days, but after 5 days, alive cells are reduced on all biomaterials, just HAp-1 that showed more attached cells after 5 days. After 5 days, cell reduction could be because of normal leukocyte death during 5 days and detachment from biomaterial. On the other hand, as most of the leukocytes are mature and terminally differentiated cells, they do not have proliferation ability, so we did not see proliferation on HAp-1 of attached viable cells.

After trypsinizing cells attached to the biomaterial, Giemsa staining was done to detect attached cells, and according to the data, there are some lymphocyte and monocyte detectable in pictures. According to the differential cell type percentage in rat's blood, lymphocytes and monocytes are about 90% of leukocytes in the blood, and they have more chance to contact biomaterials and attachment.

Part II Vein Attachment to Microporous Calcium Phosphate Biomaterial

VII. Chapter 1; Overview of Blood Vessel Structure and Interaction to Biomaterial

1.1 Introduction

Tissue engineering is intended to regenerate damaged tissues or organs using engineered transplantable tissues by combining engineering and life sciences. Limitations in utilizing tissue transplantation, such as host body immune response and shortage of supply, have made tissue engineering an attractive potential approach to compensate for the lack of transplantable organs (93, 94).

Nutrition and oxygen delivery using perfusion to artificial tissues are essential concepts that can lead to tissue engineering success in limited tissues that are avascular (cartilage) or have a slow metabolism that relies on either diffusion or quick angiogenesis (skin or bladder). To develop more complicated and functional tissues and, the vascularization after implantation should be evoked as it is necessary for the viability of tissues larger than 200µm due to diffusion insufficiency (54). Furthermore, only a few studies worked on developing real tissue-like systems (96-98).

In bone tissue engineering that needs to be vascularized, blood vessel cells will contact biomaterials to be grown and make tissues. Tissue-biomaterial interactions that finally may lead to graft failure is the most critical challenge overcome. All interactions between tissues and implanted biomaterials and all effects they may put on each other should be studied to prevent health complications for patients or implant failure. Optimizing tissue-biomaterial interactions to attain the desired results is thus a significant emphasis on research (99, 100). Blood vessels in contact with implanted materials in the body react, and the material characteristics angiogenesis around the implant. Blood vessels have a heterogeneous structure that their outer layer will be in contact with the implant.

1.2 Blood Vessels Structure

Blood vessel walls are composed of heterogeneous three-layered structures called intima, media, and adventitia. Each layer has its specific histological, biochemical, and biological characteristics, and all layers work together for the same goal of maintaining vascular homeostasis and stress response regulation. The thickness of each of the layers varies among individual blood vessel types. The tunica intima, the innermost layer of the arteries and veins, consists of a monolayer of polygonal

endothelial cells with a thickness of about 2 µm and a basal lamina connective tissue of collagen with a thickness of about 1 µm. There may also be a supportive internal elastic lamina, especially in the arterial wall. The media layer is thicker and is composed of alternate layers of smooth muscle cells, elastic laminae, and a minor amount of collagen and ground substances. The tunica media of arteries contains more smooth muscle than the tunica media of the veins, and this causes the arteries to constrict and dilate to adjust the volume of blood needed by the tissues they feed. The adventitia consists of fibroblast cells, collagen connective tissue, ground substances, and external elastic lamina in arteries. However, the first two inner layers are extensively investigated in vascular biology concepts, Adventitia importance in the function of blood vessels is unnoticed.

1.3 Adventitia

As the outermost connective tissue of most blood vessels wall, the tunica adventitia is the most complex compartment of the vessel wall and comprises various cells, including fibroblasts, immunomodulatory cells, resident progenitor cells, vasa vasorum endothelial cells, adrenergic nerves, and collagen-rich extracellular matrix. Adventitial fibroblasts, the most common cell type in the adventitia, produce the ECM fibers, including types I and III fibrillar collagen (108). In addition to these, a normal adventitia contains leukocytes such as resident macrophage, mast cells, T cell, B cell, and dendritic cells. The cellular structure of tunica Adventitia makes it a complex and dynamic part of the vessel wall that is important in diseases, repair, inflammation, and immune response (109-111).

The adventitia is separated from the media by the external elastic lamina. In large vessels, the media and the adventitia are easily distinguishable, whereas in small vessels, the media is thin, and the external elastic lamina is not well developed, so adventitial cells are much closer to medial and intimal tissue (108).

Adventitial compartments are demonstrated to have a critical rule in regulating blood vessel function by processing regulatory, integratory, and healing factors in both pulmonary and systemic circulations. This blood vessel layer is sensitive to any environmental stresses such as hypoxia or vascular dilatation. In response to vascular stress or injury, resident adventitial cells such as fibroblasts are often the first to be activated and reprogrammed to influence the vessel wall's tone

and structure. Experimental data indicate that the adventitial fibroblast, the most abundant cellular constituent of adventitia, is a critical regulator of vascular wall function.

The adventitia is considered a highly active part of blood vessel tissue associated with different types of vascular disorders.

Other than pericytes, adventitial cells represent another perivascular cell type. Pericytes and adventitial cells are two perivascular cell compartments with distinct phenotypes and anatomical locations in situ and different culture behaviors. These perivascular cells can be prospectively purified by flow cytometry using a well-defined surface marker combination, standard in all human organs tested.

More recently, several studies have reported a dynamic role for the tunica adventitia in vascular remodeling. These studies provided evidence indicating the activation of adventitial fibroblast cells in response to injury and their phenotypic characterizations, contributing to pulmonary vascular remodeling.

The adventitial fibroblast has been suggested to be the most appropriate cell for "sensing" hypertensive states. In hypertension, atherosclerosis, and vascular injury, adventitial fibroblast proliferation increases while SMC proliferation decreases, leading to the increased adventitial thickness and cell number long before changes in the media or intima by decreases in SMC density.

These observations of adventitial mechanical characteristics and early increases in fibroblast proliferation have stimulated the hypothesis that the adventitia plays an essential role in the regulatory systems that control vascular remodeling and vascular tone, at least under high wall conditions stress.

Progenitor cells (MPCs) are described as descendants of stem cells that can differentiate into at least one mesenchymal lineage, such as an adipocyte lineage, osteoblast lineage, or myofibroblast lineage.

Apart from tunica intima, in which the subendothelial area has been suggested as one of the sources of endothelial progenitor cells (EPCs), the possibility that other structural layers of the blood vessels harbor stem/progenitor cells were recently demonstrated in tunica media and adventitia.

The importance of the tunica adventitia in regenerative vascular medicine is highlighted by the numerous reports describing the presence of multipotent progenitors within the wall of arteries and veins. In a vascular remodeling setting following an injury, it has been shown that adventitial cells (ACs) start a proliferation process, migration into the tunica media and intima, and differentiation into smooth muscle cells. The adventitia's contribution to tissue repair and blood vessels' hemostasis is related to the presence of mesodermal progenitors (23, 112, 113).

Fibroblast cells have the fundamental rule in regulating extracellular matrix (ECM) in adventitia during stress and injury situations. Adventitial fibroblast manages the stress response by adjustment of ECM components production. Collagens types I and II are the major components and principal proteins of the adventitial matrix produced by fibroblast cells. Under normal conditions, adventitial fibroblast cells are undifferentiated and environmental stress or diseases lead to activation of them, contributing to cell growth and differentiation changes besides alteration of matrix contents (114, 115).

VIII. Chapter 2; Methods

2.1 Vein Attachment to Microporous Bioceramic

The femoral vein was harvested from a healthy mal rat after animal euthanasia. The fat and connective tissue around the vein was removed, and after cleaning vein was dissected. The dissected vein was put on the concave surface of cylindric bioceramic in order that the outer layer of the vein was in contact with biomaterial. The attached vein pieces on biomaterial were incubated with DMEM medium in cell culture conditions for three and five days.

2.2 Live and Dead Assay; Cell Viability and Cell Proliferation

The cell viability of attached cells on the materials was assessed using a LIVE&DEAD® Viability/Cytotoxicity Kit to observe leukocyte attachment on biomaterials. The two-color assay discriminates vital from dead cells by simultaneously staining with green fluorescent (494–517 nm) calcein-acetoxymethyl (calcein-AM) to indicate intracellular esterase activity and red fluorescent (528–617 nm) ethidium homodimer-1 to predict the loss of plasma membrane integrity.

Five days after culturing vein on biomaterial, DEMEM was removed samples and were washed three times with PBS 1X. The Live & dead assay was performed as recommended by the manufacturer. A 1:100 dilution of each dye was prepared. Hoechst dye (blue fluorescent stain specific for DNA (i.e., nuclei of eukaryotic cells)) with 2:1000 was added to the staining solution. Stain solution was added to materials and incubated at room temperature for 15-20 minutes. Imaging was done with a fluorescence microscope (Zeiss 416).

IX. Chapter 3; Results

3.1 Live and Dead Assay

Vein attached to the biomaterial was cultured in DMEM medium with 10% FBS for five days. Cell viability and proliferation were tested. Live and Dead assays were accomplished to detect vein cell attachment to the cylindric bioceramic. Figure IX.1 shows vein attachment on biomaterial and cell proliferation after five days. Cells are spreading around the vein attached to the biomaterial.

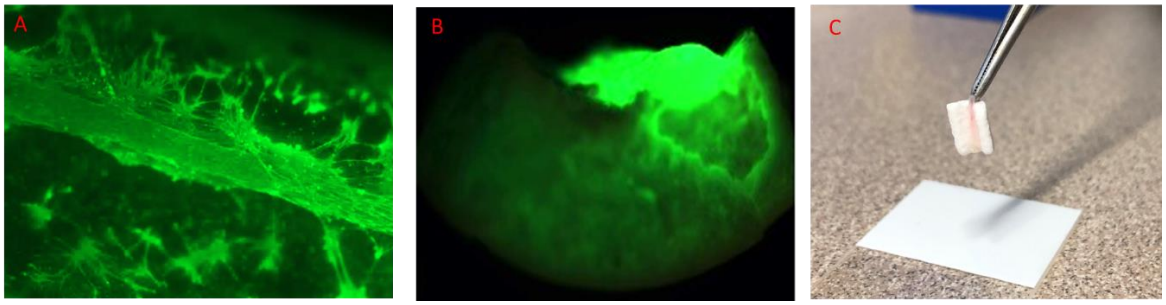


Figure IX.1.Vein cultured on the scaffold for Five Days (stain Calcein AM) B: cross-section of cut scaffold showing viable cells' fluorescence throughout 1mm thickness. C: vein well attach with the scaffold.

3.2 SEM Imaging

FESEM (Field Emission Scanning Electron Microscope) microscopy examined cell vein tissue attachment and cells' penetration inside the scaffold. Cell detection through the material was investigated by investigated with SEM FEG (Inspect F50, FEI Company, Hillsboro, ORA, USA) High voltage 5kv. IX.2 depicts a FESEM image of a vein attached to cylindric biomaterial after five incubation days in cell culture condition. An extracellular matrix of growing cells is shown penetrating inside the porous material in FESEM imaging.

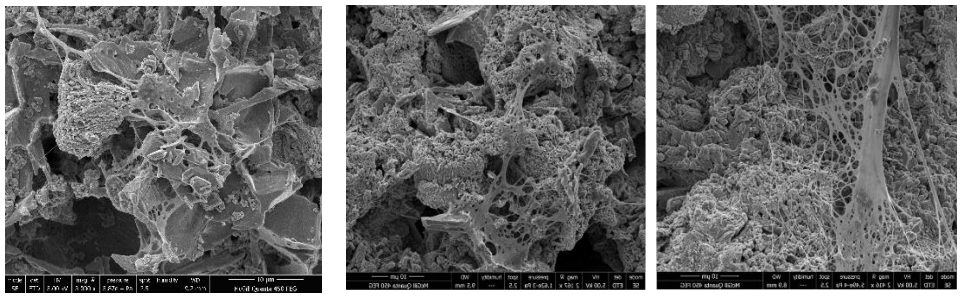


Figure IX.2 FESEM image of vein tissue cultured in the porous scaffold. The extracellular matrix shows tissue spreading into the material

Discussion

Characterization of the vessel–biomaterial interactions is crucial for the development of biomedical devices and implants. The vessel wall has three layers with different cellular composition, which the outermost layer is adventitia. The adventitia is the first layer of blood vessels in contact with the implanted biomaterial, and its interaction with biomaterial tried to be studied in this project. According to the adventitia cell profile, it has a collagen-rich extracellular matrix beside fibroblasts and some progenitor cells. Effect of external synthetic stents and sheaths in vein into artery interposition grafting was studied before and found them to affect vein graft remodeling and thicken profoundly. This study showed that a series of immunological and molecular reactions activated and affected angiogenesis after implanting external devices (116).

Our SEM images depicted penetrating ECM through the material pores after 5 days of culturing vein on cylindric biomaterial. Our live and dead assay also approved cell proliferation and attachment into and on the biomaterial surface. This pilot project indicated that contact of the material with the vein could stimulate cell proliferation and production of the extracellular matrix, and the material was colonized by cells, likely fibroblasts or smooth muscle cells. Further work is required to determine if new blood vessels were beginning to form. As decalcification takes several weeks, there was insufficient time to determine this, but in vivo experiments have indicated that this is the case (117).

Conclusion

According to obtained data from different experiments with blood and vein tissue in this study, the leukoreduction effect of hydroxyapatite materials on whole blood and adventitial cell viability and proliferation on calcium phosphate bioceramic were confirmed. The Leukoreduction effect of HAp is mass-dependent and time-dependent. Increasing the amount of material enhances efficiency by increasing the surface area. Leukocytes can stay attached and survive on HAp biomaterials for 5 days, compared to cotton, and commercial filter cell attachment to HAp-1 was greater. Identification of the attached cells on HAps demonstrated that lymphocytes and monocytes stayed attached to biomaterials more than other types, and this could be because of their higher population (> 90 % of leukocytes in rat's blood), which gave them more chance to be presented.

On the other hand, vein tissue can be well attached to cylindrical microporous calcium phosphate biomaterial, and according to SEM and fluorescence imaging, cells were growing through the biomaterial, and extracellular matrix was penetrating the pores.

In conclusion, calcium phosphate biomaterial showed good affinity to leukocytes; besides that, blood cells and vein tissue cells can be well attached and survive, proliferate and differentiate.

For future studies, cells' proliferation and survival on biomaterial could be investigated for more than five days. As the leukocytes' life span is different, a specific type of leukocytes such as neutrophils could be monitored for more than five days by isolation and activation of them and increased cell numbers. Another factor that should be considered for further work is the gender of the studied animal. Although the total white blood cell number in male and female rodents are not significantly different, hormonal fluctuations cause significant changes in differential WBC counting in females (118). In addition to these, *in vivo* studies will provide a more comparable situation with disorders in the human body that could be targeted for biomaterial implantation.

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