Universidade de Trás-os-Montes e Alto Douro

BIOLOGICAL CHARACTERIZATION OF HEMOSTATIC AGENTS – *in vitro* studies

Master's dissertation in Clinical Laboratory Biology

Maria Guerra Gomes

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Maria Guerra Gomes

Advisor: Professor Bruno Jorge Antunes Colaço, Universidade de Trás-os-Montes e Alto Douro

Co-advisor: Professor Pedro de Sousa Gomes, Faculdade de Medicina Dentária da Universidade do Porto

Jury Composition

President: José Albino Gomes Alves Dias

Members: Sara Filipa Oliveira Durão

Bruno Jorge Antunes Colaço

Vila Real, 2020

Scientific Orientation

Professor Bruno Jorge Antunes Colaço

Universidade de Trás-os-Montes e Alto Douro

Professor Pedro de Sousa Gomes

Faculdade de Medicina Dentária da Universidade do Porto

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Resumo

A hemorragia é uma das principais causas de mortalidade evitável e o reconhecimento deste facto levou a grandes avanços no âmbito do controlo da hemóstase, assim como no crescimento de agentes hemostáticos disponíveis em ambientes clínicos, que são uma opção terapêutica bastante importante para o tratamento de hemorragias quando os processos fisiológicos demonstram ser ineficientes.

No entanto, como estes agentes hemostáticos não passam por uma avaliação completa e regular, assim como outros biomateriais ou fármacos, atualmente não existe uma abordagem sistemática para a aquisição e documentação do uso destes agentes em contextos clínicos humanos. Assim, este trabalho tem como objetivo abordar a caracterização biológica de agentes hemostáticos à base de gelatina clinicamente disponíveis, através de estudos *in vitro* com várias linhas celulares humanas.

As células fibroblásticas e as células osteoblásticas foram cultivadas por 24 horas em meio de crescimento (α-MEM com 10% de soro bovino fetal (SBF), 100 UI/mL de penicilina, 100 UI/mL de estreptomicina e 2,5 μg/mL de anfotericina B); após este tempo foram adicionados os extratos, previamente diluídos em α-MEM, a 50%, 25% e 12,5%. Células sem extratos foram semeadas como grupos de controlo negativo. As culturas celulares foram então avaliadas quanto à morfologia celular, proliferação celular, viabilidade celular, atividade metabólica e atividade da fosfatase alcalina, bem como histoquímica, especificamente coloração da fosfatase alcalina e conteúdo de colágenio, em diferentes timepoints.

De um modo geral, os resultados não mostraram citotoxicidade significativa em nenhuma das células, exibindo de facto resultados semelhantes às células do controlo; no entanto, houve algumas diferenças nos ensaios realizados que poderiam demonstrar que, embora as esponjas não afetem a proliferação celular, elas podem ter algum efeito na sua viabilidade. Além disso, também foi possível detetar que esponjas diferentes obtiveram melhores resultados para cada tipo celular. Por estes motivos, são necessários mais estudos para elucidar os mecanismos subjacentes a estes resultados.

Abstract

Hemorrhage is a leading cause of preventable mortality and recognition of this has led to progressive advances in hemostasis control and in a growth of clinically available topical hemostatic agents, which are an important therapeutic option for the management of bleeding, in which physiological processes are inefficient.

However, because these hemostatic products do not undergo a thorough regulatory evaluation as do other biomaterials or substances, there is currently no systematic approach for the acquisition and documentation of use of these agents in human clinical settings. As such, this work aims to address the biological characterization of clinically available gelatin-based hemostatic agents, through *in vitro* studies with various human cell lines.

Fibroblastic cells and osteoblastic-like cells were cultured for 24 hours in growth medium (α-MEM with 10% FBS, 100 UI/mL penicilin, 100 UI/mL streptomycin and 2,5 μg/mL amphotericin B) after which the leachables, previously diluted in α-MEM, were added at 50%, 25% and 12,5%. Cells without leachables were seeded as negative control groups. The cell cultures were then evaluated on cellular morphology, cell proliferation, cell viability, metabolic activity and alkaline phosphatase activity, as well as histochemistry, specifically alkaline phosphatase staining and collagen content, at different timepoints.

Generally speaking, the results didn't show any significant cytotoxicity in any of the cells, exhibiting in fact similar results to the control cells; however, there were some differences in the assays performed that could demonstrate that while the sponges' may not affect cell proliferation, they could have some effect on their viability. Aside from this, it could also be detected that different sponges had better results for each cell type. For these reasons, further studies are needed in order to elucidate the mechanisms underlying these results.

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List of Abbreviations

ADP – Adenosine diphosphate

ALP – Alkaline phosphatase

BC – Before Christ

BSA – Bovine serum albumin

CO_2 – Carbon Dioxide

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

FBS – Fetal Bovine Serum

FVa – Activated factor V

FV – Factor V

FVIIa – Activated factor VII

FIXa – Activated factor IX

FIX – Factor IX

FXa – Activated factor X

FX – Factor X

FXIa – Activated factor XI

FXI – Factor XI

FXIIa – Activated factor XII

FXII – Factor XII

FXIIIa – Activated factor XIII

FXIII – Factor XIII

GPIa – Glycoprotein Ia

GPIb – Glycoprotein Ib

GPIIb – Glycoprotein IIa

GPIIIa – Glycoprotein IIb

hADAS – Human adipose derived adult stem cells

HUVEC – Human umbilical vein endothelial cells

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

PBS – Phosphate buffered saline

PDGF – Platelet-derived growth factor

pNPP – p-nitrophenyl phosphate

PT - Protein

Pt – Prothrombin

RPM – Rotations per minute

RT – Room temperature

SBF – Soro bovino fetal

TF – Tissue factor

TXA_2 – Thromboxane A2

VEGF – Vascular endothelial growth factor

vWF – von Willebrand factor

α-MEM – Alpha-Minimum Essential Medium

Chapter 1 - Introduction

For millennia, humankind has attempted to stop the bleeding of a wound by applying a variety of topical agents. As long ago as 10,000 BC, evidence suggests that primitive man undertook surgical procedures and recognized the hazards of uncontrolled bleeding; leading to progressive advances in hemostasis control that, in the last two decades, resulted in a growth of clinically available topical hemostatic agents (Eyre and Gamlin, 2010). Which agent to use depends on the type of bleeding, its specific mechanism of action and interaction with the environment, and the patient's individual coagulation abnormalities (Achneck *et al*., 2010).

To better understand the interactions between medical devices and the extravascular tissues of the body, an understanding of the wound healing process is critical (Padera, 2017).

Hemostasis, the act of stopping bleeding, is very important to maintain homeostasis, a stable internal environment; it is a complex process that is contingent on the interaction of platelets, plasma coagulation cascades, fibrinolytic proteins, blood vasculatures, and cytokine mediators (Periayah *et al.*, 2017).

There are two main components of hemostasis. Primary hemostasis refers to platelet aggregation and platelet plug formation. Secondary hemostasis refers to the deposition of insoluble fibrin, which is generated by the proteolytic coagulation cascade. This insoluble fibrin forms a mesh that is incorporated into and around the platelet plug. This mesh serves to strengthen and stabilize the blood clot. These two processes happen simultaneously and are intertwined. The fibrinolysis pathway also plays a significant role in hemostasis (Gale, 2011).

Vascular damage triggers the clotting cascade, so as to produce a localized platelet-fibrin plug to prevent blood loss. This process is accompanied by rapid and sequential processes leading to clot containment, wound healing, clot dissolution, tissue regeneration, and remodeling. In a healthy person, all of these occur continuously and in a balanced manner, allowing the bleeding to stop but also keeping the blood vessels open so as to maintain adequate blood flow. When any of these hemostatic processes are disrupted, either by inherited defects or acquired abnormalities, faulty hemostasis may result in a cycle of positive feedback, which consists of a decrease in blood volume and blood pressure, which in turn disturbs the homeostasis and can prove fatal (Kriz *et al.*, 2009; Periayah *et al.*, 2017; Seeley *et al.*, 2011).

1.1 - Hemostasis

The first step in hemostasis is carried out both by cellular (platelets) and humoral (clotting factors) components (Padera, 2017). Platelets, which circulate within the blood, are the essential mediators that trigger the mechanical pathway of the coagulation cascade upon encountering any damage to the blood vessels (Periayah *et al*., 2017).

The platelets encourage primary hemostasis via three major processes: activation, adhesion, and aggregation. When the vascular endothelium is damaged, various macromolecular elements of the vascular subendothelium become exposed and, consequently, accessible to platelets (Periayah *et al*., 2017).

Upon tissue injury, the hemostatic mechanism makes use of several vascular and extravascular receptors to seal off the cuts or ruptures to the blood vessels and closing them off from the surrounding tissues. Normal hemostatic responses involve the intricate interplay of four key components: the vascular endothelium, platelets, the coagulation pathway and finally fibrinolysis, which fall under three major categories of hemostasis: vasoconstriction, platelet plug formation and coagulation (Eyre and Gamlin, 2010).

Vasoconstriction is the immediate, but temporary, closing of a blood vessel by contraction of the vessel wall's smooth muscle. This constriction, when it happens in the smaller vessels, can collapse them and obstruct the blood flow. If the vessels are medium to large-sized, the spasms slow down immediate blood flow, lessening the damage but still preparing the vessel for the later steps of hemostasis. The vascular spasm is produced by reflexes of the nervous system and chemical agents and it becomes stronger and lasts longer in more severe injuries (Seeley *et al.*, 2011).

1.1.1 - Primary hemostasis

A platelet plug is the accumulation of platelets that can close small ruptures in blood vessels. The formation of this plug is essential to maintain the integrity of the circulatory system because small tears occur frequently, every day, in the smaller vessels and capillaries, and the platelet plug seals them quickly. People with an abnormal number of platelets tend to develop numerous small hemorrhages in their skin and internal organs (Seeley *et al.*, 2011).

Normal vascular morphology comprises three distinct layers: the intima which consists of a monolayer of non-thrombogenic endothelial cells and an internal basement membrane; the media, made up of smooth muscle; and the adventitia, which consists of an external membrane and supporting connective tissue. The balance of coagulation is

characterized by continuous factor activation and coordinated assembly of enzyme complexes, which are down regulated by circulating inhibitor proteins. The endothelial cells of intact vessels prevent clotting by expressing the fibrinolytic heparin molecule and thrombomodulin, which prevent platelet aggregation and stop the coagulation cascade; in the absence of blood vessel disruption, enzyme complex formation and the resultant thrombin generation are minimal and slow. Circulating endogenous anticoagulants are sufficient to inactivate these procoagulant complexes and prevent inappropriate clot formation, in physiological conditions. When a procoagulant stimulus occurs, the endothelial cells stop secreting coagulation and aggregation inhibitors and instead secrete von Willebrand factor (vWF), which causes platelet adherence during the initial formation of a clot, sealing off the injured area. Platelet adhesion occurs when the platelets adhere to the exposed collagen from the wound on the blood vessel. Platelets bind to this collagen via GPIa, and further binding is facilitated by the vWF, which forms a bridge between subendothelial microfibrils and platelets via the membrane GPIb. Binding of GPIa and GPIb exposes GPIIb/IIIa, which binds fibrinogen and vWF (Eyre and Gamlin, 2010; Kriz *et al.*, 2009; Minors, 2007).

The platelets, after having adhered to the collagen, are then activated. When activated, platelets undergo changes in terms of the organization of proteins that form the cytoskeleton. The platelets go from a discoid shape to an irregular shape and the formation of multiple cytoplasmic projections, which adhere to the injured vessel wall. The granules distributed throughout the platelet undergo centralization and fusion with the plasma membrane and secrete their contents by exocytosis. The released chemicals include adenosine diphosphate (ADP), vWF, thromboxane A2 (TXA_2), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), serotonin, and coagulation factors.

When they are activated, platelets release surface receptors that bind to the fibrinogen, a plasma protein. During platelet aggregation, the fibrinogen forms a bridge between the surface receptors of the different platelets, resulting in the formation of a platelet plug. The activated platelets accelerate the phospholipids (platelet factor III) and the coagulation factor V (fV), an important part of the clot formation (Eyre and Gamlin, 2010; Kriz *et al.*, 2009; Seeley *et al.*, 2011).

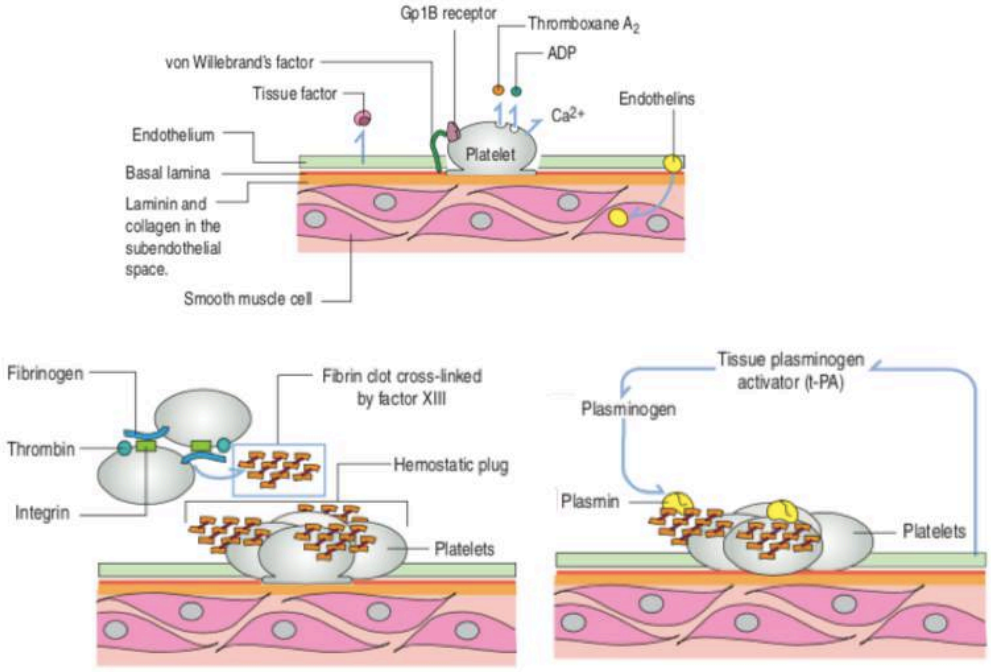

Figure 1 - Primary Hemostasis (adapted from Kierszenbaum and Tres, 2016)

1.1.2 - Secondary hemostasis

When the damage to the vessels is too extensive, the secondary hemostasis, known as the blood coagulation cascade, is triggered. The blood clotting process can be classified into three important steps: (i) a complex cascade, triggering the chemical reactions that are mediated by the coagulation factors, to form fibrin strands for consolidating the platelet plugs, (ii) the conversion of prothrombin (Pt) into thrombin, and (iii) conversion of fibrinogen into fibrin, which eventually embroils the plasma, platelets and blood cells to build a firmer clot. The coagulation cascade is classically divided into three pathways: the intrinsic (contact activation pathway), the extrinsic (tissue factor pathway), and the common pathway (Periayah *et al*., 2017).

Thrombin is a proteolytic enzyme derived from prothrombin, which aids in the process of forming blood clots by catalyzing the conversion of fibrinogen to fibrin. All the coagulation factors involved in the hemostasis process feed into the regulation and control of thrombin generation, which then forms clots at the sites of injury.

1.1.2.1- Extrinsic pathway

The extrinsic pathway is the major initiator of blood coagulation. Clotting by this pathway is triggered when circulating blood comes in contact with tissue factor (TF). TF is a cell-bound transmembrane glycoprotein and nonenzymatic protein cofactor that is

expressed in adventitial cells of the vasculature and is not normally in contact with the circulation. Exposure of blood to TF occurs as a result of vascular damage. Circulating molecules of activated factor VIIa (fVIIa) then escape the circulation and bind to the exposed TF generating a potent procoagulant complex. A limited proteolysis process extends to the TF/fVIIa complex, which activates factor X (fX) or factor IX (fIX), further activating factor X/fIX and activating serine proteases via cleaving an activation peptide. Proteolysis is the hydrolysis process that involves the breakdown of proteins into smaller polypeptides. The generated fVIIa–TF complex propagates and amplifies coagulation via the proteolytic activation of fIX (intrinsic pathway) and fX (common pathway) (Neuenschwander, 2006; Periayah *et al.*, 2017).

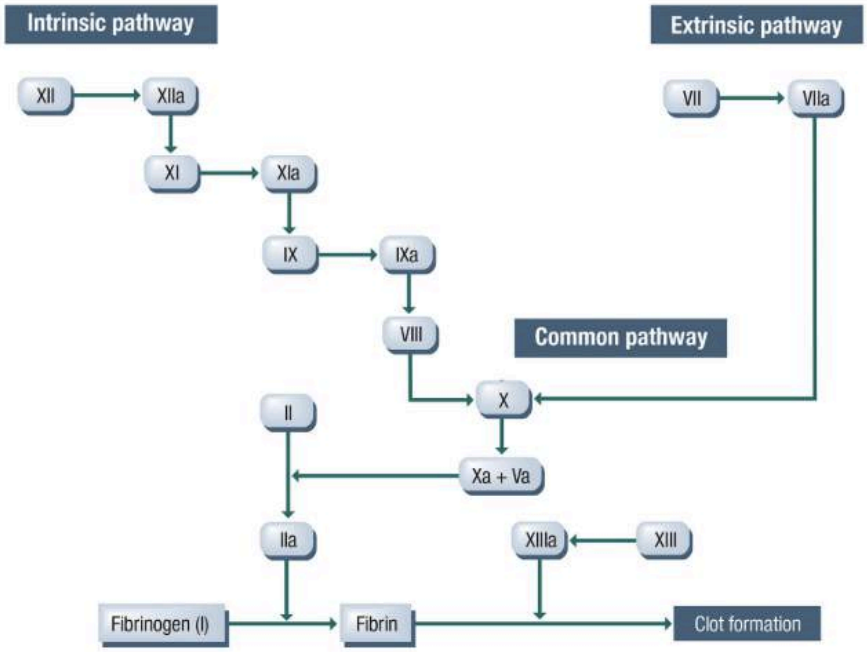

Figure 2 - Secondary Hemostasis (adapted from Overbey *et al.*, 2014)

1.1.2.2 - Intrinsic pathway

The intrinsic pathway of the coagulation cascade involves factors that are present in normal plasma and thus do not require a vascular surface to form fibrin. This process begins with factor XII (fXII), which is activated to become factor XIIa (fXIIa). FXIIa converts factor XI (fXI) into activated factor XIa (fXIa), which converts factor IX (fIX) into activated factor IXa (fIXa), which in turn is responsible for cleaving factor X (fX) into activated factor Xa (fXa), at the junction with the extrinsic pathway. (Overbey *et al.*,

2014). To form a stable blood clot, thrombin needs to activate factor XIII (fXIII) to the transglutaminase enzyme activated factor XIIIa (fXIIIa); this factor is the fibrin stabilizing factor of the blood coagulation system that crosslinks with fibrin. Furthermore, fXIIIa also plays a significant role in tissue repair and the angiogenesis process (Periayah *et al*., 2017).

1.1.2.3 - Common Pathway

The common pathway for thrombin activation is initiated via the activation of fX, which can happen by either the extrinsic or intrinsic pathway and produces the identical product, activated factor Xa (fXa). The activated fXa merges with its cofactor, activated factor V (fVa) and calcium on the phospholipid surfaces to construct the prothrombinase complex that cleaves prothrombin into the active form of thrombin. Thrombin then cleaves the fibrinogen that is present within the circulating blood to create fibrin. Fibrin then links together with platelets to form a clot. FVa is a cofactor to fXa. FV must be first converted to fVa before it can be applied as a cofactor (Neuenschwander, 2006; Overbey *et al.*, 2014; Periayah *et al.*, 2017).

1.2 - Biomaterials for hemostasis enhancement

Hemorrhage is a leading cause of preventable morbidity and mortality; at least 3 factors have an impact on bleeding-related complications: progressively widespread use of anticoagulant therapeutics, technologic advances that allow for more complex and lengthier surgical procedures, and the advancing age of the population and associated comorbidities. Recognition of the importance of bleeding-related complications has resulted in an explosive use of topical hemostatic agents throughout the health care environment (Shander *et al*., 2014).

Topical hemostatic agents are an important means of controlling or preventing bleeding where conventional methods are inefficient or impractical (Heher *et al*., 2018). They are employed for intraoperative hemostasis, for hemorrhage control in several situations, such as dental extractions and in odontostomatology (Elisabettacenni *et al*., 2000). These agents differ in mechanism of action, efficacy, safety profile, and type of formulations (Hajosch *et al*., 2010).

There are several ways of classifying them, and one approach is based on three different categories regarding the activity of the materials: active, non-active, and agents with combinations of both active and non-active characteristics. Active hemostatic agents

display biologic activity and directly participate in the coagulation cascade to induce a fibrin clot; these include products containing fibrinogen and thrombin (fibrin sealants) and are known as adhesive hemostatics because of their tissue sealing action. Non-active hemostatic agents, also known as passive hemostatic agents, do not contain any clotting factors; they function via mechanical or synthetic components, providing a framework where platelets can aggregate and activate so that a stable clot can form. These include collagen, gelatines, and regenerated oxidized cellulose. Agents with both non-active and active characteristics include thrombin plus mechanical hemostatic agents (sometimes described as 'flowable hemostatic agents') and fibrin sealants (Emilia *et al.*, 2011; Heher *et al.*, 2018; Kamoh and Swantek, 2012).

1.2.1 - Non-active (Passive) hemostatic agents

Mechanical hemostats form physical barriers that create an inert scaffold that exerts direct pressure on vessels and provide a surface for fibrin deposition and platelet adherence. They achieve hemostasis by relying on individuals' own circulating coagulation factors. Therefore, they are optimal for patients with an intact coagulation system. Mechanical hemostats are primarily used in cases of minimal bleeding. These agents are low cost, easy to use, and are available in several different forms. Additionally, these agents can be stored at room temperature and typically have a longer shelf life than the biologically active agents, making them cheaper and theoretically safer to use because of their inert nature. They are also biodegradable, with decomposition rates ranging from 24 hours (polysaccharide spheres) to 6 weeks (porcine gelatin). There are 4 general categories of mechanical hemostats: porcine gelatin, bovine collagen, oxidized regenerated cellulose, and polysaccharide spheres (Overbey *et al.*, 2014; Shander *et al.*, 2014).

1.2.1.1 - Gelatin hemostatic agents

Gelatin-based hemostatic agents are denatured collagen, prepared from purified pork skin gelatin, which is is obtained through methods that break the natural triple-helix structure of collagen to single strand molecules by thermal denaturation or physical/chemical degradation of the collagen; they have gained much attention as a hemostatic material due to their biocompatibility and biodegradability. They provide a mechanical, porous matrix that supports platelet enmeshing and fibrin clot formation. Gelatin-based agents are available in sponge, powder or granular forms. Gelatin sponges,

in particular, are extremely hygroscopic and can absorb a large amount of blood and other fluids, about 40 times its weight and able to expand to 200% of the initial volume. Gelatin matrix induces a small tissue reaction and liquefies in the oral cavity within a week, fully absorbing within 4-6 weeks. It is very useful in managing post-operative bleeding after dental extractions. Absorbable gelatin sponges do not need to be removed before wound closure; however, surgeons often remove them, when possible, to prevent compression of adjacent structures from the gelatin's swelling (Emilia *et al.*, 2011; Imani *et al.*, 2013; Kumar, 2016; Vezeau, 2016; Yazdimamaghani *et al.*, 2014).

1.2.1.2 - Microfibrillar collagen

Microfibrillar collagen is derived from purified bovine dermal collagen; it is a fibrous, water-insoluble partial hydrochloric salt. It is available in a loose fibrous form and also as sheets or sponges. In contact with blood, collagen causes aggregation of platelets, which bind in large numbers to the collagen fibrils. The aggregated platelets degranulate, releasing factors that assist in the formation of a clot. The sponge also provides a matrix for strengthening the blood clot. Microfibrillar powder or sheet collagen does not significantly swell when exposed to fluids. It is absorbed in approximately 8 weeks. This hemostatic agent is useful in the management of moderate to severe bleeding, i.e., capillary, venous, or small arterial bleeding. This form is generally less useful for oral surgical procedures (Kumar, 2016; Vezeau, 2016).

1.2.1.3 - Oxidized cellulose

Produced from wood pulp cellulose, oxidized cellulose is a chain of loosely connected cellulose chains. It is available in sheets and has an acidic pH, causing red cell lysis and formation of a pseudo-clot, achieving hemostasis by mechanical pressure. It provides an absorbable physical matrix for clotting initiation, expands on contact with blood 7-10 times its own weight; however, the rate of absorption is greatly dependent on the amount used, the extent of blood saturation, and the tissue bed. These hemostatic agents are used to control capillary, venous, and small arterial bleeding (Kumar, 2016; Vezeau, 2016).

1.2.1.4 - Polysaccharide spheres

Polysaccharide spheres are derived from vegetable starch and contain no human or animal components; they are processed into microporous spheres, which are applied

topically to the surgical site. They are used to control capillary, venous, and small arterial bleeding by producing a hydrophilic effect, dehydrating the blood, and concentrating erythrocytes and platelets, thereby aiding in hemostasis. These are resorbed in 24 to 48 hours by tissue amylases and are nonpyogenic (Kumar, 2016; Vezeau, 2016).

1.2.2 - Active hemostatic agents

Topical thrombin products are derived from either bovine or human plasma, or they are manufactured using recombinant DNA techniques (i.e., recombinant thrombin) (Kumar, 2016). They are indicated for cases of minor bleeding and oozing from accessible microvasculature. These topical hemostatics agents promote fibrin clot formation by converting fibrinogen into fibrin. In contrast to the mechanical agents, thrombin-containing topical agents do not rely on patients' intrinsic clotting mechanism as the mechanical hemostatic agents do and can be used in patients with impaired coagulation in the presence of adequate fibrinogen levels. The most commonly used types of thrombin agents are bovine thrombin, pooled human plasma thrombin, and recombinant thrombin (Shander *et al*., 2014).

Fibrin sealants are dual-component products that combine high concentrations of human fibrinogen with human, bovine, or recombinant thrombin. Fibrin sealants exert their effects at the end of the coagulation cascade, where fibrinogen is converted to fibrin in the presence of thrombin and calcium. Thrombin also activates fXII to stabilize the clot. A fibrin sealant forms both a hemostatic plug and a matrix to enhance wound healing. Because this process is independent of earlier steps in the coagulation cascade, fibrin sealants are effective in patients with defects in other parts of their coagulation pathways. Fibrin sealants may be applied to small blood vessels and to places that cannot be reached by conventional sutures (Shander *et al*., 2014).

1.2.3 - "Flowable" hemostatic agents

Flowable hemostatic agents combine thrombin with a mechanical hemostat, mostly gelatin, to form a "flowable" paste-like mixture. These agents work on actively bleeding tissue and have the advantage of conforming to the shape of irregular wound surfaces. Flowables can be applied either focally or spread to cover larger areas of bleeding. When applied to a bleeding site, the gelatin granules swell by about 10-20% as it comes into contact with blood, causing a seal at the bleeding site. The thrombin portion of the product activates the common pathway of the coagulation cascade and converts

fibrinogen to a fibrin polymer, forming a clot around the stable matrix. It is then absorbed by the body within 6-8 weeks, consistent with the time frame of normal wound healing (Kumar, 2016; Shander *et al.*, 2014).

1.3 - Biological characterization of hemostatic agents

The use of topical hemostatic agents within surgical procedures is a very common situation, within different clinical scenarios. Because these hemostatic products tend to go around the thorough regulatory process as other drugs or biomaterials for tissue implantation, there is currently no systematic approach to acquisition and documentation of use of these agents in human clinical settings. In addition, their place in clinical practice is not always evidence-based, and they are not systematically evaluated and managed in many institutions. Moreover, the abundance of agents that are available allows for duplicative products that use the same mechanism of action to be set within the same class, leading to an amazing array of choices for clinicians (Shander *et al*., 2014).

Given this situation, cytotoxicity testing of materials and/or devices is the first step in the assessment of biological compatibility of devices (Elisabettacenni *et al*., 2000). Genotoxicity tests are also important because a foreign material could release substances that induce gene mutation and even long-term neoplastic transformation (Elisabettacenni *et al*., 2000).

In vivo toxicity is a dynamic and complex sequence of physiological events. The use of animals in science is a global practice, and is still very much in use, and the main purposes of animal experiments are to gain basic biological knowledge for fundamental medical research, to test the toxicity of xenobiotics and ultimately contribute toward the discovery and development of novel drugs, and the development of vaccines and medical devices. Processes that lead to tissue damage as a result of exposure to foreign substances may vary or be specific to the different organs and, although much is written about general celular mechanisms of apoptosis, such as DNA fragmentation and oxidative stress, very little is known about specific organ toxicities and these events are seldom repeated in terms of molecular detail, dynamics or cellular metabolic processing in simplified *in vitro* models (Astashkina *et al.*, 2012; Kyffin *et al.*, 2018).

However, the outcomes of animal testing regarding its predictive power for *in vivo* human response exhibit certain limitations as well, mainly due to physiological and biochemical differences between species. In addition to the ethical considerations, the principle of the 3Rs – Replacement, Reduction and Refinement – has become an

increasing public and legal demand in scientific research, which is shifting the emphasis on producing more relevant and representative *in vitro* (human cell and cell line) models (Drasler *et al.*, 2017; Kyffin *et al.*, 2018).

The main aim of an *in vitro* model, cell culture in particular, is to be able to understand and obtain relevant and useful end points, such as assessing the toxicity potential of novel xenobiotics and efficacy (Kyffin *et al*., 2018). Benefits associated with these techniques are, being easy to follow, less time consuming and less expensive than *in vivo* and pre-clinical trials, as well as avoid excessive animal use and, consequently, sacrifice (Doke and Dhawale, 2015).

Modern technology enables us to cultivate *in vitro* almost every type of cell from all animal species, including human. These cells can provide very useful preliminary information or help us understand how certain substances interact with cell metabolism functions, such as secretion of proteins, motility or enzyme activity (Garattini and Grignaschi, 2017).

The principal purpose of any *in vitro* model is to simplify experimental variables to effectively isolate different components of organs or organ structures for study under well-controlled and easily assessed conditions, which will allow reproduction of important aspects of the whole process. Not every *in vitro* assay must necessarily recapitulate *in vivo* physiology; different *in vitro* models may reflect different levels of cellular organization and behavior, and provide different degrees of *in vivo*-relevant information, therefore the extrapolation of the results has to be made in the context of inherent characteristics of the system and of different physiological processes (Astashkina *et al.*, 2012; Turpeinen, 2007).

Over the years, exploitation of *in vitro* cell culture systems has proven to be a valuable method to study biological, physiological and pathological processes and that good risk predictions can be made based on *in vitro* data for human exposure to foreign substances and materials, but, as any tool, is subject to limitations, artifacts, and misleading results when removed from physiological context without validation or justification (Campbell *et al.*, 2012).

In this assignment I'll be determining the toxicity of four gelatin-based hemostatic sponges, that are routinetly used in the dental clinic of Faculdade de Medicina Dentária da Universidade do Porto, through various *in vitro* tests. The sponges in question are Hemospon, Clinix, Roeko and Octocolagen.

Chapter 2 - Aim

Hemostatic sponges are an important therapeutic option for the management of bleeding, in which physiological processes are inefficient or impractical. However, because these hemostatic products do not undergo a regulatory evaluation as, for instance, drugs, there is currently no systematic approach for the acquisition and documentation of use of these agents in human clinical settings.

In accordance, this work aims to address the biological characterization of clinically available gelatin-based hemostatic agents, through *in vitro* studies with various human cells.

We can list as specific aims:

- Preparation of hemostatic sponges leachables and thorough characterization through various experimental techniques.
- Establishment of distinct *in vitro* models – cell cultures, specifically human gingival fibroblasts and human osteoblasts, and subsequent exposure to the aforementioned leachables.
- Cytotoxicity assays will be performed, at different timepoints, in accordance with the cell lines, to evaluate cell viability, proliferation and functional activity.

Chapter 3 – Materials and Methods

3.1 - Preparation of the Hemostatic Sponges' Leachables

In this work, hemostatic sponges of 4 commercial brands were used, specifically Hemospon, Clinix, Octocolagen, and Roeko.

For the preparation of the hemostatic sponges' leachable, the sponges were soaked in 4 mL of simple alpha-Minimum Essential Medium (α-MEM, Gibco) and incubated for 24 hours at 37 ºC, 5% CO_2, 95% humidity and 100 rpm. Afterwards, the leachables were collected and stored in eppendorfs at - 20 ºC.

3.2 - Establishment of the Cell Cultures

Frozen primary human gingival fibroblast cells, passage 14, and frozen osteoblastsic-like cells, passage 94, were harvested by rapid thawing in a 37 °C water bath and seeded in T-flasks (Corning) with growth medium (α-MEM with 10% Fetal Bovine Serum (FBS), 100 UI/mL penicilin, 100 UI/mL streptomycin and 2,5 μg/mL amphotericin B, all from Gibco).

The cell cultures were maintained at 37 °C, 5% CO_2 and 95% humidity, under constant observation with an inverted phase contrast microscope (Nikon DN100), until achieving a confluence of 70%. The medium was changed 2 times a week.

Afterwards, the cultured cells were washed twice with phosphate buffered saline (PBS, pH 7,4, Sigma-Aldrich) and then incubated with trypsin (0,05% trypsin in 0,25% EDTA, both from Sigma-Aldrich), to detach the cells from the bottom of the flask, for 5 min at 37 ºC. The trypsin was neutralized with double the volume of growth medium and the liquid was transferred to a tube, which then underwent centrifugation at 1200 rpm for 10 minutes.

The pellets were resuspended in fresh growth medium and then the cells were cultured, in triplicate, at a density of 10^4 cells/cm^2 and $5x10^3$/cm^2 for fibroblasts and osteoblasts cells, respectively. The cells were incubated at 37 °C, 5% CO_2 and 95% humidity. After 24 h of incubation, the medium was replaced by the hemostatic sponges' leachable, previously diluted in α-MEM, at 50%, 25% and 12,5%. Cells incubated with only growth medium (without leachables) were seeded as negative control.

3.3 - Characterization of the Cell Cultures

The established cell cultures were characterized, at different periods, through the analysis of the cellular morphology and biochemical techniques, specifically cell proliferation, cell viability, metabolic activity and quantification of alkaline phosphatase activity, as well as by histochemical techniques, such as alkaline phosphatase staining and collagen content.

The different cell cultures used, fibroblastic and osteoblastic-like cells, were submitted to different tests at different times.

3.3.1 - Analysis of Mitochondrial and Cellular Morphology - MIF

The cellular and mitochondrial morphology was analyzed in the MG-63 cells on the 3rd, and 5th days.

For mitochondrial staining, the live cells were stained with MitoSpy^{TM} Red CMXRos (250 nM, Biolegend) for 30 minutes at 37 ºC, 5% CO_2 and 95% humidity, after which they were washed with PBS and fixed with 3,7% formaldehyde (Sigma-Aldrich) for 10 minutes and washed once more with PBS.

To stain the cell's cytoskeleton, the cell cultures were incubated with 50 µL of 0,1% Triton X-100 (Sigma-Aldrich) in PBS for 30 minutes at room temperature, after which it was discarded and 50 µL of 1% bovine serum albumin (BSA, Sigma-Aldrich) were added to each well and allowed to act for another 30 minutes at room temperature (RT). The next steps were performed in the dark, given the fact that some of the reagents are photosensitive. To the cells was added 50 µL of Alexa Fluor 488 – conjugated phalloidin (1:100, Molecular Probes), to stain the actin filaments, and left to incubate for 30 minutes at RT, after which time it was discarded, and the cells washed twice with PBS. Next, it was added 2 µg/mL of Hoescht (BioLegend) in PBS to stain the nucleus, for 10 minutes at RT.

The fluorescence images were obtained using a digital image system Celena S (Logos Biosystems).

3.3.2 - Biochemical Techniques

3.3.2.1 - Analysis of Cell Proliferation – DNA Quantification

This cell proliferation assay was performed for MG-63 cells on the 3rd, 5th, and 9th days.

This assay was performed using the Quant-iT™ PicoGreen™ dsDNA Assay kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The fluorescence was the measured at 485 nm and 528 nm, for excitation and emission, respectively.

3.3.2.2 - Analysis of Functional Activity/Metabolic Activity – MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was the first widely accepted method and is still today one of the most exploited approaches to quantitatively assess cytotoxicity and cell viability. In the presence of living cells, the MTT tetrazolium salt is internalized by the cells and enzymatically reduced to a crystalline water-insoluble purple/blue formazan compound, the amount of which is proportional to the number of viable cells (Lupu and Popescu, 2013; Pascua-Maestro *et al.*, 2018; Stepanenko and Dmitrenko, 2015).

This assay was performed for both the fibroblasts, on the 1st, 3rd, and 7th days, and MG-63 cells, on the 1st, 3rd, 5th and 9th days

To the growth medium of each cell culture, 10 μL of the MTT stock solution (5 mg/mL, Sigma-Aldrich) were added. Cells were incubated for 3 h at 37 ºC, 5% CO_2 and 95% humidity, after which the medium was removed. For the viability assay, the formazan product was dissolved in 100 μL of dimethylsulfoxide (DMSO, Panreac) and left to incubate at RT for 15 minutes, after which the absorbance was measured at 550 nm with a microplate reader (Synergy HT, BioTek).

3.3.2.3 – Analysis of Cell Viability - Live/Dead Assay

The LIVE/DEAD assay provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent cell-permeating calcein acetoxymethyl (AM) to the bright green-fluorescent calcein. Propidium iodide enters cells with damaged

membranes and binds to nucleic acids, thereby producing a bright red fluorescence in dead cells (Maltaris *et al.*, 2006).

This cell viability assay was performed on fibroblasts on the 1st day.

After incubation, cells were washed with PBS twice and incubated in 50 µL of the assay solution (25 µL calcein-AM (2 µL/mL, BioLegend) and 25 µL of propidium iodide (50 µL/mL, BD Biosciences) for 10 minutes at 37 ºC. The live/dead fluorescence images were captured using a digital image system Celena S (Logos Biosystems).

3.3.2.4 - Analysis of Alkaline Phosphatase Activity

The enzyme alkaline phosphatase (ALP) is an important serum analyte and its elevated state is associated with the presence of bone, liver, and other diseases, as well as active bone formation occurring, considering that ALP is a byproduct of osteoblast activity (Sharma *et al.*, 2014).

To date, several classical colorimetric methods have been developed for ALP detection, based on its biological function. In these methods, few substrates, such as disodium phenyl phosphate and p-nitrophenyl phosphate (pNPP), are dephosphorylated by ALP, resulting in a colorimetric reaction that can be correlated with ALP activity (Bzura *et al.*, 2018; Tang *et al.*, 2019).

This assay was performed on MG-63 cells, on the 1st, 3rd, 5th and 9th days.

At each time period, the medium was discarded, and the cells were washed twice with PBS. Afterwards, the cells were incubated, with 100 µL of 0,1% of Triton X-100 in H_2O, for 30 min at room temperature, after which time, it was added 80 µL of the enzyme's substrate, pNPP 25mM (Sigma-Aldrich), to each well and left to incubate for one hour at 37 ºC, 5% CO_2 and 95% humidity. Next, 20 µL of NaOH 5M (Sigma-Aldrich) were added to stop the reaction. The absorbance was measured at the wavelength of 400 nm on a microplate reader (Synergy HT, BioTek).

The amount of ALP in each well was calculated and normalized by the total protein content, which was measured using the DC™ Protein Assay (Bio-Rad), according to the manufacturer's protocol.

3.3.2.5 – Analysis of Cell Viability/Apoptosis - Flow Cytometry

Apoptosis consists of a cascade of events and many of the changes during the progression of this cascade can be followed using flow cytometry.

At a late stage of apoptosis, cells lose the integrity of their plasma membrane; as the DNA degrades, the membrane undergoes blebbing leading to the release of apoptotic bodies. Another hallmark of apoptosis is the breakdown of the nucleus, which includes the condensation of chromatin and associated fragmentation of the DNA (Hollville, 2013; Ormerod, 2002).

This assay was performed on fibroblasts on the 1st day.

At day 1, the levels of apoptosis were determined by staining the cells with Annexin V-FITC Apoptosis Detection Kit (640914, BioLegend), according to the manufacturer's protocol. Stained cells were immediately evaluated by flow cytometry (FACScalibur cytometer) and the data were analysed using FlowJo software.

3.3.3 - Histochemical Techniques

These techniques were done for the MG-63 cells and the cells were fixed at specific periods, specifically the 1st, 3rd, 5th, and 9th days. The histochemical staining techniques were performed later.

The medium was removed, and the cells were washed twice with PBS; after, the cells were fixed with 1,5% glutaraldehyde in sodium cacodylate buffer (pH 7,3, Sigma-Aldrich) for 15 minutes and then washed twice with PBS. The well-plates were stored at 4 ºC with enough sodium cacodylate buffer so as to prevent dehydration.

3.3.3.1 - Alkaline Phosphatase Staining

For the alkaline phosphatase (ALP) staining, the buffer in which the cells were kept was discarded and to them was added 50 μL of Tris buffer with Fast Blue RR salt and naphthyl sodium phosphate (all from Sigma-Aldrich). The cells were incubated for 1 hour in the dark at RT, after which time the staining solution was discarded and each well washed several times with distilled water, to remove the excess staining. The cells were photographed with an inverted phase contrast microscope (Nikon DN100).

3.3.3.2 - Collagen Synthesis

To measure the collagen synthesis, the buffer in which the cells were kept was discarded and to them was added 100 μL of Sirius Red stain. The cells were incubated for 1 hour in the dark at room temperature, after which time the staining solution was discarded and each well washed several times with 200 μL of 0,01M HCl (Panreac), to

remove the excess staining. The cells were photographed with an inverted phase contrast microscope (Nikon DN100).

3.3 - Statistical Analysis

The results here presented are from two independent experiments. All experimental data are expressed as the mean ± standard deviation, unless otherwise stated. The statistical analysis was done using Graph Pad Prism 8 software and the significance of differences between groups was analyzed using Bonferroni's multiple comparisons test. $P < 0.05$ was considered statistically significant.

Chapter 4 – Results

4.1 – Preparation of the hemostatic sponges' leachables

As was described in the previous chapter, to prepare the leachables the sponges were soaked in 4 mL of simple α-MEM for 24 hours at 37ºC, 5% CO_2, 95% humidity and 100 rpm.

To determine the different sponges' rate of degradation, the protocol described in the previous chapter was followed but, instead of being incubated for only 24 hours, the sponges were left in these conditons for several days, until they had completely dissolved. To document this process, photographs were taken on specific days at approximately the same time, which are represented here in figures 3 through 9.

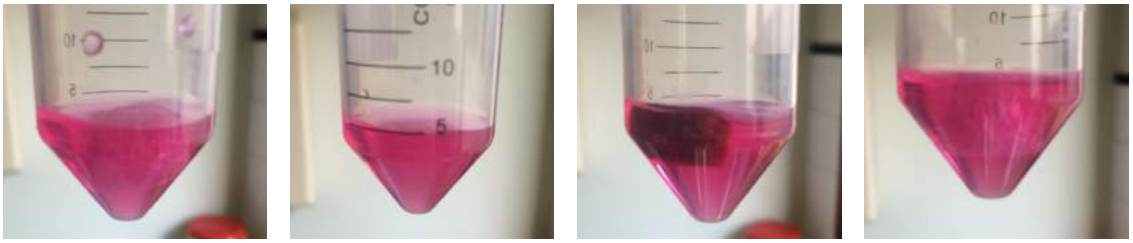

Figure 3 - Hemostatic sponges, soaked in simple α-MEM, on day 0. From left to right, Hemospon, Clinix, Roeko, Octocolagen.

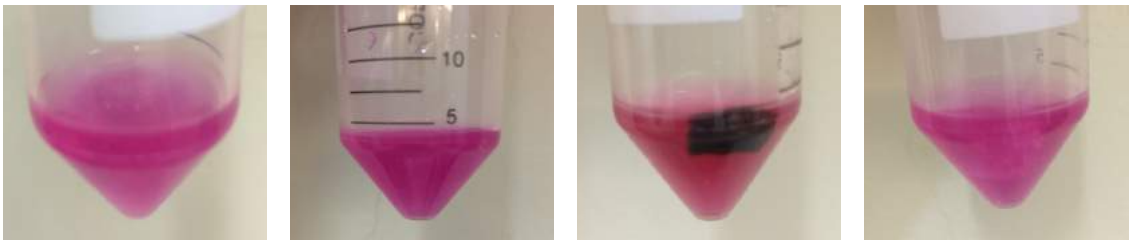

Figure 4 - Hemostatic sponges, soaked in simple α-MEM, on day 1. From left to right, Hemospon, Clinix, Roeko, Octocolagen. The Roeko sponge had already started to dissolve, as can be seen by the slightly more orange α-MEM, in comparison with the others.

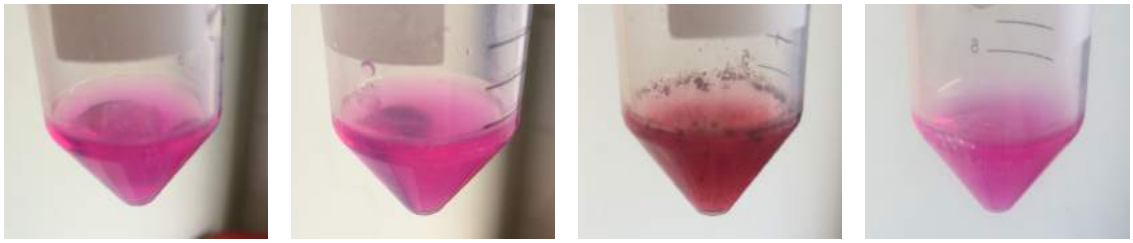

Figure 5 - Hemostatic sponges, soaked in simple α-MEM, on day 6. From left to right, Hemospon, Clinix, Roeko, Octocolagen. The Clinix and Roeko sponge had already dissolved.

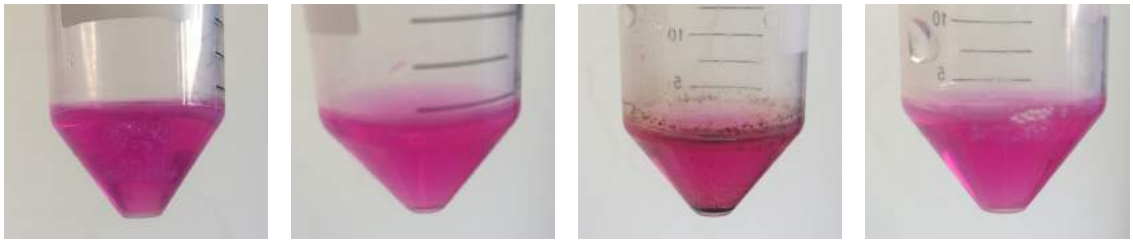

Figure 6 - Hemostatic sponges, soaked in simple α-MEM, on day 12. From left to right, Hemospon, Clinix, Roeko, Octocolagen. The Clinix and Roeko sponge had already dissolved.

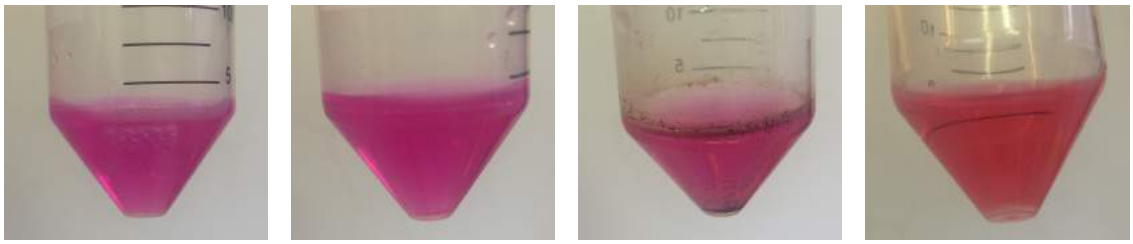

Figure 7 - Hemostatic sponges, soaked in simple α-MEM, on day 21. From left to right, Hemospon, Clinix, Roeko, Octocolagen. The Clinix, Roeko and Ocotolagen sponge had already dissolved.

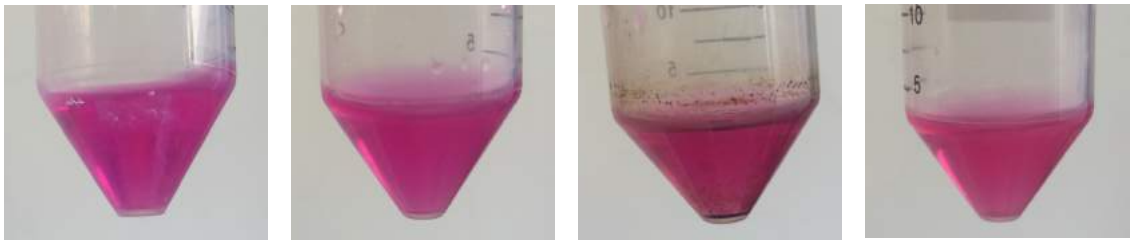

Figure 8 - Hemostatic sponges, soaked in simple α-MEM, on day 34. From left to right, Hemospon, Clinix, Roeko, Octocolagen. The Clinix, Roeko and Ocotolagen sponge had already dissolved.

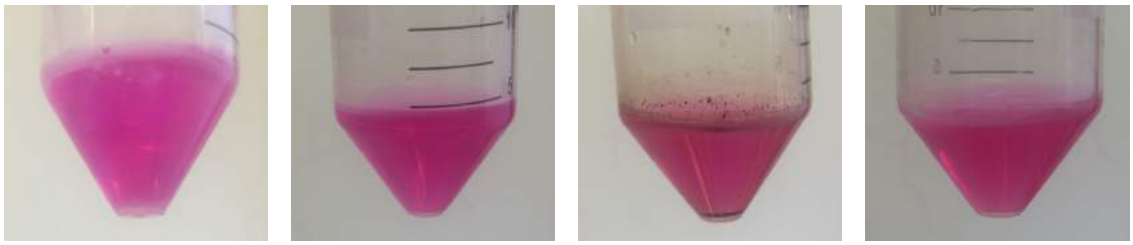

Figure 9 - Hemostatic sponges, soaked in simple α-MEM, on day 41. From left to right, Hemospon, Clinix, Roeko, Octocolagen. All the sponges had already dissolved by then.

4.2 – Characterization of the Human Gengival Fibroblastic cell cultures

This study aims to evaluate the toxicity of various hemostatic sponges' leachables in human gingival fibroblasts.

The cells were cultured for 24 hours in growth medium (α-MEM with 10% FBS, 100 UI/mL penicilin, 100 UI/mL streptomycin and 2,5 μg/mL amphotericin B) after

which time the leachables, previously diluted in α-MEM, were added at 50%, 25% and 12.5%. Cells without leachables were seeded as negative control groups.

The cell cultures were evaluated for cell proliferation, specifically metabolic activity (MTT), and cell viability, including flow cytometry and LIVE / DEAD, over the course of 7 days.

4.2.1 – Analysis of Functional Activity/Metabolic Activity – MTT Assay

The metabolic activity was assessed by the MTT assay (Figure 10).

On the 1st day there was an increase in metabolic activity of cells exposed to sponge extracts when compared to the control cells; the metabolic activity decreased on day 3 in relation to the control, and recovered on the 7th day, exhibiting metabolic activity similar or superior to the control cells.

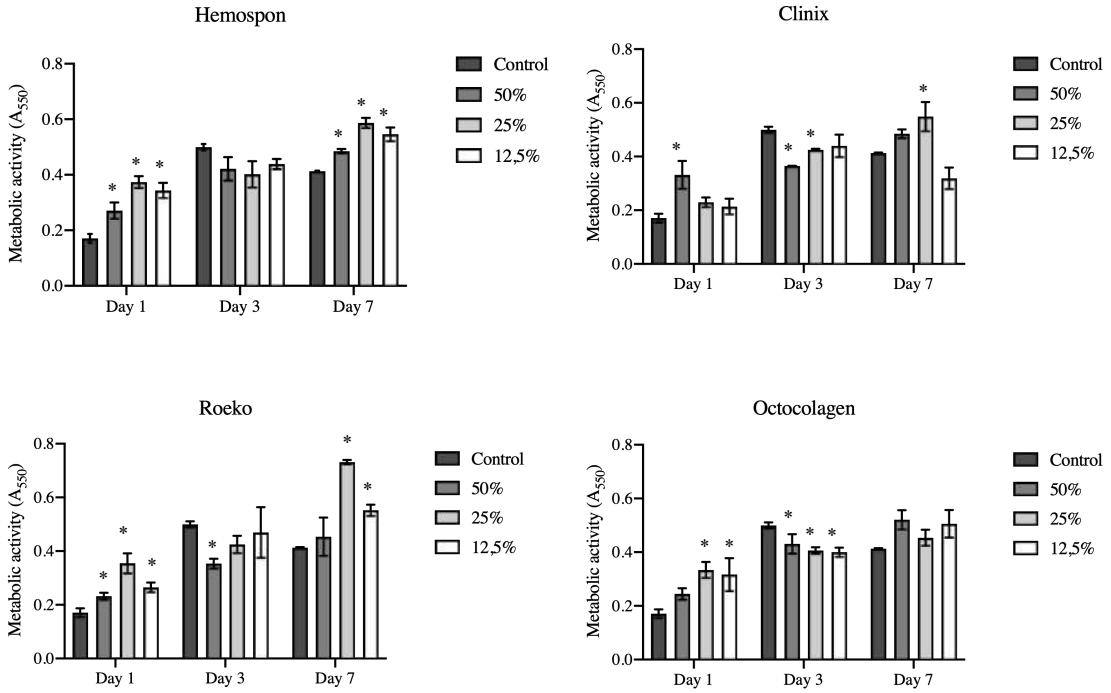

Figure 10 - Metabolic activity of human gingival fibroblasts, at different days, when exposed to different concentrations (50%, 25% and 12,5%) of several sponges' leachables (Hemospon, Clinix, Roeko and Octocolagen).

4.2.2 – Analysis of Cell Viability/Apoptosis - Flow Cytometry

Cell viability was assessed by flow cytometry, to distinguish between viable and apoptotic cells.

As can be ascertained by analyzing the graphs (Figure 11) and table (Table 1) below, the cells exposed to the sponges' leachables all had a viability greater than 90%, however both the Clinix and Roeko sponges had higher percentages of late apoptosis and necrotic cells, respectively, compared to the other two sponges, Hemospon and Octocolagen, which had quite high viability percentages.

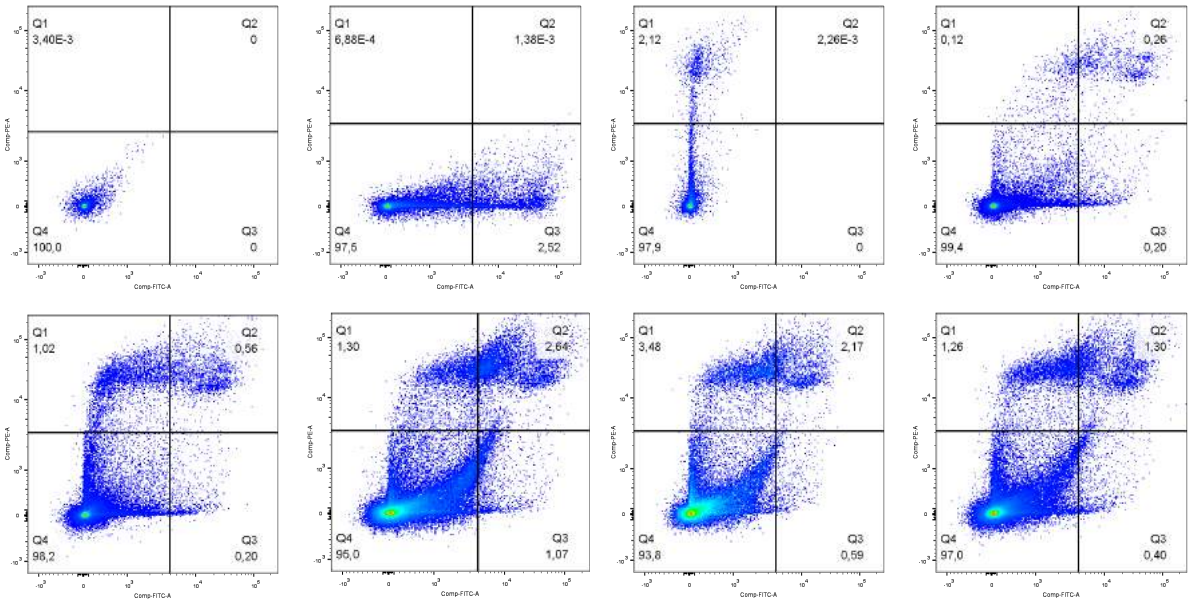

Figure 11 - Measuring apoptosis of human gingival fibroblasts exposed for 24 hours at a concentration of 12,5% of different sponges' leachables (Hemospon, Clinix, Roeko and Octocolagen).

Table 1 - Percentages of viable, early and late apoptotic, and necrotic cells of human gingival fibroblasts exposed for 24 hours at a concentration of 12,5% of different sponges' leachables (Hemospon, Clinix, Roeko and Octocolagen).

	UNSTAINED	ANNEXIN	PI	CONTROL ALL	HEMOSPON	CLINIX	ROEKO	OCTOCOLAGEN
Viable	100	97,5	97,9	99,4	98,2	95	93,8	97
Early	0	2,52	0	0,2	0,2	1,07	0,59	0,4
Late	0	0	0	0,26	0,56	2,64	2,17	1,3
Necrotic	0	0	2,12	0,12	1,02	1,3	3,48	1,26

4.2.3 – Analysis of Cell Viability - Live/Dead Assay

The live/dead double staining was performed to assess the viability on cells treated with different concentrations of four hemostatic sponges' leachables (Hemospon, Clinix, Roeko and Ocotocolagen), specifically 50%, 25% and 12,5%, after one day of incubation.

As observed, all groups of cells exhibited high cell viability with no significant differences between them (Figure 12).

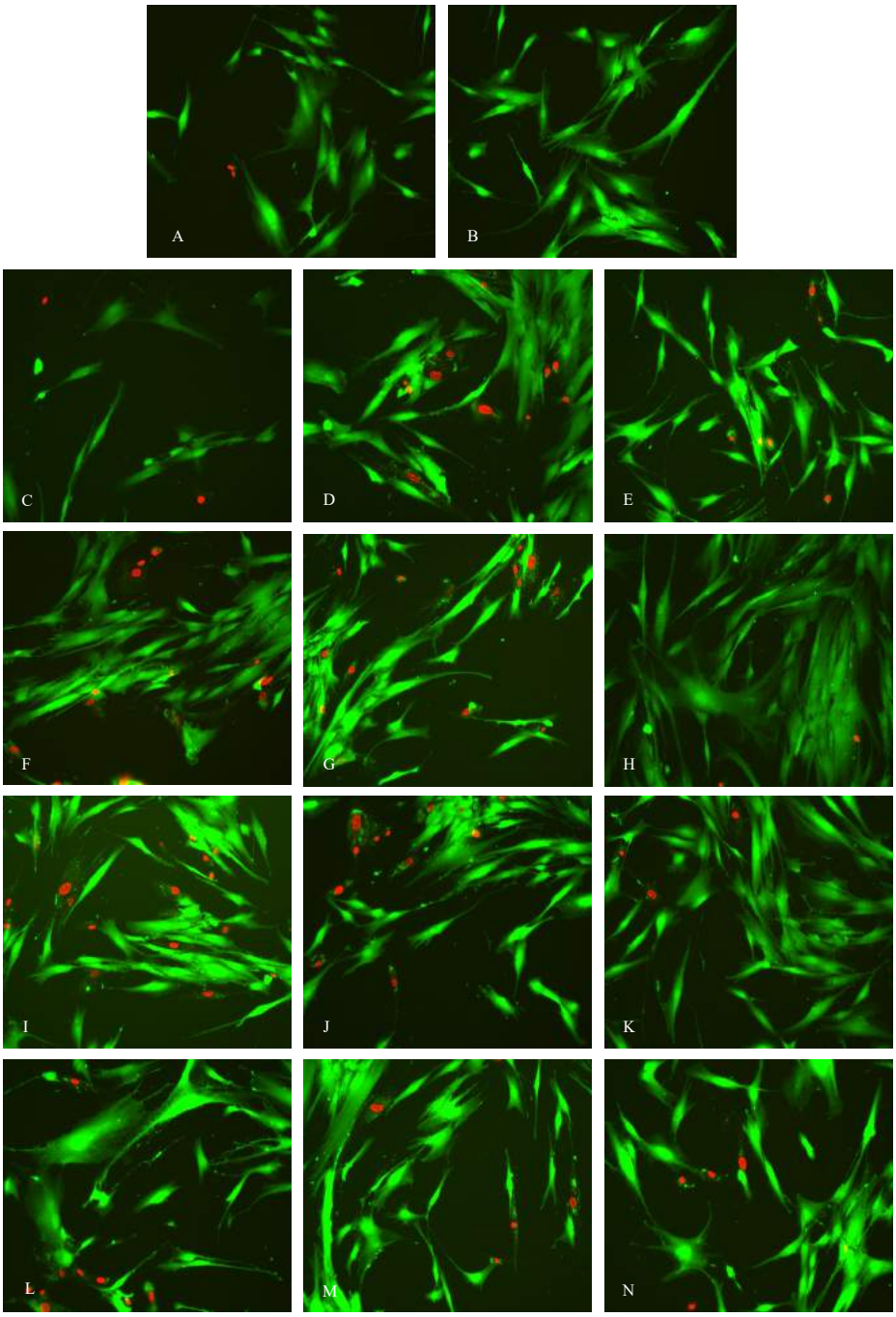

Figure 12 - Fluorescence images for live/dead double staining tests on the 1st day after the cells were cultured with different concentrations (50%, 25% and 12,5%) of 4 hemostatic sponges' leachables (Hemospon, Clinix, Roeko and Ocotocolagen) in growth medium. Live and dead cells were stained by the calcein AM and the propidium iodide respectively. Green color represents viable cells while red color represents dead cells.
The images here represented are Control (A and B), Hemospon 50% (C), Hemospon 25% (D) Hemospon 12,5% (E), Clinix 50% (F), Clinix 25% (G), Clinix 12,5% (H), Roeko 50% (I), Roeko 25% (J) Roeko 12,5% (K), Octocolagen 50% (L), Octocolagen (M) and Octocolagen 12,5% (N).

4.3 – Characterization of the Osteoblastic MG-63 cell cultures

This study aims to evaluate the toxicity of various hemostatic sponges' leachables in osteoblastic MG-63 cells.

The cells were cultured for 24 hours in growth medium (α-MEM with 10% FBS, 100 UI/mL penicilin, 100 UI/mL streptomycin and 2,5 μg/mL amphotericin B) after which time the leachables, previously diluted in α-MEM, were added at 50%, 25% and 12.5%. Cells without leachables were seeded as negative control groups.

The cell cultures were evaluated for cell proliferation, specifically metabolic activity (MTT) and DNA quantification, ALP activity, cellular morphology, ALP histochemical staining and collagen content over the course of 9 days.

4.3.1 – Analysis of Functional Activity/Metabolic Activity – MTT Assay

The metabolic activity was assessed by the MTT assay (Figure 13).

The three sponges followed a very similar pattern over time; on the 1st day after the leachables were added to the cells there was a reduction in the metabolic activity in comparison to the control cells, with the 3rd day showing higher metabolic activity in the treated cells than the control cells. On the 5th and 9th days the cells showed metabolic activity similar to the control cells.

On the 1st, 3rd and 5th days, the metabolic activity increased gradually, but on the 9th day it shot up dramatically.

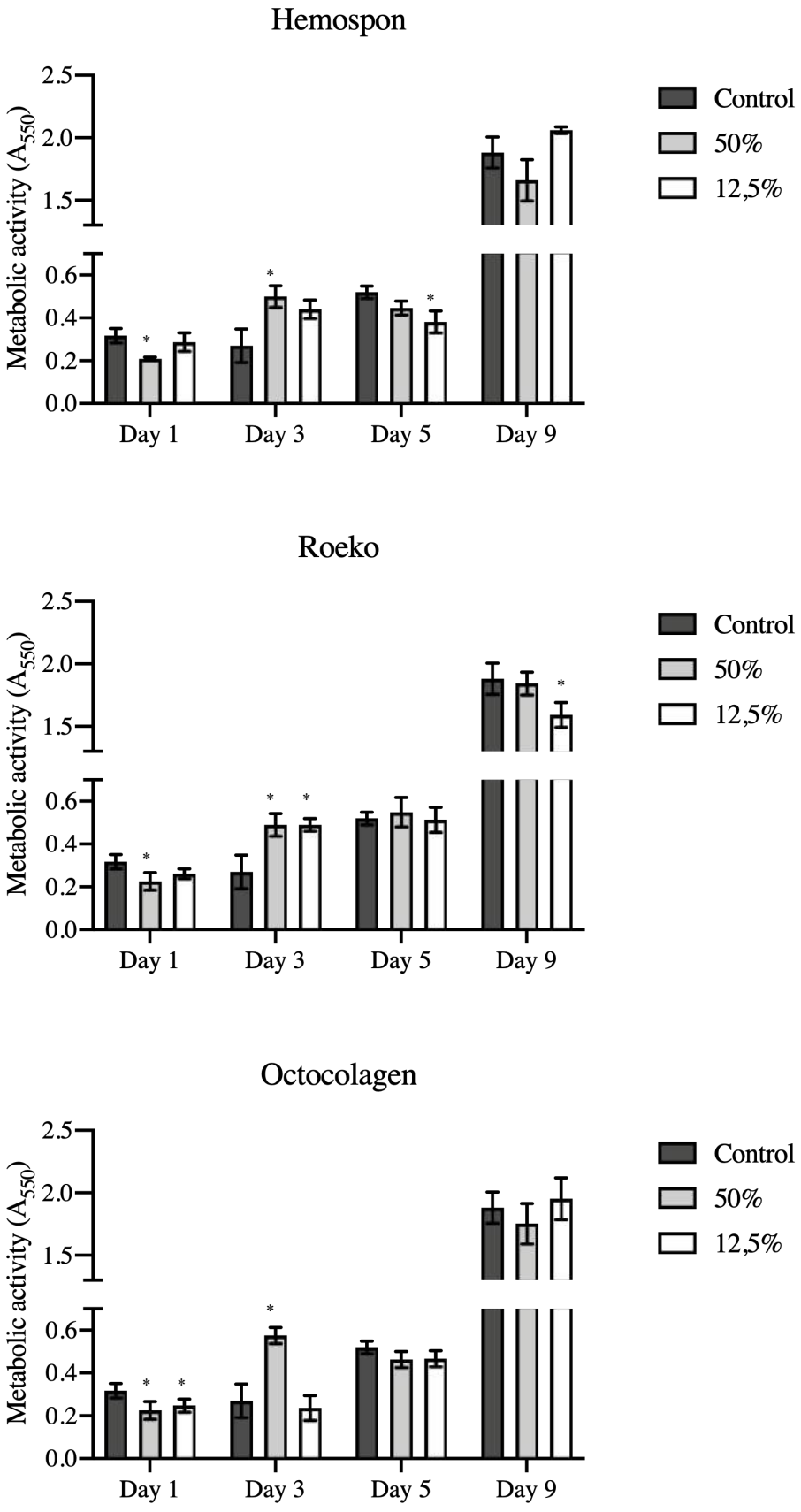

Figure 13 - Metabolic activity of osteoblastic-like cells, at different days, when exposed to different concentrations (50% and 12,5%) of several sponges' leachables (Hemospon, Roeko and Octocolagen).

4.3.2 – Analysis of Cell Proliferation – DNA Quantification

Cell proliferation, evaluated by DNA quantification, is shown in figure 14.

On the 5th day, the cells exposed to the different sponges' leachables showed a similar rate of proliferation as the control cells, with the cells of the Roeko group being only slightly higher than the control.

On the 9th day, the cells of the Hemospon and Octocolagen groups exhibited a lower rate of proliferation, when compared to the control cells, at a concentration of 50%; at a concentration of 12,5%, the treated cells showed a similar rate as the control cells. The cells of the Roeko group, however, showed a similar rate to the control cells at both concentrations.

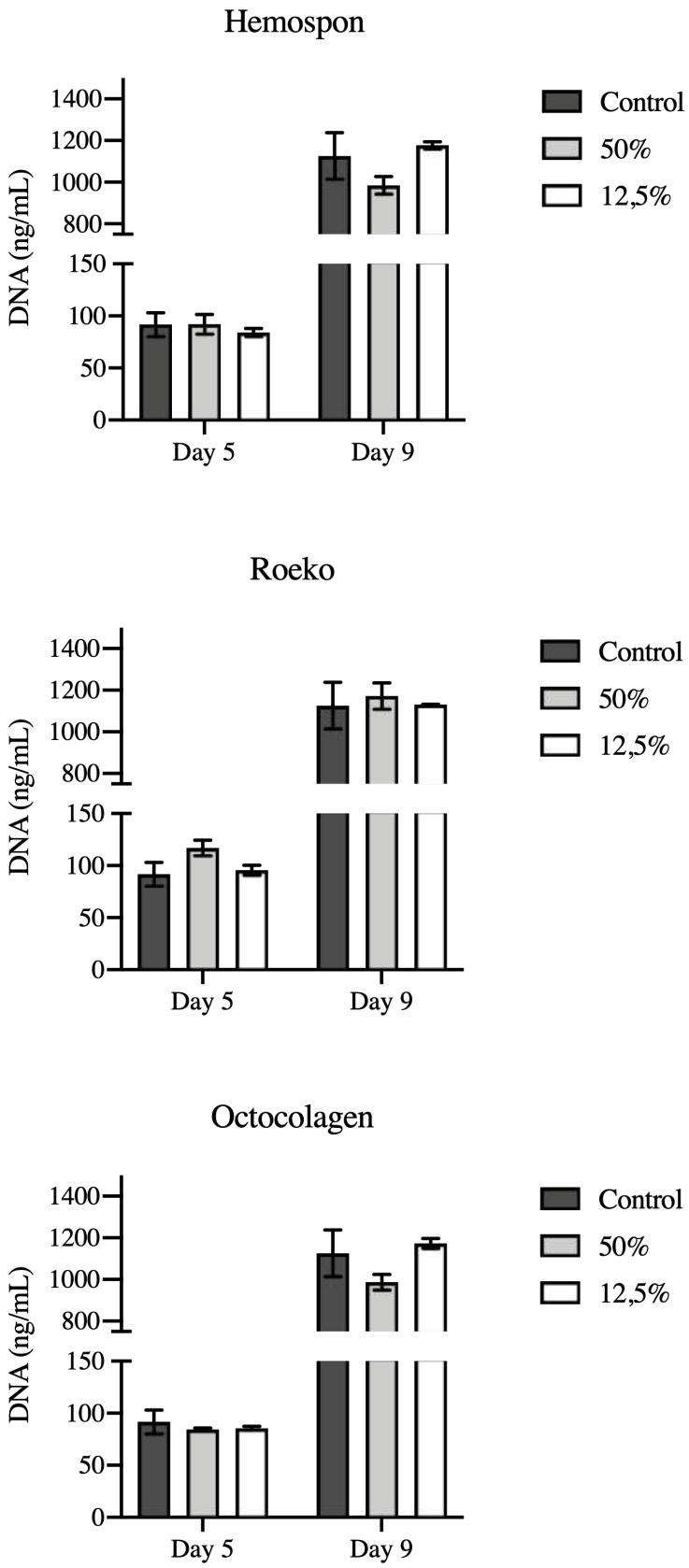

Figure 14 - Cell proliferation of MG-63 osteoblastic cells, at different days, when exposed to different concentrations (50% and 12,5%) of several sponges' leachables (Hemospon, Roeko and Octocolagen).

4.3.3 – Analysis of Mitochondrial and Cellular Morphology

In order to evaluate the mitochondrial and cellular morphology, the actin filaments that make up the cell cytoskeleton, nuclei and mitochondria were fluorescently labeled (Figures 15). After 3 days of culture, the cells showed an elongated morphology, exhibiting well-organized F-actins and well-defined nuclei and mitochondria, as shown in figure 15A.

On the 5th day, the morphology of the treated cells appeared similar to the control cells, however the groups exposed to the leachables appeared to exhibit a higher number of cells compared to the control group, as shown on figure 15B.

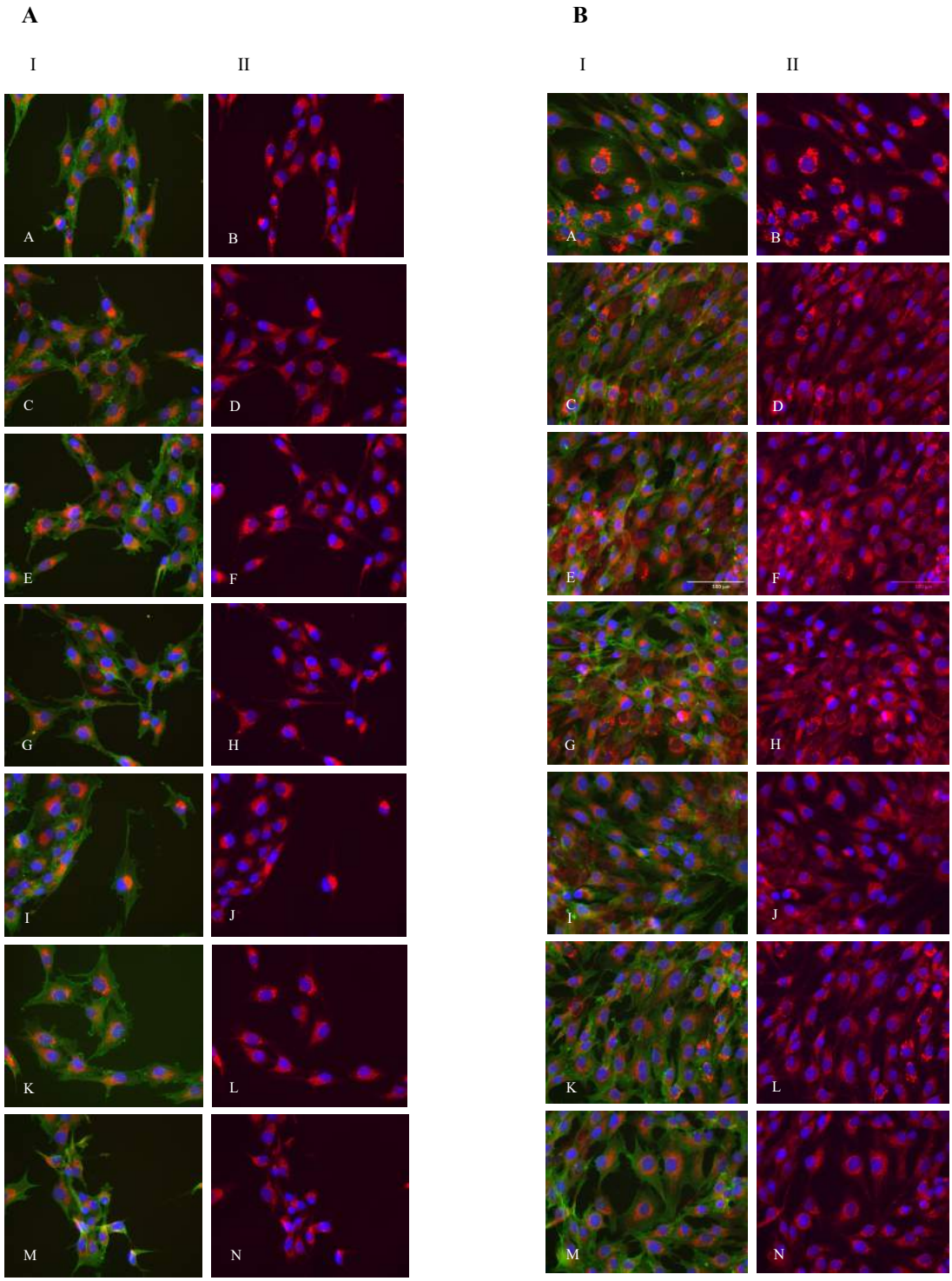

Figure 15 - Representative images of cellular morphology (I) and expression of mitochondrial activity (II) of MG-63 osteoblastic cells via confocal microscopy after 3 days (A) and 5 days (B) of incubation. After fluorescent labeling, the mitochondria appear red, the cell cytoskeleton F-actin filaments appear green and the nuclei appear blue. Scale bar length = 100 μm.
The images here are represented are Control (A and B), Hemospon 12,5% (C and D), Hemospon 50% (E and F), Roeko 12,5% (G and H), Roeko 50% (I and J), Octocolagen 12,5% (K and L), Octocolagen 50% (M and N).

4.3.4 – Analysis of Alkaline Phosphatase Activity

The alkaline phosphatase (ALP) activity, shown in figure 16, was normalized by the amount of total protein.

The cells showed a maximum peak of ALP activity on day 3 in all sponges, with a decrease on day 5 and a very slight increase on day 9.

On days 3 and 5, the ALP values of the treated cells were similar to the control on all sponges. On day 9 the cells of the Hemospon and Octocolagen groups showed a slight decrease compared to the control when exposed to a concentration of 50%; cells exposed to a concentration of 12,5% showed values similar to those of the control. On the other hand, the cells of the Roeko group presented higher values than the control, being that the values of the 12,5% concentration were superior to the 50% concentration.

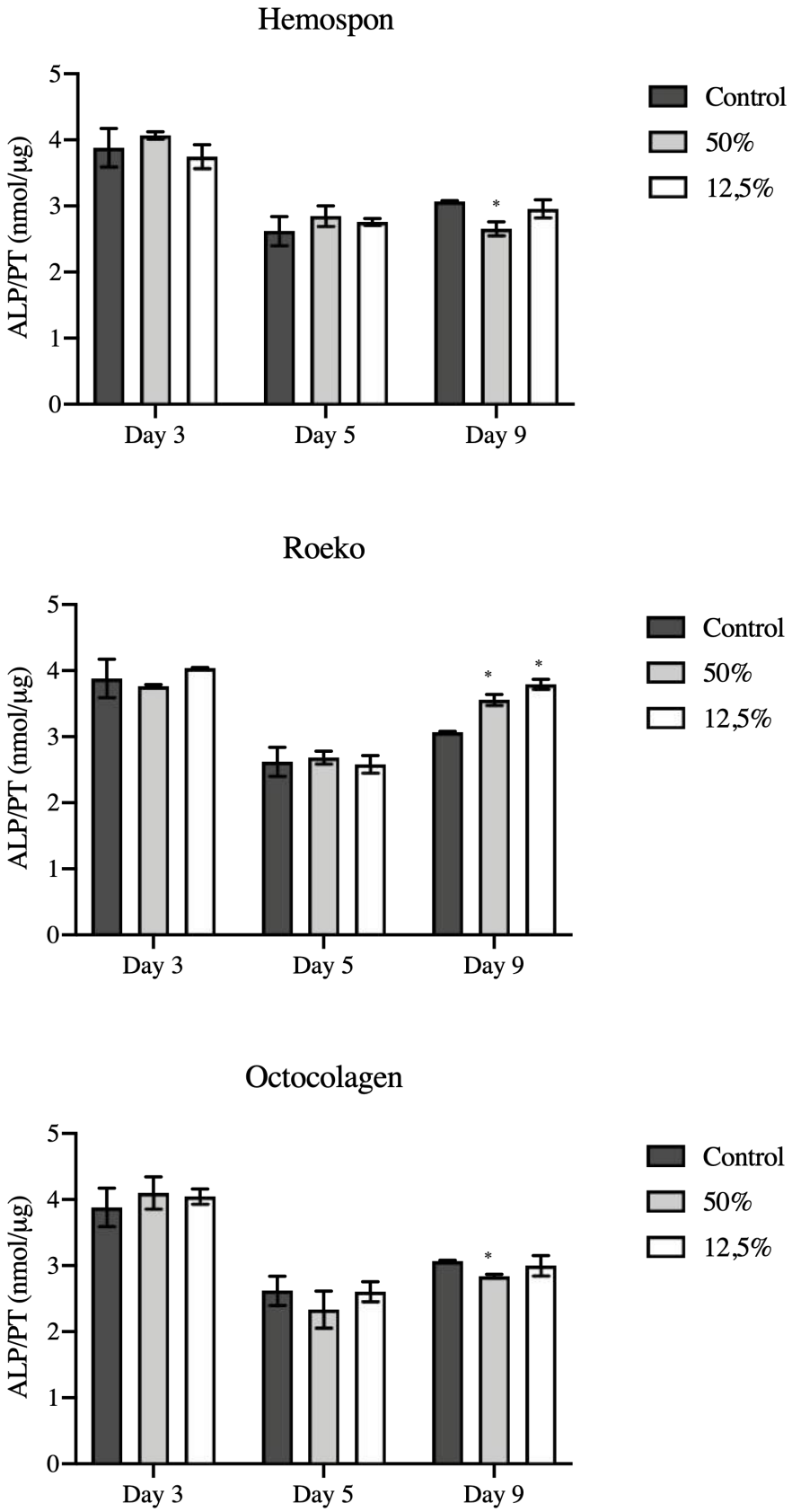

Figure 16 - Alkaline phosphatase activity, normalized by the amount of total protein, of MG-63 osteoblastic cells, at different days, when exposed to different concentrations (50% and 12,5%) of several sponges' leachables (Hemospon, Roeko and Octocolagen).

4.3.5 – Alkaline Phosphatase and Collagen Histochemistry

Optical microscopy images of alkaline phosphatase activity and collagen production, identified by histochemistry, are presented in the figures 17, 18 and 19.

On the 3rd day, regarding ALP activity, the cell cultures showed no differences in staining intensity compared to the control. The same was verified with the collagen staining (Figure 17 I and II).

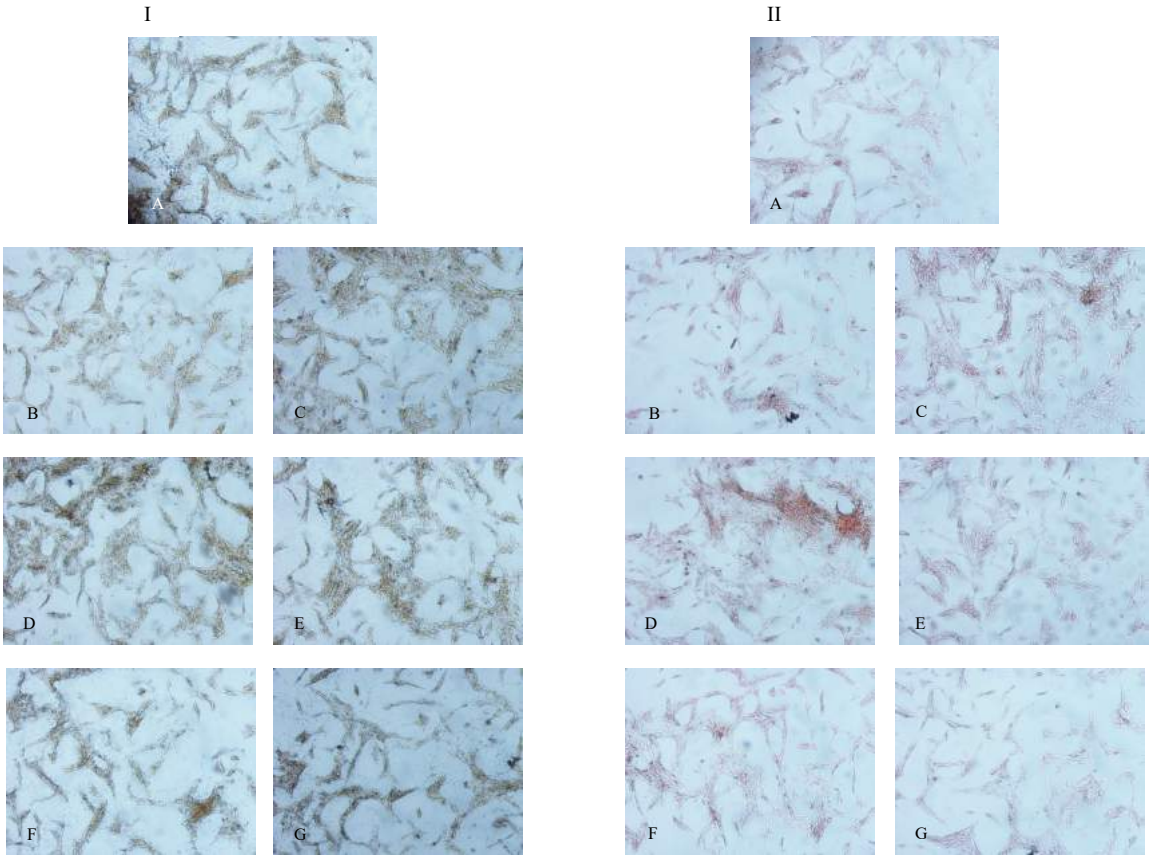

Figure 17 - Representative images of the microscopic appearance of MG-63 osteoblastic cell cultures on the 3rd day of incubation. On the left side the cells are stained histochemically for the presence of ALP; on the right side the cells are stained histochemically to detect collagen production. 40x magnification.
The images here are represented are Control (A), Hemospon 12,5% (B), Hemospon 50% (C), Roeko 12,5% (D), Roeko 50% (E), Octocolagen 12,5% (F), Octocolagen 50% (G).

On the 5th day, there was a considerable increase in the ALP staining without, however, the cells exposed to the leachables showing any significant differences in comparison to the control (Figure 18 I). In the collagen staining, the cells exposed to a concentration of 12,5% of the different sponges' leachables exhibited a slighlty more intense stain than the cells exposed to a concentration of 50%, as shown in the figure 18 II.

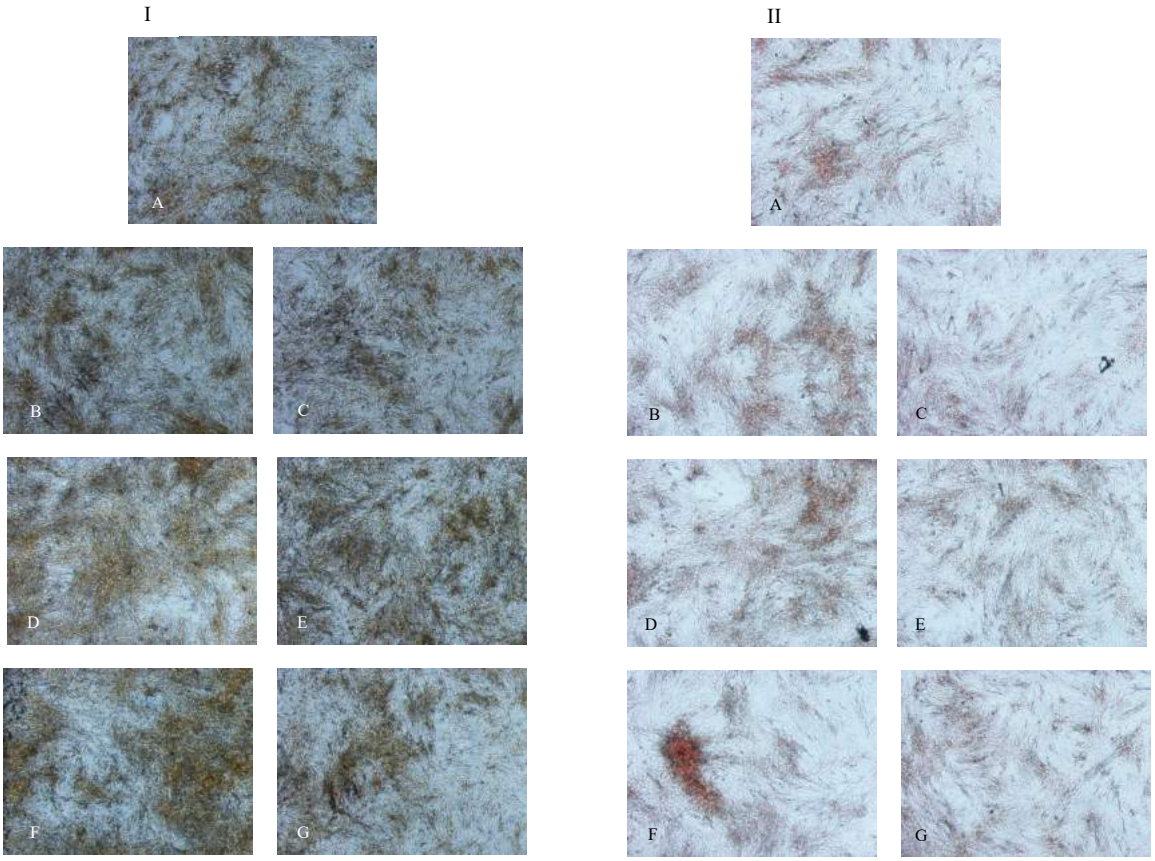

Figure 18 - Representative images of the microscopic appearance of MG-63 osteoblastic cell cultures on the 5th day of incubation. On the left side the cells are stained histochemically for the presence of ALP; on the right side the cells are stained histochemically to detect collagen production. 40x magnification.
The images here are represented are Control (A), Hemospon 12,5% (B), Hemospon 50% (C), Roeko 12,5% (D), Roeko 50% (E), Octocolagen 12,5% (F), Octocolagen 50% (G).

As had happened on the 5th day, on the 9th day there was a considerable increase in the ALP staining without, however, there being any significant differences in the treated cells and the control (Figure 19 I). In the collagen staining, the treated cells appeared similar to the control, with the cells of the Roeko group exhibiting a more intense stain when exposed to a concentration of 12,5% in comparison with the cells exposed to a concentration of 50%, as shown in the figure 19 II.

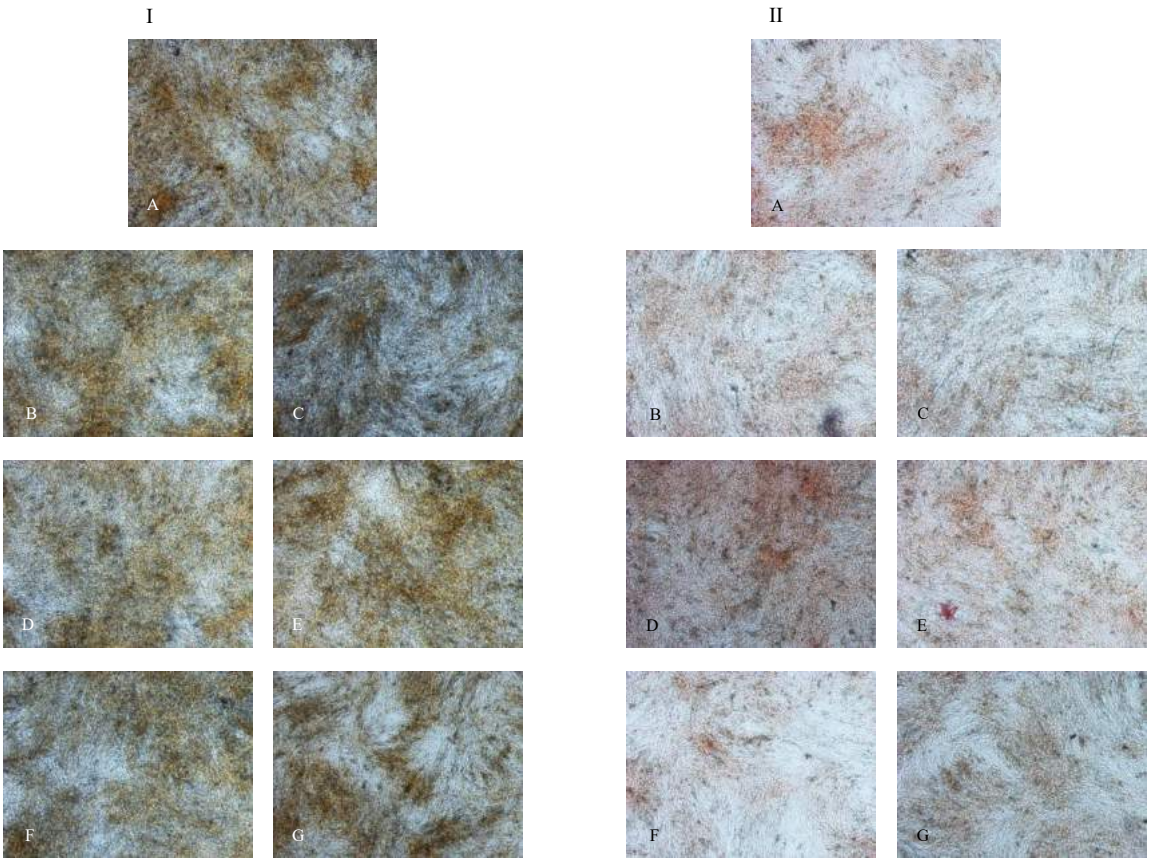

Figure 19 - Representative images of the microscopic appearance of MG-63 osteoblastic cell cultures on the 9th day of incubation. On the left side the cells are stained histochemically for the presence of ALP; on the right side the cells are stained histochemically to detect collagen production. 40x magnification.
The images here are represented are Control (A), Hemospon 12,5% (B), Hemospon 50% (C), Roeko 12,5% (D), Roeko 50% (E), Octocolagen 12,5% (F), Octocolagen 50% (G).

Chapter 5 – Discussion

Hemorrahge is a leading cause of preventable morbidity and mortality, and recognition of bleeding-related complications has resulted in progressive advances in hemostasis control and in a growth of clinically available topical hemostatic agents, which are an important therapeutic option for the management of bleeding (Heher *et al.*, 2018; Shander *et al.*, 2014).

Basic treatment for bleeding wounds consists of the application of absorbent dressings combined with efforts to stop blood flow, such as pressure, wound packing, etc. Hemostasis normally occurs within minutes (with the exception of severe wounds or bleeding- associated disorders), and during this, the damaged tissue space is rapidly filled with a blood clot stopping fluid loss and re-establishing a barrier to the outside (Wiegand *et al.*, 2019).

Topical hemostatic agents can induce or accelerate the coagulation cascade, as well as improve blood conservation by reducing fluid loss, shorten the time of hemostasis and avoid the potential adverse effects of hemostatic drugs (Wiegand *et al.*, 2019).

Different types of hemostatic agents are available, including plant-derived (ex. cellulose, polysaccharides), synthetic (ex. polyethylene glycol, glutaraldehyde), animal-derived (ex. gelatin, collagen), and human-derived (ex. thrombin, fibrinogen), and these agents can be divided into three categories: active, which display biologic activity and participate directly in the coagulation cascade, non-active, which simply provide a framework where platelets can aggregate and activate, and agents with both active and non-active characteristics (Emilia *et al.*, 2011; Heher *et al.*, 2018; Kamoh and Swantek, 2012; Wiegand *et al.*, 2019).

The most widely used topical hemostatic agents nowadays are collagen and gelatin (Wiegand *et al.*, 2019).

However, because these hemostatic agents do not undergo a thorough regulatory evaluation as, for instance, drugs, there is the need of a biological evaluation for the use of these agents in human clinical settings. In addition, their place in clinical practice is not always evidence-based, and they are not systematically evaluated and managed for different clinical applications (Elisabettacenni *et al.*, 2000; Shander *et al.*, 2014). Therefore, application of controlled *in vitro* techniques might serve as an effective screening tool in the monitoring and even development of new hemostatic agents and allow quantitative and qualitative assessment of their overall effects *in vitro*, with

potential translational data for the subsequent stages of biological evaluation (Wiegand *et al.*, 2019).

Aside from this point, the studies done so far in an effort to understand the effects and efficacy of hemostatic agents have shown varying results; some conclude that hemostatic agents have no negative effects on the tissues, some even sustaining that they might assist on the regeneration (Finn *et al*., 1992; Salgado *et al.*, 2016), while others claim that there is a delay in healing when these materials are present within the healing milieu (Armstrong *et al.*, 2010; Rocha *et al.*, 2012)

There are also studies that differ in their claims of which types of materials are better, specifically collagen and gelatin agents; *Wagner et al., 1996* claimed that collagen sponges perform better than gelatin ones, whereas *Renati et al., 2017* reported that a microfibrillar collagen sponge, specifically Avitene, induced a granulomatous reaction. *Kuo et al., 2016*, however, concluded the suitability of gelatin hemostatic sponges as a scaffold for bone tissue engineering.

After some research it was determined that reports in this field have focused a lot on *in vivo* animal trials using specific organ lacerations and artery puncture models to evaluate hemostatic efficacy, such as the assessment of coagulation, platelet activation, as well as fluid uptake and biodegradation (Lan *et al.*, 2015; Wagner *et al.*, 1996; Wiegand *et al.*, 2019), with some literature reports focusing on the biological functionality of these materials and on the assessment of potential modulatory effects of the materials on the cellular functionality, many of which study the effects and efficacy of cross-linked products with the hemostats.

In accordance, this work aims to address the biological characterization of clinically available gelatin-based hemostatic agents, through *in vitro* studies with various human cells.

The cells used in this study were gingival fibroblasts and human osteoblastic-like cells. The gingival fibroblasts were chosen because they are the first cells to come into contact with the sponges, which means it is of extreme importance that their reaction be evaluated; the osteoblasts, while not being in direct contact with the sponges, are cells that could be affected by the degradation products, upon sponge placement in alveolar sockets.

The sponges used were Hemospon, a porcine gelatin sponge, Clinix, a sponge manufactured from highly purified first extract grade porcine gelatin, Roeko, a porcine gelatin sponge with 5% colloidal silver, for the purpose of providing an antimicrobial

effect, according to the manufacturer, and Octocolagen, a sponge of lyophilized porcine gelatin. The sponges are all gelatin-based because, unlike collagen, which has antigenicity due to its animal origin, gelatin exhibits low-levels of immunogenicity under physiological conditions because of its denatured characteristics, giving it, theoretically, an advantage over collagen-based sponges; it is also more inexpensive than collagen while still exhibiting similar characteristics such as high biocompatibility and biodegradability and low cytotoxicity (Peter *et al*., 2010; Yazdimamaghani *et al.*, 2014).

The first assay conducted was the degradation study; to determine the degradation rate of the sponges.

Although this *in vitro* assay is far from representing real physiological conditions, specially considering that the medium in which the sponges were immersed was devoid of many of the components present in saliva, which would predictably exacerbate even further their dissolution, it may provide a quick and alternative method to understand gelatin sponge degradation in a short period (Kuo *et al.*, 2016), as well as illucidate whether or not it could have any effect on the cells' functionality; the sponges' degradation rate will affect the preparation of the leachables, in sense that the leachables of the sponges that degrade faster will inevitably be more concentrated than the sponges that degrade at a slower rate. Degradation patterns of absorbable gelatin sponges are also important because gelatin behaves differently if it lies on top of a cavity or just on the surface of the tissue (Goncalves *et al.*, 2015).

As determined by the observation of the sponges, which was achieved by taking photographs at different times, every sponge had different rates of degradation. The Hemospon sponge was the one that took the longest to dissolve, at 41 days; the Octocolagen sponge came in second, having dissolved at 21 days; the Clinix sponge appeared to have dissolved after only 6 days; the Roeko sponge showed signs of dissolution on the 1st day, having dissolved completely on the 6th day.

Goncalves et al., 2015 performed a degradation study in 2 different gelatin sponges, GelitaSpon and Gelfoam, where they cut the sponges into and immersed them in 0.9% saline solution or 3% BA; they observed degradation of Gelfoam by day 14, while GelitaSpon showed complete degradation by the third day; this allowed the authors to reach the conclusion that the structure of the sponge plays a very important role in their degradation, meaning if the sponge has more cross-linkages, the degradation process is slower and could predictably last longer, whereas finely produced sponges with fewer cross-linkages and a lower mass will degrade faster. *Whu et al., 2013* came to a similar

conclusion when they evaluated gelatin-based sponges' degradation rate, by immersing them in PBS, and observed that non-crosslinked gelatin degraded very fast, even in the absence of an enzyme, while gelatin crosslinked with chitosan prevented disintegration of the scaffolds during material degradation.

The results of these articles are consistent with the ones obtained in our study, specifically the differences between the sponges. While these sponges are strictly gelatin and are not crosslinked with any other substance, the varying rates of degradation could be explained by each sponge's form of production.

The effect of the hemostatic agents on the cells' behaviour was determined by an indirect method so as to ascertain what effects the sponges' degradation products may have on gingival fibroblasts and osteoblasts. This was done by the analysis of the cellular morphology, biochemical techniques, specifically cell proliferation, cell viability, metabolic activity and quantification of alkaline phosphatase activity, as well as histochemical techniques, such as alkaline phosphatase staining and collagen content, at different times.

Regarding the biological evaluation with human gingival fibroblasts, the metabolic activity results, that were obtained by the MTT assay, demonstrated a similar pattern for the 4 sponges over the course of 7 days.

On the 1st day, after cells incubation with different concentrations of the sponges' extracts, specifically 50%, 25% and 12,5%, a higher metabolic activity was achieved, as comparing to those cells not exposed to the extracts, which served as a control; despite this these results do not allow for the conclusion that the sponges are non toxic and it is more likely that the cells were compensating for being exposed to a foreign agent.

On day 3 there was an increase in the metabolic activity of the control cells that was not accompanied by the groups cultured in the presence of sponges' extracts, leading to the belief that the sponges may have a slightly inhibitory effect when the cells are exposed a little longer to the leachables. However, on 7th day the cells appeared to have recovered and exhibited metabolic activity similar or superior to the control.

These results lead to the assumption that sponges are non-toxic, which matches with the conclusions of *Lan et al., 2015* and *Aydemir Sezer et al., 2018,* who performed MTT assays on murine fibroblast cells line (L929) and human umbilical vein endothelial cells (HUVEC), respectively, exposed to gelatin-based materials and reported good cell compatibility without cytotoxicity.

Cell viability on the fibroblasts was assessed by flow cytometry and a live and dead assay, on cells treated with different concentrations of four hemostatic sponges' leachables (Hemospon, Clinix, Roeko and Octocolagen), specifically 50%, 25% and 12,5%, after one day of incubation.

The Live and Dead assay did not reveal any cytotoxicity when compared to the control cells; although the cells exposed to the leachables did show more dead cells than the control, the differences were not significant.

Similar results were obtained by *Aydemir Sezer et al., 2018,* who tested different gelatin based agents on HUVEC cells and found no significant difference among the groups in terms of dead cells. *Awad et al., 2004* also tested several different hemostats, one of them being a gelatin sponge, on human adipose derived adult stem (hADAS) cells, and performed a Live and Dead assay that showed relatively uniform distribution of cells with viability greater than 95%.

To further add to the conclusions already drawn from the Live and Dead assay, a flow cytometry assay, to evaluate viability and cell death, was performed.

This assay showed a viability higher than 90% on all the cell groups, which on its own already allows the conclusion that none of the sponges are cytotoxic; there are however differences when it comes to late apoptotic and necrotic cells, with both the Clinix and Roeko sponges presenting higher percentages of late apoptosis and necrotic cells, respectively, compared to the other two sponges, Hemospon and Octocolagen.

These results can be correlated with the MTT assay; while the sponges' leachables may not affect the cells' proliferation, they might affect their viability. Despite this, these differences were not significant, which disallows the conclusion that the Clinix and Roeko sponges are more cytotoxic than the Hemospon and Octocolagen ones.

Regarding the assessment of the biological functionality of the sponges' extracts in human osteoblastic-like cells, the metabolic activity followed a very similar pattern for the three sponges studied, Hemospon, Roeko and Octocolagen; on the 1st day after the leachables were added to the cells, there was a reduction in the metabolic activity in comparison to the control cells, which can be explained as the initial shock at being exposed to a foreign agent. The 3rd day showed higher metabolic activity in the treated cells than the control cells, which could possibly be the cells compensating for the leachables. On the 5th and 9th days the cells showed metabolic activity similar to the control.

On the 1st, 3rd and 5th days, the metabolic activity increased gradually, but on the 9th day it increased significantly. *Peter et al., 2010* perfomed viability assays, specifically an MTT assay, on MG-63 cells incubated with chitosan-gelatin/nanophase hydroxyapatite composite scaffolds for 24 and 48 hours, and obtained similar results, with the authors concluding that the gelatin-based scaffolds were cytocompatible. *Kim et al., 2005* studied MG-63 cells' metabolic activity and ALP activity when incubated with gelatin-hydroxyapatite, and showed similar results to ours, with slightly higher metabolic and ALP activities than the control.

The MTT results obtained in these cells, on the 1st and 3rd days, were the opposite of the results obtained in the fibroblasts, and this inversion could be explained by the fact that the cells used in this study are phenotypically different.

To supplement this last assay, cell proliferation was evaluated by DNA quantification.

On the 5th day, the cells exposed to the different sponges' leachables showed a similar rate of proliferation as the control cells, with the cells of the Roeko group being only slightly higher than the control. On the 9th day, the cells of the Hemospon and Octocolagen groups exhibited a lower rate of proliferation, when compared to the control cells, at a concentration of 50%; at a concentration of 12,5%, the treated cells showed a similar rate as the control cells. The cells of the Roeko group, however, showed a similar rate to the control cells at both concentrations.

A similar result pattern was observed in the ALP activity assay. On days 3 and 5, the ALP values of the treated cells were similar to the control on all sponges; on day 9 the cells of the Hemospon and Octocolagen groups showed similar results to the control at a 12,5% concentration, with slight decrease at 50%, whereas, the cells of the Roeko group presented higher values than the control at both concentrations.

The cells showed a maximum peak of ALP activity on day 3 in all sponges, with a decrease on day 5 and a very slight increase on day 9.

Rohanizadeh et al., 2008 studied osteoblast proliferation and differentiation in gelatine sponges, and they reported different results than ours in regards to the DNA quantification, with the group cells showing a higher DNA content that the control after one week, but similar results in the ALP activity assay, meaning no significant differences between the control and the gelatin sponge after 7 days. *Awad et al., 2004* obtained similar results, with hADAS cells on various hemostats, one of them being gelatin-based, where the DNA content was significantly higher than their control conditions counterparts after

one week. It is worth mentioning that the authors cultured the cells onto the sponges, as opposed to this study, in which leachables were used at different concentrations; this could account for the differences in results.

While these assays appear to demonstrate that none of the sponges studied are cytotoxic, the cell proliferation and the ALP activity assays showed that the Hemospon and Octocolagen groups, at a 50% concentration, performed poorly in comparison to the Roeko group, which did not happen in the MTT assay, where all sponges had similar results.

The biochemical results can be correlated to the histochemical results, both regarding assessment of ALP activity and collagen. The ALP staining demonstrated a steady increase in intensity over the 9 days, without demonstrating significant differences in comparison to the control. The collagen staining showed the same gradual increase in stain intensity, with the cells exposed to a 12,5% concentration already showing a slightly more intense colouration after the 5th day; *Moscato et al., 2008* studied PVA/gelatine spongy matrices so as to evaluate their biocompatibility with gingival fibroblasts; they determined that the fibroblasts interacted positively with the sponges and were able to create a biological microenvironment rich in extracellular matrix molecules such as fibronectin, type I collagen and laminin, which matches our results that show that the gelatin sponges here studied did not hinder the osteoblasts' collagen production.

To evaluate the cellular and mitochondrial morphology of the cells, the actin filaments that make up the cell cytoskeleton, nuclei and mitochondria were fluorescently labeled. The cells showed an elongated morphology, exhibiting a well-organized F-actin structure and well-defined nuclei and mitochondria, appearing only to exhibit a higher number of cells compared to the control group on the 5th day.

As was mentioned above, there are many authors who choose to focus on *in vivo* animal trials to evaluate hemostatic efficacy, more specifically the inflammatory response which can, on some level, be correlated with an impaired biocompatibility.

The majority of the authors conclude that the the sponges do not induce any inflammatory response, at least not significantly; for example, *Goncalves et al., 2015* performed *in vitro* and *in vivo* studies of gelatin-based sponges crosslinked with different agents on the middle ear of rats to study the reabsorption rate and the inflammatory response generated by absorbable gelatin sponges, as well as the effect of the crosslinked agents upon the sponges' degradation; the histological staining of each hemostatic

material tested showed no acute inflammation or other adverse effects, while *Ibarrola et al., 1985,* in a rat experimental bone defect model, tested different hemostatic agents and denoted that Gelfoam, a gelatin-based sponge, showed little inflammation and allowed for complete healing.

However some other studies do paint a different picture, like *Lied et al., 2019* who reported the case of a 71 year old man who had intraoperative anaphylaxis due to the use of a topical gelatin-based hemostatic agent, Surgiflo, who, after undergoing an allergological examination reported a history of red meat allergy, and *Ji and Barrett, 2015* who also reported the case of a 2 year old girl with no known allergies, who had intraoperative anaphylaxis, and the authors cautiously concluded that it was due to the gelatin-based hemostat, Gelfoam, after undergoing skin prick and intradermal testing for all of the drugs administered and suspected agents during the dental rehabilitation.

Despite all of these contradictory results and studies, a value of 70% for cell viability after exposure to biomaterials provides a reasonable threshold to discriminate between toxic and non-toxic materials (Elisabettacenni *et al*., 2000) and these results, when taken into account as one, can reasonably be considered as having a viability higher than 70%, which leads to the conclusion that these sponges are not cytotoxic.

Chapter 6 - Conclusion

Hemostatic sponges are an important therapeutic option for the management of bleeding, in which physiological processes are inefficient or impractical.

This work aims to address the biological characterization of clinically available gelatin-based hemostatic agents, through *in vitro* studies with various human cells.

In this experimental work, the results showed no significant cytotoxicity to the cells studied; however, there were some differences in the assays performed that could demonstrate that while the sponges' may not affect cell proliferation, they could have some effect on their viability. Aside from this, it could also be detected that different sponges had better results for each cell type. For these reasons, further studies are needed in order to elucidate the mechanisms underlying these results.

Bibliographic References

Achneck, H. E., Sileshi, B., Jamiolkowski, R. M., Albala, D. M., Shapiro, M. L., & Lawson, J. H. (2010). A Comprehensive Review of Topical Hemostatic Agents: Efficacy and Recommendations for Use. *Annals of Surgery*, *251*(2), 217–228. Retrieved from https://journals.lww.com/annalsofsurgery/Fulltext/2010/02000/A_Comprehensive_Review_of_Topical_Hemostatic.8.aspx

Armstrong, J. K., Han, B., Kuwahara, K., Yang, Z., Magyar, C. E., Dry, S. M., … Fisher, T. C. (2010). The effect of three hemostatic agents on early bone healing in an animal model. *BMC Surgery*, *10*(37), 1–12. https://doi.org/10.1186/1471-2482-10-37

Astashkina, A., Mann, B., & Grainger, D. W. (2012). A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. *Pharmacology & Therapeutics*, *134*(1), 82–106. https://doi.org/10.1016/J.PHARMTHERA.2012.01.001

Awad, H. A., Wickham, M. Q., Leddy, H. A., Gimble, J. M., & Guilak, F. (2004). Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials*, *25*(16), 3211–3222. https://doi.org/10.1016/j.biomaterials.2003.10.045

Aydemir Sezer, U., Kocer, Z., Sahin, İ., Aru, B., Yanıkkaya Demirel, G., & Sezer, S. (2018). Oxidized regenerated cellulose cross-linked gelatin microparticles for rapid and biocompatible hemostasis: A versatile cross-linking agent. *Carbohydrate Polymers*, *200*, 624–632. https://doi.org/10.1016/j.carbpol.2018.07.074

Bzura, J., Fiedoruk-Pogrebniak, M., & Koncki, R. (2018). Photometric and fluorometric alkaline phosphatase assays using the simplest enzyme substrates. *Talanta*, *190*, 193–198. https://doi.org/10.1016/j.talanta.2018.07.052

Campbell, J. L., Andersen, M. E., & Clewell, H. J. (2012). Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results AU - Yoon, Miyoung. *Critical Reviews in Toxicology*, *42*(8), 633–652. https://doi.org/10.3109/10408444.2012.692115

Doke, S. K., & Dhawale, S. C. (2015). Alternatives to animal testing: A review. *Saudi Pharmaceutical Journal*, *23*(3), 223–229. https://doi.org/10.1016/J.JSPS.2013.11.002

Drasler, B., Sayre, P., Steinhäuser, K. G., Petri-Fink, A., & Rothen-Rutishauser, B.

(2017). In vitro approaches to assess the hazard of nanomaterials. *NanoImpact*, *8*, 99–116. https://doi.org/10.1016/J.IMPACT.2017.08.002

Elisabettacenni, Ciapetti, G., Stea, S., Corradini, A., & Carozzi, F. (2000). Biocompatibility and performance in vitro of a hemostatic gelatin sponge. *Journal of Biomaterials Science, Polymer Edition*, *11*(7), 685–699. https://doi.org/10.1163/156856200743959

Emilia, M., Luca, S., Francesca, B., Luca, B., Paolo, S., Giuseppe, F., … Mauro, L. (2011). Topical hemostatic agents in surgical practice. *Transfusion and Apheresis Science*, *45*, 305–311. https://doi.org/10.1016/j.transci.2011.10.013

Eyre, L., & Gamlin, F. (2010). Haemostasis, blood platelets and coagulation. *Anaesthesia & Intensive Care Medicine*, *11*(6), 244–246. https://doi.org/10.1016/J.MPAIC.2010.02.015

Finn, M. D., Schow, S. R., & Schneiderman, E. D. (1992). Osseous regeneration in the presence of four common hemostatic agents. *Journal of Oral and Maxillofacial Surgery*, *50*(6), 608–612. https://doi.org/10.1016/0278-2391(92)90443-4

Gale, A. J. (2011). Continuing education course #2: current understanding of hemostasis. *Toxicologic Pathology*, *39*(1), 273–280. https://doi.org/10.1177/0192623310389474

Garattini, S., & Grignaschi, G. (2017). Animal testing is still the best way to find new treatments for patients. *European Journal of Internal Medicine*, *39*, 32–35. https://doi.org/10.1016/J.EJIM.2016.11.013

Goncalves, S., Chiossone-Kerdel, J. A., Bianco, A. S., Ercolino, J. M., & Hernandez-Rojas, J. (2015). Effect of absorbable gelatin sponge in the middle ear: In vitro and in vivo animal model. *Acta Oto-Laryngologica*, *135*(1), 14–25. https://doi.org/10.3109/00016489.2014.951455

Hajosch, R., Suckfuell, M., Oesser, S., Ahlers, M., Flechsenhar, K., & Schlosshauer, B. (2010). A novel gelatin sponge for accelerated hemostasis. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *94B*(2), 372–379. https://doi.org/10.1002/jbm.b.31663

Heher, P., Monforte, X., Keibl, C., Redl, H., Spazierer, D., & Gulle, H. (2018). Efficacy of Topical Hemostatic Agents: A Comparative Evaluation of Two Gelatin/Thrombin-Based Hemostatic Matrices in a Porcine Kidney Surgical Model AU - Slezak, Paul. *Journal of Investigative Surgery*, 1–8. https://doi.org/10.1080/08941939.2018.1447619

Hollville, E. (2013). Measuring apoptosis by microscopy and flow cytometry. *Methods*, *61*(2), 90–97. https://doi.org/10.1016/J.YMETH.2013.01.008

Ibarrola, J. L., Bjorenson, J. E., Peter Austin, B., & Gerstein, H. (1985). Osseous reactions to three hemostatic agents. *Journal of Endodontics*, *11*(2), 75–83. https://doi.org/10.1016/S0099-2399(85)80123-0

Imani, R., Rafienia, M., & Emami, S. H. (2013). Synthesis and characterization of glutaraldehyde-based crosslinked gelatin as a local hemostat sponge in surgery: an in vitro study. *Bio-Medical Materials and Engineering*, *23*(3), 211–224. https://doi.org/10.3233/BME-130745

Ji, J., & Barrett, E. J. (2015). Suspected intraoperative anaphylaxis to gelatin absorbable hemostatic sponge. *Anesthesia Progress*, *62*(1), 22–24. https://doi.org/10.2344/0003-3006-62.1.22

Kamoh, A., & Swantek, J. (2012). Hemostasis in Oral Surgery. *Dental Clinics of North America*, *56*(1), 17–23. https://doi.org/https://doi.org/10.1016/j.cden.2011.06.004

Kierszenbaum, A. L., Tres, L. L. (2016). *Histology and Cell Biology An Introduction to Pathology.* 4th edition. Elsevier, Philadelphia, USA. 978-0-323-31330-8

Kim, H. W., Knowles, J. C., & Kim, H. E. (2005). Hydroxyapatite and gelatin composite foams processed via novel freeze-drying and crosslinking for use as temporary hard tissue scaffolds. *Journal of Biomedical Materials Research - Part A*, *72*(2), 136–145. https://doi.org/10.1002/jbm.a.30168

Kriz, N., Rinder, C. S., & Rinder, H. M. (2009). Physiology of Hemostasis: With Relevance to Current and Future Laboratory Testing. *Clinics in Laboratory Medicine*, *29*(2), 159–174. https://doi.org/https://doi.org/10.1016/j.cll.2009.06.004

Kumar, M. P. S. (2016). Local hemostatic agents in the management of bleeding in oral surgery. *Asian Journal of Pharmaceutical and Clinical Research*, *9*(3), 35–41.

Kuo, Z.-K., Lai, P.-L., Toh, E. K.-W., Weng, C.-H., Tseng, H.-W., Chang, P.-Z., … Cheng, C.-M. (2016). Osteogenic differentiation of preosteoblasts on a hemostatic gelatin sponge. *Scientific Reports*, *6*, 32884. Retrieved from http://dx.doi.org/10.1038/srep32884

Kyffin, J. A., Sharma, P., Leedale, J., Colley, H. E., Murdoch, C., Mistry, P., & Webb, S. D. (2018). Impact of cell types and culture methods on the functionality of in vitro liver systems – A review of cell systems for hepatotoxicity assessment. *Toxicology in Vitro*, *48*, 262–275. https://doi.org/10.1016/J.TIV.2018.01.023

Lan, G., Lu, B., Wang, T., Wang, L., Chen, J., Yu, K., … Wu, D. (2015).

Chitosan/gelatin composite sponge is an absorbable surgical hemostatic agent. *Colloids and Surfaces B: Biointerfaces*, *136*, 1026–1034. https://doi.org/10.1016/j.colsurfb.2015.10.039

Lied, G. A., Lund, K. B., & Storaas, T. (2019). Intraoperative anaphylaxis to gelatin-based hemostatic agents: a case report. *Journal of Asthma and Allergy*, *12*, 163–167. https://doi.org/10.2147/jaa.s202784

Lupu, A. R., & Popescu, T. (2013). The noncellular reduction of MTT tetrazolium salt by TiO2 nanoparticles and its implications for cytotoxicity assays. *Toxicology in Vitro*, *27*(5), 1445–1450. https://doi.org/10.1016/J.TIV.2013.03.006

Maltaris, T., Kaya, H., Hoffmann, I., Mueller, A., Beckmann, M. W., & Dittrich, R. (2006). Comparison of xenografting in SCID mice and LIVE/DEAD assay as a predictor of the developmental potential of cryopreserved ovarian tissue. *In Vivo*, *20*(1), 11–16. Retrieved from https://pdfs.semanticscholar.org/e6f8/d3f1162f3532a1d6763fa6f20af969a11e6c.pdf

Minors, D. S. (2007). Haemostasis, blood platelets and coagulation. *Anaesthesia & Intensive Care Medicine*, *8*(5), 214–216. https://doi.org/10.1016/J.MPAIC.2007.02.008

Moscato, S., Mattii, L., D'Alessandro, D., Cascone, M. G., Lazzeri, L., Serino, L. P., … Bernardini, N. (2008). Interaction of human gingival fibroblasts with PVA/gelatine sponges. *Micron*, *39*(5), 569–579. https://doi.org/10.1016/j.micron.2007.06.016

Neuenschwander, P. F. (2006). COAGULATION CASCADE | Overview. In *Encyclopedia of Respiratory Medicine* (pp. 478–486). Academic Press. https://doi.org/10.1016/b0-12-370879-6/00081-8

Ormerod, M. G. (2002). Investigating the relationship between the cell cycle and apoptosis using flow cytometry. *Journal of Immunological Methods*, *265*(1–2), 73–80. https://doi.org/10.1016/S0022-1759(02)00071-6

Overbey, D. M., Jones, E. L., & Robinson, T. N. (2014). How Hemostatic Agents Interact With the Coagulation Cascade. *AORN Journal*, *100*(2), 148–159. https://doi.org/10.1016/J.AORN.2013.12.012

Padera, R. F. (2017). A perfect storm: Understanding hemostasis, coagulation and inflammation with artificial material. *Progress in Pediatric Cardiology*, *47*, 21–26. https://doi.org/https://doi.org/10.1016/j.ppedcard.2017.10.001

Pascua-Maestro, R., Corraliza-Gomez, M., Diez-Hermano, S., Perez-Segurado, C.,

Ganfornina, M. D., & Sanchez, D. (2018). The MTT-formazan assay: Complementary technical approaches and in vivo validation in Drosophila larvae. *Acta Histochemica*, *120*(3), 179–186. https://doi.org/10.1016/J.ACTHIS.2018.01.006

Periayah, M. H., Halim, A. S., & Mat Saad, A. Z. (2017). Mechanism Action of Platelets and Crucial Blood Coagulation Pathways in Hemostasis. *International Journal of Hematology-Oncology and Stem Cell Research*, *11*(4), 319–327. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/29340130

Peter, M., Ganesh, N., Selvamurugan, N., Nair, S. V., Furuike, T., Tamura, H., & Jayakumar, R. (2010). Preparation and characterization of chitosan-gelatin/nanohydroxyapatite composite scaffolds for tissue engineering applications. *Carbohydrate Polymers*, *80*(3), 687–694. https://doi.org/10.1016/j.carbpol.2009.11.050

Renati, S., Kaur, S., Kresak, J. L., Wicklund, M., & Malaty, I. (2017). Granulomatous meningitis secondary to Avitene (microfibrillar collagen). *Neurology. Clinical Practice*, *7*(5), 384–386. https://doi.org/10.1212/CPJ.0000000000000305

Rocha, A. L., Shirasu, B. K., Hayacibara, R. M., Magro-Filho, O., Zanoni, J. N., & Araújo, M. G. (2012). Clinical and histological evaluation of subepithelial connective tissue after collagen sponge implantation in the human palate. *Journal of Periodontal Research*, *47*(6), 758–765. https://doi.org/10.1111/j.1600-0765.2012.01491.x

Rohanizadeh, R., Swain, M. V., & Mason, R. S. (2008). Gelatin sponges (Gelfoam®) as a scaffold for osteoblasts. *Journal of Materials Science: Materials in Medicine*, *19*(3), 1173–1182. https://doi.org/10.1007/s10856-007-3154-y

Salgado, C. L., Grenho, L., Fernandes, M. H., Colaço, B. J., & Monteiro, F. J. (2016). Biodegradation, biocompatibility, and osteoconduction evaluation of collagen-nanohydroxyapatite cryogels for bone tissue regeneration. *Journal of Biomedical Materials Research Part A*, *104*(1), 57–70. https://doi.org/10.1002/jbm.a.35540

Seeley, R. R., Stephens, T. D., & Tate, P. (2011). *Anatomia e Fisiologia*.

Shander, A., Kaplan, L. J., Harris, M. T., Gross, I., Nagarsheth, N. P., Nemeth, J., … Ferraris, V. A. (2014). Topical hemostatic therapy in surgery: Bridging the knowledge and practice gap. *Journal of the American College of Surgeons*. https://doi.org/10.1016/j.jamcollsurg.2014.03.061

Sharma, U., Pal, D., & Prasad, R. (2014). Alkaline Phosphatase: An Overview. *Indian*

Journal of Clinical Biochemistry, *29*(3), 269–278. https://doi.org/10.1007/s12291-013-0408-y

Stepanenko, A. A., & Dmitrenko, V. V. (2015). Pitfalls of the MTT assay: Direct and off-target effects of inhibitors can result in over/underestimation of cell viability. *Gene*, *574*(2), 193–203. https://doi.org/10.1016/J.GENE.2015.08.009

Tang, Z., Chen, H., He, H., & Ma, C. (2019). Assays for alkaline phosphatase activity: Progress and prospects. *TrAC - Trends in Analytical Chemistry*, *113*, 32–43. https://doi.org/10.1016/j.trac.2019.01.019

Turpeinen, M. (2007). In vitro–in vivo extrapolation of hepatic clearance: Biological tools, scaling factors, model assumptions and correct concentrations AU - Pelkonen, O. *Xenobiotica*, *37*(10–11), 1066–1089. https://doi.org/10.1080/00498250701620726

Vezeau, P. J. (2016). Topical Hemostatic Agents: What the Oral and Maxillofacial Surgeon Needs to Know. *Oral and Maxillofacial Surgery Clinics of North America*, *28*(4), 523–532. https://doi.org/10.1016/J.COMS.2016.06.007

Wagner, W. R., Pachence, J. M., Ristich, J., & Johnson, P. C. (1996). Comparative in vitro analysis of topical hemostatic agents. *Journal of Surgical Research*, *66*(2), 100–108. https://doi.org/10.1006/jsre.1996.0379

Whu, S. W., Hung, K. C., Hsieh, K. H., Chen, C. H., Tsai, C. L., & Hsu, S. H. (2013). In vitro and in vivo evaluation of chitosan-gelatin scaffolds for cartilage tissue engineering. *Materials Science and Engineering C*, *33*(5), 2855–2863. https://doi.org/10.1016/j.msec.2013.03.003

Wiegand, C., Abel, M., Hipler, U. C., Elsner, P., Zieger, M., Kurz, J., … Stoppelkamp, S. (2019). Hemostatic wound dressings: Predicting their effects by in vitro tests. *Journal of Biomaterials Applications*, *33*(9), 1285–1297. https://doi.org/10.1177/0885328219831095

Yazdimamaghani, M., Vashaee, D., Assefa, S., Walker, K. J., Madihally, S. V, Köhler, G. A., & Tayebi, L. (2014). Hybrid macroporous gelatin/bioactive-glass/nanosilver scaffolds with controlled degradation behavior and antimicrobial activity for bone tissue engineering. *Journal of Biomedical Nanotechnology*, *10*(6), 911–931. https://doi.org/10.1166/jbn.2014.1783