UNIVERSIDADE CATÓLICA PORTUGUESA | INSTITUTO DE CIÊNCIAS DA SAÚDE

THE EFFECTS OF CHITOSAN IN THE HEALING PROCESS OF THE ORAL MUCOSA

Dissertação apresentada à Universidade Católica Portuguesa para obtenção do grau de Mestre em Medicina Dentária

Por:

Gonçalo João Pereira de Jesus

Viseu, Ano Letivo 2014/2015

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Sob a orientação do Professor Doutor Rui Amaral Mendes

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“The world lies in the hands of those that have the courage to dream and who take the risk of living out their dreams”

Paulo Coelho

Acknowledgments

To my supervisor, Professor Rui Amaral Mendes, for all the learning, share of knowledge, essential support and care during my journey up to here.

To my family, in special to my parents and brother for the trust, support and care, which were fundamental throughout my path until now.

To the Universidade Católica Portuguesa - Centro Regional das Beiras, to teachers, staff, with whom I had the pleasure to work during the last five years, for the knowledge transmitted.

To the Escola Superior de Biotecnologia da Universidade Catolica Portuguesa - Centro regional do Porto, mainly to Professor Manuela Pintado and her PhD Student Eduardo Costa, for being available and for having processed and provided us with the chitosan.

To all the patients in the study who accepted to collaborate in this investigation.

To my roommates, for the friendship, care and share during my academic journey.

To the Centro de Alto Rendimento do Fontelo, my training partners, who have accompanied me in my sport career for the last years.

To all my friends, my sincere regards.

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RESUMO

Introdução: A cicatrização de tecidos é um processo dinâmico, caracterizado por uma sequência de fases que se manifestam através de sinais e sintomas inflamatórios entre os quais o rubor, o edema, a dor e a perda de função[1].

O quitosano é um polímero natural derivado da desacetilação da quitina com a capacidade de facilitar o processo regeneração e cicatrização de tecidos[2]. Possui, também, outras propriedades fundamentais tais como a biocompatibilidade, biodegrabilidade, capacidade hemostática e antimicrobiana[3]. O quitosano é solúvel em meio ácido e possui uma boa afinidade com os tecidos, podendo ser usado na forma de gel[4,5].

Objetivos: O objetivo deste estudo experimental foi avaliar a influência do quitosano no processo de cicatrização da mucosa oral.

Materiais e Métodos: Este estudo experimental é do tipo *split-mouth design*. A população em estudo é constituída por 8 pacientes que foram submetidos a duas cirurgias, uma utilizando o quitosano e outra de controlo. A informação foi registada fotograficamente e recorrendo a uma ficha de controlo pós-operatório.

Resultados: Neste estudo, foram verificadas diferenças no processo de cicatrização da mucosa oral entre os casos de controlo e os casos em que foi utilizado o quitosano. Numa fase inicial, verificaram-se sinais e sintomas inflamatórios em ambos os casos, sendo que, naqueles em que foi administrado o quitosano, estes observaram-se num menor período de tempo. Nestes mesmos casos, também se observou uma diminuição da dor e ausência de hemorragia.

Conclusões: De acordo com a bibliografia e os resultados observados, o quitosano acelera a cicatrização através da atração e ativação dos neutrófilos e macrófagos e da estimulação da agiogénese[6]. Também devido às suas propriedades antimicrobiana e hemostática, conclui-se que o quitosano pode melhorar a qualidade pós-operatória dos pacientes, permitindo uma rápida cicatrização com menos complicações.

Palavras chave: Quitosano, cicatrização, mucosa oral, cirurgia oral.

ABSTRACT

Introduction: The healing process is a dynamic procedure, characterized by a sequence of phases and accompanied by some classical symptoms of inflammation such as redness, swelling, pain and loss of function[1].

Chitosan, a naturally derived polymer from the deacetylation of chitin, has appropriate proprieties for the use in several biomedical fields that contributes to the tissue healing and regeneration[2]. Proprieties such as biocompatibility, biodegradability, hemostatic, antimicrobial activity and the capacity to influence wound healing indicate that it could be applied in periodontal therapy such as the wound healing of the oral mucosa[3]. Chitosan is soluble in an acidic environment and exhibits excellent tissue affinity and consequently it could be used in hydrogel forms[4,5].

Aim: The aim of this experimental study is to assess possibilities of the chitosan to influence the healing process of the oral mucosa.

Materials and Methods: This experimental pilot study is a split-mouth design of 8 patients where the studied group was subjected to two oral surgeries, one with chitosan hydrogel into the socket and other without biomaterial. The data was collected by means of a questionnaire and photography.

Results: There were differences in the healing process of the oral mucosa in sites where chitosan was used compared to the control. Some classic signs of inflammation were observed in a short period of time where chitosan acts. In these cases it was observed a decrease in pain and absence of bleeding.

Conclusions: According to the references and the obtained results chitosan favoured the healing process by attraction and activation of neutrophils and macrophages and stimulation of angiogenesis[6]. Other proprieties such as hemostatic and antimicrobial activity also play an important role in wound healing. All these peculiar abilities make the chitosan essential in a postoperative quality of patients, allowing a rapid healing of the wound, with less complications.

Key-words: Chitosan, wound healing, oral mucosa, oral surgery

INTRODUCTION

1. INTRODUCTION

1.1. Wound Healing

A wound is a type of injury defined as damage or disruption to the normal anatomical structure and function which may extend from a simple break in the epithelial integrity of skin or oral mucosa or extending into tissue with damage to other structures such as muscles, nerves, parenchymal organs and even bone which can be caused by a drilling, trauma or even a cut due to a surgical incision[7].

Whatever the cause of the wound damage is, it will always disrupt the environment with it and a physiological response will start in a dynamic and complex process involving several events, including bleeding, coagulation, initiation of an acute inflammatory response, regeneration, migration and proliferation of connective tissue and parenchyma cells[7].

1.1.1. Phases of the Healing Process

Wound healing is a dynamic process initiated at the time of physical injury and it proceeds continuously throughout the repair process that is characterized by a sequence of orchestrated phases: hemostasis, inflammation, cellular migration and proliferation, protein synthesis, wound contraction and remodelling[6,8,9]. Besides, only inflammatory, proliferative and remodelling phase are mainly present due to the overlap of phases[8].

1.1.2. Coagulation and Hemostais Phase

The coagulation and hemostasis begins immediately after injury with the objective to prevent exsanguination and consequently protect the vascular system, keeping it intact, so that the function of the vital organs can remain unharmed. In a long term it supplies a matrix for invading cells that are needed in the other phases of healing[7].

When there is an injury to a tissue a neuronal reflex mechanism occurs that leads to a rapid constriction of vessels due to contraction of a vascular smooth muscle cells in the circular muscle layer. During the reparative

process the amount of fibrin is deposited at the wound site and through a dynamic balance between endothelial cells, thrombocytes, coagulation and fibrinolysis regulates hemostasis[7].

Together with hemostatic events a clot formation and a platelet aggregation occur in order to limit blood loss[7].

When blood spills into the site of injury contact with exposed collagen and other extracellular matrix components it results in a release of clotting factors from the platelets and the formation of a blood clot, composed by fibronectin, fibrin, vitronectin and thrombospondin. These events happen by an extrinsic and intrinsic pathways[7]

Besides hemostasis, the coagulum is also important for other next phases to provide a provisional matrix for cell migration. The cytoplasm of platelets contains α-granules with growth factors and cytokines (such as platelet derived growth factor [PDGF]), transforming growth factor-β (TGF-β), epidermal growth factor and others which promote a cascade of wound healing through activation and attraction of neutrophils and, later, macrophages, endothelial cells and fibroblasts[8].

The first phase involves infiltration of neutrophils and monocytes into the clot which clean the wounded area of necrotic tissue and bacteria and then monocytes release fibroblast in growth factor-2 and vascular endothelial initiating the formation of granulation tissue[7].

1.1.3. Inflammatory Phase

The inflammation stage begins immediately after injury and the main aim is the removal of debris, damage tissue, and bacteria by neutrophils and macrophages[10].

The first phase involves vasoconstriction, platelet aggregation at the injury site and then infiltration of leukocytes and the T lymphocytes to the wound area. This stage is commonly accompanied by some classical symptoms such as pain, redness and oedema[1]. In a more specific analysis T velnar et al divide this stage into two separate phases, an early inflammatory phase and a late inflammatory phase[7,8].

1.1.1.1. Early Inflammatory Phase

This stage starts during the late phase of coagulation with the aim of activating the complement cascade and initiating molecular events. In order to prevent the infection of the wound various chemoattractive agents including TGF-β, complement components such as C3a and C5a, and formylmethionyl peptides produced by bacteria and platelet products attract by neutrophils (within 24-36 of injury) to the wound site to destroy and remove bacteria, foreign particles and damaged tissue, through phagocytosis[7,8].

Intercellular adhesion molecule-1 (ICAM-1), an immunoglobulin cell expressed by several cell types including leukocytes and endothelial cells plays an important role in recruiting and retaining neutrophils, and it is involved in neutrophil migration into the gingival sulcus in periodontal tissue making it a key regulator of wound healing in the periodontium[11].

After all the contaminating bacteria has been removed, neutrophil activity gradually changes and they should be removed before the progression to the next phase of healing[8].

1.1.1.2. Late Inflammatory Phase

This phase corresponds approximately to 48-72h of the inflammatory phase which macrophages appear and continue the process of phagocytosis. They are attracted to the wound by chemoattractive agents as clotting factors, complement components, cytokines (PDGF, TGF-β, leukotriene B4 and platelet factor IV), elastin and collagen breakdown products[7].

Macrophages have a longer lifespan than neutrophils and they have an important role in late stages of the inflammatory response activating keratinocytes, fibroblasts and endothelial cells and providing an abundant reservoir of potent tissue growth factors (TGF-β, TGF-α, heparin binding epidermal growth factor, fibroblast growth factor [FGF], collagenase)[7].

Lymphocytes, last cells to enter in the wound site in the inflammatory phase, are attracted 72h after injury by the action of interleukin-1 (which has an important role in collagenase regulation), complement components and immunoglobulin G breakdown product[7].

1.1.4. Proliferative Phase

After several hours of injury, proliferative phase begins with the migration and proliferation of epithelial cells. This phase proceeds over the next 5-14 days and involves a new tissue formation, fibroblast migration and deposition of newly synthesized extracellular matrix (that replace the provisional network composed of fibrin and fibronectin), granulation and re-epithelialization and restores vascular network[3,6,7].

1.1.1.1. Fibroblast Migration

In the first three days after injury, fibroblasts and myofibroblast are stimulated and attracted into the wound by factors like TGF-β and PDGF (released by inflammatory cells and platelets). On the third day after injury fibroblasts appear in the wound, produce the matrix proteins hyaluronan, fibronectin, proteoglycans and type 1 and type 3 procollagen, which allow in the end of the first week an abundant accumulation of extracellular matrix to supports cell migration. After this, fibroblasts change their phenotype and differentiate in myofibroblasts (which contain thick actin bundles below the plasma membrane and actively extend pseudopodia, attaching to fibronectin and collagen in the extracellular matrix). This cells helps wound repair by contracting the edges of the wound using their smooth muscle type actin-myosin complex. After this, fibroblasts are eliminated by apoptosis[7].

1.1.1.2. Collagen synthesis

Collagen is synthesized by fibroblasts and it is an important element of tissues for their integrity and strength because it acts as a structural scaffold in tissues. It influences cell behaviour and their functions including cell shape differentiation, migration, synthesis of a number of proteins and the regulation of the quantity and quality of matrix deposition[7,12].

1.1.1.3 Angiogenisis and granulation tissue formation

In an initial situation there is no vascular supply in wound centre. The margins of the wound, which are constituted by viable tissue are perfused by

uninjured vessels which allows the angiogenic capillary sprouts to invade the fibrin / fibronectin-rich wound clot and within a few days to compose and organize a microvascular network through the granulation tissue[7].

The attraction of neutrophils and macrophages in the hemostatic phase increases the segregation of angiogenic factors such as FGF, vascular endothelial growth factor (VEGF), PDGF, angiogenin, TGF-α and TGF-β that promote angiogenesis and have an important role as modulator of cell growth and differentiation[7].

The actin network acts as a mechano-effector in the dynamic reorganization and in coordinator of cell migration. Cellular mobility requires three distinct actions: protrusion of the leading edge of the cell, adhesion of the leading edge and de-adhesion of the cell body and rear, and cytoskeletal contraction to pull the cell forward[7,12].

Contractile forces are transmitted through the integrin-cytoskeletal connections and they allow the cell to pull cytoplasm forward by generating traction to the substratum and these forces can be high enough to deform the extracellular matrix and rearrange it significantly[7].

The granulation tissue is composed by macrophages, proliferating fibroblasts, vascularized stroma, collagen matrix, fibrinogen, fibronectin and hyaluronic acid that replaces the fibrin based provisional matrix and with collagen accumulation it gradually matures to produce a scar[7].

In periodontology, this is an important phase because the population of gingival fibroblasts is stimulated to proliferate and several growth factors may promote cell division to reconstruction of injured gingival and periodontal tissues[3].

1.1.5. Remodelling phase

At last remodelling phase is characterized by a reorganization and contraction of newly formed matrix[8]. This phase is responsible for the formation and the development of new epithelium which involves remodelling of collagen from type III to I, the cellular activity reduces and the number of blood vessels in the wounded area regresses and decreases[7].

An acute wound is regulated by mechanisms that maintain a delicate balance between degradation and synthesis to accomplish normal healing. While there is a maturation of intracellular matrix, collagen bundles increase in diameter and hyaluronic acid and fibronectin are degraded. Neutrophils, macrophages and fibroblasts produce matrix metalloproteinase enzymes that are responsible for the degradation of collagen[7].

Initially the deposition of collagen bundles is disorganized however, in the final stages of the remodelling phase, the new collagen matrix becomes more oriented and cross linked due to the wound contraction which the underlying connective tissue shrinks in size and brings the wound margins closer together (owing to fibroblast interactions with the extracellular matrix)[7].

With time, metabolic activity at the wound site decreases along with the growth arrest of capillaries and decline of blood and the final results are a fully matured scar with decreased number of cells and blood vessels and a high tensile strength[7].

The acquired final strength of the tissue depends on the localization of the repair and its duration, but the strength achieve can never be the same compared to unwounded tissue, because collagen fibres may regain approximately 80% of the original strength[7].

1.2. Classification of wounds

In the literature we can find different classifications for the wounds. The healing time is an important parameter to consider in that we can classify wounds accordingly to three types: acute wounds, chronic wounds or complicated wounds. In the first case, which will be more frequent in our experimental work, wounds repair themselves and that proceed normally by following a timely and orderly healing pathway with the anatomical and functional restauration which the time course of healing usually ranges from 5 to days, or within 30 days. This type of wound may be the result of traumatic loss of tissue or a surgical procedure such as an operation for removing tumour or a tooth extraction[7].

Chronic wounds are characterized by failure in the normal healing process in which the wound is not repaired in an orderly and timely manner and the stages of healing process will be prolonged and repeated several times. Several factors such as infection, tissue hypoxia, necrosis, excess levels of inflammatory cytokines will result in a continuous state of inflammation that creates a sequence of tissue responses that together perpetuate a non-healing state[7].

Complicated wounds are defined as a combination of an infection and a tissue defect which poses a constant threat to the wound. Such wounds may result due to the traumatic or post-infectious aetiology, or a wide tissue resection[7].

Other criteria taken into account in wound classification include the degree of contamination (Aseptic wounds, contaminated wounds and septic wounds), aetiology (according to the trigger factor into contusions – abrasions, avulsions, lacerations, stab wounds, shot wounds and burns) and morphological characteristics[13].

Other classifications are established for wound healing accordingly to the type of closure: primary intention (when it's possible an approximation of wound margins or by placement and all layers are close), second intention (describe deep layers closed but superficial layers are left to heal from the inside out) or third intention (also referred to as delayed primary closure). An example of healing by primary intention is the wounds created and closed in

the operative room where the wound is usually healed at a minimum of time with minimal oedema, no local infection, without separation of the wound edges and with minimal scar formation[13].

1.2.1. Closure Technique

Primary closure technique is the complete closure of extraction socket by way of repositioning of the flaps by sutures aimed at wound healing by primary intention which avoid the contamination from the oral cavity . This technique prevents drainage of the socket, preserving vital coagulum that acts as a space-occupying agent[14].

Secondary closure technique involves healing of the socket by secondary intention. Egbor P. et al observed a significant reduction in post-operative swelling and trismus in subjects that had dressing of the sockets compared with those with primary closure but the effects on pain reduction were not statically significant[14].

One of the inclusion criteria for our study is the healing by primary intention in order to achieve the preservation of the biomaterial within the alveolus.

1.3. Chitin and Chitosan

Nowadays, multiple biomaterials are being investigated for wound healing whose function is to preserve hydration of the wound in order to optimize regeneration, protect against infection, guarantee uniform cell distribution, maintain cell viability and phenotype, and induce migration and proliferation of epithelial cells, fibroblasts and endothelial cells, while fulfilling prerequisites concerning structure and biocompatibility to promote wound healing[8,9]. This has increased the interest in chitosan to produce specialized biomaterials in wound healing.

1.3.1. Chitin

Chitin, a natural polysaccharide of major importance, is synthesized by an enormous number of living organisms and it is considered the most abundant polymer after cellulose. Chitin is a structural component in the exoskeleton of arthropods or in the cell walls of fungi and yeast and it is extracted from crustaceans like crab and shrimp shells by acid treatment. This treatment allows to dissolve calcium carbonate and then, by alkaline extraction, to solubilize proteins. By partial deacetylation of chitin, under alkaline conditions, we can obtain chitosan[4,15,16].

1.3.2. Chitosan

Chitosan, a linear biopolymer extracted from N-acetylated of chitin, is a cationic polysaccharide containing copolymers of glucosamine and its molecular structure comprises a linear backbone linked through glycosidic bonds with random copolymer of β-(1–4)-linked D-glucosamine and N-acetyl-D-lucosamine[5,15,17,18].

Glucosamine is the major component of chitosan and it's a natural substance produced in the body from glucose and it is involved in manufacture of glycosaminoglycan which forms cartilage tissue in the body and it is also present in tendons and ligaments[19].

Chitosan is a weak base insoluble in water but becomes soluble in aqueous acidic media[15,20]. Its insolubility in neutral or basic solutions occurs due its

slightly crystalline character but an acidic environment enables the free amino groups of chitosan to become protonated. The high positive charge of chitosan permits the formation of a polyelectrolyte complex hydrogel with polyanionic species in an acidic environment[4].

Tissue engineering has shown interest in chitosan to culture hepatocytes, fibroblasts and cartilage cells due to its scalloped structure and the ability to promote cell attachment and growth[10].

Chitosan has shown a minimal body reaction and may be applied in periodontal therapy for tissue-reconstruction purposes due to their ability to promote cell proliferation of human gingival fibroblasts and stimulate wound healing. It actives macrophages and mononuclear cell and induces the production of various growth factors that enhance wound healing and bone formation [4,21]. Chitosan membranes have been used to promote skin, tendon, bone and periodontal wound healing which allows a significant histological evidence of tissue regeneration reported but the precise role of chitosan in modulating cell-regenerative responses in periodontal cells remains to be studied[3].

No data on the carcinogenic potential of chitosan was found in literature and the N-acetylglucosamine, a constituent of chitosan, also showed no evidence of carcinogenicity when dosed at low levels in the diet of rats during 2 years[22].

A very small portion of the human population exhibits allergies to marine crustaceans but chitosan and chitin-derived products are shown to be safe and well tolerated in patients with crustacean allergy[23]. This allergy is caused by IgE antibodies to antigens in the flesh of the shellfish and not to the shell, so the use of chitosan is safe for patients with crustaceans allergy[24].

Chitosan has attracted great attention in dentistry because of its biological properties such as biocompatibility, biodegradability, bioadhesion, antimicrobial, antibacterial, hemostatic and non-toxic effect[25].

1.3.3. Properties

Biocompatible

In this area it's important to understand the definition of biocompatibility which according to The European Society for Biomaterial corresponds to the biomaterial's ability to perform a desired function with respect to a medical

therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy. This propriety allows the study of the chitosan like a material in interact with the surrounding environment, instead of being ignored by them[26].

The positive charges of chitosan usually provides a great interaction with cell membranes (which are negatively charged) due to ionic interchanges between the intercellular and extracellular medium. This means that highly deacetylated chitosan which has more number of positively charged amino groups has a naturally higher propensity for cell interaction[26].

The effects of chitosan have been the subject of several toxicological studies in a medical field and in general it showed its biocompatibility[18]. For example, in vitro studies showed that chitosan with a degree of deacetylation ranging from 53-97,5%DD have been biocompatible with human neonatal primary keratinocytes with fibroblasts grown (fully deacetylate chitosan does not serve as a substrate for enzymatic degradation by lysozyme)[18,24]. Similar results were seen with primary human fibroblasts, each cell line adhered to either side of the chitosan membranes, proliferating and remaining viable for up to 14 days[18].

Human volunteers have been orally taken levels of 6.75g of chitosan daily with

no adverse effects reported and chitosan were reported as safe[27,28].

In vivo studies in rats were performed and their results showed no toxicity at up to 2000mg/kg/day in gavage dosing in the diet during of up 3 months. Other studies, where higher oral doses of chitosan were administered in a short-term human trials, reported chitosan as safe with no significant clinical symptoms and no evidence of an allergic response[18].

In vitro degradation of chitosan with human lysozyme occurs mainly through depolymerisation by enzyme. Chitosan membrane for periodontal guided tissue regeneration was tested, and the results showed that it had no toxicity, with no evidence of an inflammatory reaction[18].

Silva et Al evaluate the serum-starved human gingival fibroblast when exposed to 100, 400 and 600ug/ml of chitosan particles and the results after

measurement of LDH into the cell culture as medium as an indicator of cell injury after 48h suggesting that this molecule did not induce a cytostatic effect[3].

Biodegradability

Chitosan has been reported as highly biodegradable mainly due to the fact that under physiological condition its molecular chains can be degraded by enzymes such as lysozyme, chitinase, N-acetyl-D-glucosaminidase and lipases. The products of its degradation do not raise any critical concern because these products are oligosaccharides that are either incorporated into glycosaminoglycan and glycoprotein metabolic pathways or easily excreted in urine directly [6,26,28]. Chitosan can be degraded by lysozyme through hydrolysis of acetylated residues and the degradation products are nontoxic[28,29].

On the one hand, many studies evaluated the degradation rate of chitosan preparations by different methods and conclude that degradation rate depend on their molecular weight and the preparation methods. For example, In vivo studies in rats showed that degradation occurred less rapidly as the degree of deacetylation become higher and we can conclude that the rate of degradation is inversely proportional to the degree of deacetylation [24,28].

On the other hand, if there is a rapid degradation of chitosan, it may result in an accumulation of amino sugars, inducing an inflammatory response and, hence, affecting chitosan biocompatibility[26].

Wound Healing Effect

Chitosan promotes granulation and organization which is beneficial for the wound healing[6]. It has been reported that chitosan plays an important role in the early phase of wound healing because it increases the infiltration of Polymorphonuclear cells (PMNs) which is followed by the collagen production of fibroblasts. The stimulation of fibroblasts ensures the production of interleukon-8 (IL-8) which plays an important role in chemotaxis and angiogenesis. It performs complement activation in an alternative manner because it increases the production of C5a which increases migration and adherence of neutrophils and monocytes to vessel walls. Chitosan also acts in

the phases of the macrophage activation, cytokine production, giant cell migration and stimulation of type IV collagen synthesis[30]. Chitosan has the capacity to foster adequate granulation tissue formation accompanied by angiogenesis and regular deposition of collagen fibres[6].

In vivo and in vitro studies have indicated that an enzymatic degradation of the chitosan oligomers in a wound environment will produce stimulatory effects on macrophages and the migratory activity is significantly enhanced[4].

Silva et Al concluded that chitosan is well tolerated by gingival fibroblasts and a synergistic response with some growth factors as Platelet-derived-growth factor (PDGF is one of the numerous growth factors that regulate cell growth and division and plays a significant role in angiogenesis) may stimulate cell proliferation in gingival fibroblasts[3].

Antimicrobial/antibacterial effect

Chitosan has been investigated as an antimicrobial material against a wide range of target organisms, including, bacteria, yeasts and fungi[20].

A number of studies demonstrated a bacterial effectiveness of chitosan on Gram-negative and Gram-positive bacteria. Oral bacteria includes *Streptococcus*, *Staphylococcus*, *Actinobacillus* and various anaerobes in particular bacteroides[18,20].

A key character from other polysacharoses is that chitosan can form a polycationic structure that can bound the aninons on bacterial surface. The interaction between the anionic components of bacteria and the positive charge of chitosan could weaken the barrier function of the outer membrane of microorganisms[18].

Rejane C. Goy et al proposed three mechanisms of interaction between chitosan and microorganisms: a) the ionic surface interaction resulting in a wall cell leakage, b) the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microrganisms and c) the formation of an external barrier chelating metal and provoking the suppression of essential to microbial growth. These mechanisms can occur simultaneously but with different intensities[20].

The effect of chitosan on *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*, were evaluated in vitro and the results showed an

antimicrobial action at a very low concentration. Moreover, chitosan selectively inhibited *streptococci mutans* adsorption to hydroxyapatite. Chitosan can also penetrate into biofilms formed by the pathogenic fungus as *Cryptococcus neoformans* and damage fungal cells resulting in a reducing in the metabolic activity of the biofilms and cell viability[18].

Hemostatic effect

A range of studies has shown that chitosan acts as a hemostatic agent which clot formation appears to be due to an interaction between the cell membrane of erythrocytes and chitosan, in absence of coagulation factors or platelets.

The mechanism of this action has not been clearly well-known but it seems to be a mechanism which the reactive amino groups of chitosan interacts with blood cells. The positive charge of chitosan and negative charge of the membranes of erythrocytes and platelets are attracted leading to platelet activation and thrombus formation[28,31,32].

In vivo tests in heparinized rats showed a putative capacity to induce clot formation in the absence of coagulation factors which allows to support the idea that chitosan can be very useful in patients with coagulopathies or those who are therapeutically anticoagulated[33].

Celox, HemCon and Quikclot are chitosan-containing medical devices marketed in Europe and the US for the treatment of bleeding and have been supported by evidence of reduce bleeding and enhanced hemostasis in experimental models[28].

Bioadhesive propriety

The term bioadhesion correspond a bond or an adhesive interaction established between two biological surfaces or a biological and synthetic surface[1].

Chitosan hydrogels have a high adhesive force due to adequate water absorption capacity with the cationic nature that will promote binding to the negative surface of the mucosa. It allows to have another important propriety: the intrinsic bioadhesive propriety of chitosan hydrogel that maintain an intimate contact with oral mucosa. It's important to refer that an appropriate

swelling is the key to guarantee adhesion, however, over hydration can form slipper non-adhesive hydrogels[10].

The positive charge of chitosan has the special feature of adhering to mucosal surfaces. The variety of forms of chitosan in terms of molecular weight and degree of deacetylation affect the solubility and mucoadhesivity. If the deacetylation degree decreases, the solubilisation becomes more difficult and the mucoadhesive capacity of the polymer also decrease due less positively charged amino groups available for the interaction with negatively charged residues of the mucus. This means that highly deacetylated chitosan has more mucoadhesion which is responsible for a more prolonged retention in the site of action or absorption[26].

Bone engineering

Scaffolds for bone tissue engineering must have a highly porous and interconnected pore structure because greater porosity and pore size usually means a higher surface area/volume ratio, thus favouring cell adhesion to the scaffold and promoting bone tissue regeneration[34].

Chitosan can also be a fundamental issue of concern in bone engineering because their adhesion represents a requisite for the matrix secretion by osteoblasts, which are anchorage dependent cells[35]. It exhibits an osteocondutive effect and potentiates the differentiation of osteoprogenitor cells that facilitate bone formation[4].

In periodontal study it was possible to verify that chitosan produced a radiographic benefit to bone relative to the control treatment[24].

In vivo tests in rats showed that grafting chitosan membranes into rat subcutaneous tissue maintained their shape and space for bone regeneration for 6 weeks. Their biodegradability and the degradation rate could be managed to fit into the schedule of remodelling of tissue regeneration[18]

On the other hand, Xiaoning He et al refers that pure chitosan scaffold is fragile and lacks the bioactivity to induce hard tissue formation, which can limits it application in bone tissue engineering[34].

Chitosan membranes were tested and showed that they induced more amount of new cementum and bone, and that cementoblast and osteoblast were densely arranged along the new bone surface[18].

1.4. Pre and Postoperative management techinques

Surgeons must remain careful to use methods to avoid complications during and after surgeries, recognizing and eliminating the most possible risk factors, and these procedures must begin before any surgery. In third molar surgery risk factors that would increase the possibility of developing problems include: nerve damage, pain, swelling and infection[36].

In order to prevent nerve damage the main risk factors were found to be: the skills and the experience of the operator, the type of impaction and the proximity of the tooth to the nerve which may be radiographically provided[36].

One of the most important priorities for the dental patient visiting clinicians is that they have a pain-free experience which can be obtained with the aid of medication. The use of local anaesthetics after the surgical intervention provide an environment which is more comfortable for the patients[36].

Corticosteroids are recognised for their ability to decrease discomfort with the reduce postoperative pain, trismus and facial oedema[36].

Paracetamol (acetaminophen) is consider safe in the treatment of postoperative pain relating third molar removal, having the benefit of reducing pain intensity at both 4 and 6 hours[36].

Ibuprofen has an superior effect in providing a pain relief comparing to the paracetamol but the combination of these two medications confirms a high-quality evidence in analgesia and suggests an superior effect around 8 hours.

The use of ice has yielded positive results with the benefits of reducing the discomfort experienced with postoperative swelling due the reduce of local temperature and cellular metabolism[36].

To help reduce the incidence of alveolar osteitis (dry socket), osteomyelitis and other unwanted complications, oral surgeons will often consider the use of antibiotics following removal of third molar teeth[36].

AIM

2. AIM

Chitosan is a natural polymer that has shown a minimal body reaction and may be applied in periodontal therapy for tissue-reconstruction. The interest in the use of this polysaccharide as a pharmaceutical excipient is due to biocompatibility, biodegradable, antimicrobial, non-toxic and hemostatic proprieties[28].

This experimental study will examine the potential of chitosan in the wound healing of the oral mucosa after impacted or submucous third molar surgery.

The information collected and the conclusions of this study will be relevant in dentistry in order to improve the healing of oral tissues, decreasing postoperative complications and increasing patient comfort.

This study aims to assess the effect of chitosan on the healing process of oral mucosa by evaluating postoperative symptoms after third molar extractions in open technique and with the chance to realize a primary wound closure in Oral Surgery in the University Clinic of the University Portuguese Catholic – Viseu.

In order to do so, the effects of chitosan in the healing process of the oral mucosa will be assessed by evaluating the signs of inflammation present after the surgeries, and the following hypothesis will be addressed:

H1: Chitosan allows an improvement in the healing process of the oral mucosa;

H2: Chitosan affects negatively the healing process of the oral mucosa;

H3: Chitosan doesn’t influence the healing process of the oral mucosa.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Location and type of study

This is an experimental pilot study type split-mouth design in which the application of the hydrogel of chitosan in a socket after extraction was evaluated and the wound healing of the oral mucosa for an extraction of contralateral tooth where chitosan was not applied in the socket was compared. All surgeries were performed by the same surgeon.

3.2. Duration and study period

The study was conducted between January 2015 and June 2015.

3.3. Population study

This study included 8 patients without systemic diseases, submucous or impacted mandibular teeth with indication for extraction and which will be possible to perform a primary wound closure.

3.4. Inclusion and exclusion criteria

3.4.1. Inclusion criteria

Patients with Indications for extraction;

Submucous or impacted mandibular teeth which will be possible to perform a primary wound closure;

Patients whose contralateral tooth is indicated for extraction;

Patients without systemic diseases;

Informed consent before conducting a healthcare intervention and the study;

3.4.2. Exclusion criteria

Teeth with indication for extraction but where a primary wound closure will not be possible to be performed;

Maxillary teeth indicated for extraction;

Patients with systemic diseases;

Pregnant and lactating patients;

Patients who can't attend controls.

3.5. Preparation of chitosan hydrogel

The chitosan hydrogel was obtained by dissolving chitosan with a molecular weight 10-50 Da and 90% degree of deacetylation in 1% acetic acid with 1 N NaOH. This hydrogel has a concentration of 3% and pH 5,6.

There was an especial care in dissolving chitosan because its granules did not have enough tissue biocompatibility and were determent to regular epithelization[33].

Our biomaterial was kept within the socket through primary wound closure.

3.6. Definition of the variables in study and strategy for the treatment of data

The following variables were studied at different stages of all healing process: redness, swelling, pain, loss of function, bleeding.

The data for each variables were recorded at the following periods: 24h, 48h, 72h, 1 week and 2 weeks.

A semi-quantitative analysis of some cardinal signs of the inflammation (redness, swelling, pain and loss of function) and bleeding was made.

Preoperative and postoperative redness and swelling were evaluated taking into account the initial colour of the oral mucosa (figure 1 and 2) and the shape of the face / mandibular angle (figure 3), respectively. Loss of function was evaluated by the presence / absence of trismus.

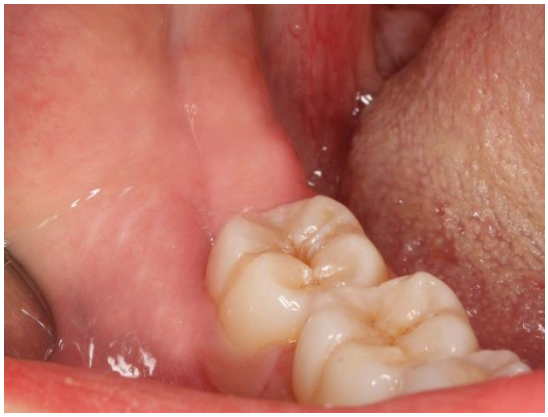


Figure 1: Preoperative redness.

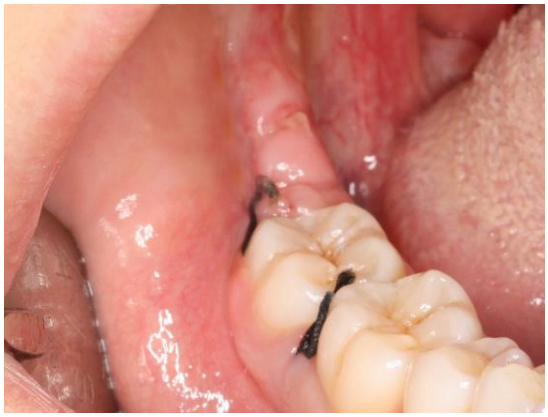


Figure 2: Postoperative redness.

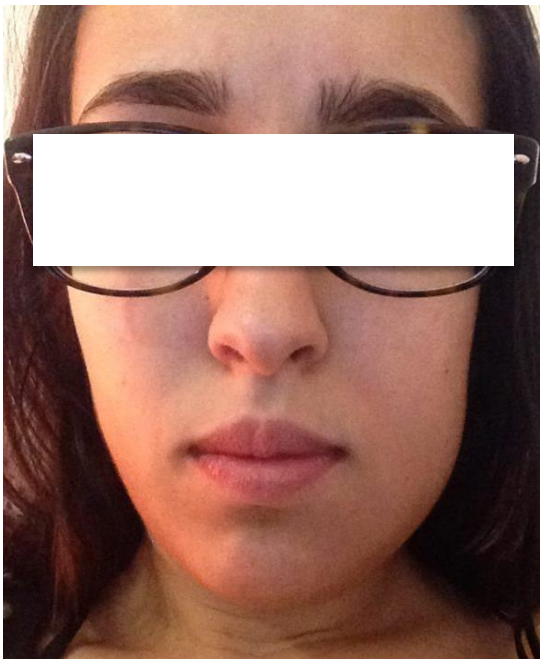


Figure 3: Postoperative swelling visible in the left side of the face of the patient after 3.8 teeth extraction.

A questionnaire was also given to evaluate the postoperative symptoms and medication. Pain was evaluated through a Visual Analogue Scale (VAS).

Data was recorded in tables accordingly to each day of follow up by the presence or absence of each sign or symptom.

Graphics were created for each variable in order to evaluate differences between chitosan and control, taking time variable into account.

3.7. Follow up and factors to consider

In this study a split-mouth design of 8 patients was used. The follow up was conducted at all healing process and it was performed photographically to be subsequently analysed. Medication and postoperative cares performed by the patient were also taken into account.

The medication used was the same for all patients: Amoxicillin + Clavulanic acid, Paracetamol and Ibuprofen. Deflazacort was prescribed only if osteotomy was performed.

Removal of suture was performed 1 week after the surgery.

3.8. Randomization of the study

Although the population is aware of the study, surgeries were performed without the patients to know what the case control would be or the case with application of the hydrogel of chitosan in a socket after extraction.

3.9. Clinical Settings

The surgical removal of third molars is one the most performed procedures in oral and maxillofacial surgery but the anatomic positions and angulations of these teeth commonly result in a high degree of dental impaction. Radiological individual anatomy, demographic aspects and operative factors are considered important variables to the determination of surgical difficulties and we adopted methods in our clinical practice in order to minimize the trans- and postoperative complication risks [37].

A structured and standardized surgical planning was devised where a pre-operative assessment of the patient's condition with a clinical examination and radiological assessment were conducted. This allowed us to obtain additional information about the associated anatomical structures and evaluate the possible intraoperative complications that may occur.

The benefits or indication of impacted tooth and third molar extraction include the relief and stop pain (that in some cases comes with unexplained origin), prevention of caries, periodontal disease, a single or multiple episodes of pericoronitis, facilitation of orthodontic treatment and orthognathic surgery and the prevention of pathological conditions such as dentigerous cyst formation and external root resorption of the adjacent teeth[38].

These surgical procedures can have some intraoperative and postoperative risks or complications. The most common complications are pain, swelling, and trismus. Besides, other risks that are described in the literature may occur as less frequent such as nerve damage leading to a temporary paraesthesia of the lips, tongue and chin, hemorrhage, alveolar osteitis (dry socket), soft tissue infection, fracture of the mandible or maxilla, damage to adjacent teeth sinus exposure or infection or anaesthetic complication.[38,39]

This experimental study was conducted in the university clinic of the University Portuguese Catholic – Viseu and the surgeries were always performed by the same surgeon.

3.10. Surgical instructions after third molar surgery

Third molar surgery is one of the most common surgical procedures performed in dental surgery and in order to minimize postoperative damage and improve quality of life of patients after surgery were given the following instructions:

- Apply a baggie filled with ice to the side of face where surgery was performed during the 12-24 hours after surgery;
- Performing a soft food diet on the day and day following surgery and avoid hard, crunchy foods after the third day till they can restore a normal diet.
- No smoking in the 3 days following surgery (however none of our patients were smokers);
- Do not consume alcoholic beverages in the 24 hours after surgery or while taking pain medications and antibiotic;
- Avoid spitting the day of the surgery;
- Do the cleaning near the surgical site smoothly;
- Do not use a straw for 3 days following surgery;
- Avoid strenuous exercise, jogging, or sporting activities that may cause an increase in blood pressure, in the next 48h after surgery;
- Keep the head elevated at a 30 - 45 degree angle (lying flat will increase the blood flow to the head, and cause more bleeding and swelling);
- Fill the prescription for a pain reliever and antibiotic at the pharmacy and take all medication as instructed: Anti-inflammatory (Ibuprofen, 600mg) 3x per day; Pain killers (Paracetamol, 1000mg) in case of S.O.S; Antibiotic (Amoxicilin + clavulanic acid, Clavamox® 875/125mg) 2x per day for 8 days; Corticosteroid (Deflazacort, 30mg) – 90-120mg in the first day and gradually reduce the dose to reach the minimum effective dose (3 mg/day).

RESULTS

4. RESULTS

In the following tables where the data of follow up was recorded, the symbol • signify presence and the - signify absence of the variable.

It was also reported an example of a follow up of a clinical case recorded photographically.

Patient 1

Radiological exam: orthopantomography

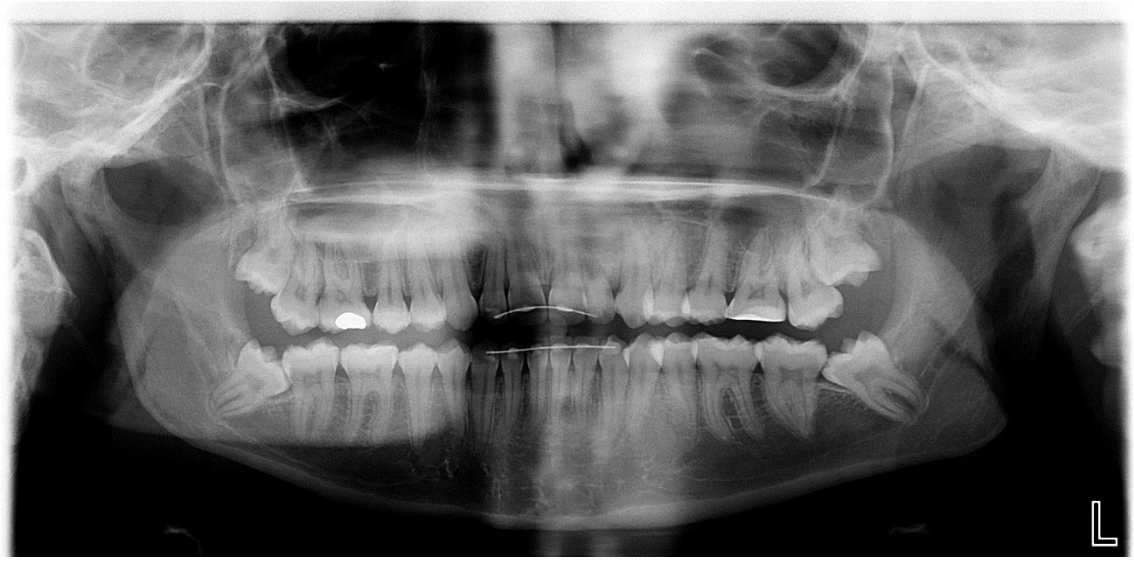


Figure 4: Patient 1 – Orthopantomography

Chitosan (Q): tooth 4.8

Pell and Gregory classification: I B

Intervention Time: <30 minutes

Suture: 3 simple points

Control (C): tooth 3.8

Pell and Gregory classification: I B

Intervention Time: <30 minutes

Suture: 3 simple points

Table 1: Patient 1 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	-	•	-	•	-	-	-	-	-	-
Redness	•	•	-	•	-	•	-	-	-	-
Swelling	-	•	-	•	-	•	-	-	-	-
Loss of function	-	•	-	•	-	•	-	-	-	-
Bleeding	-	•	-	•	-	-	-	-	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Patient 2

Radiological exam: orthopantomography

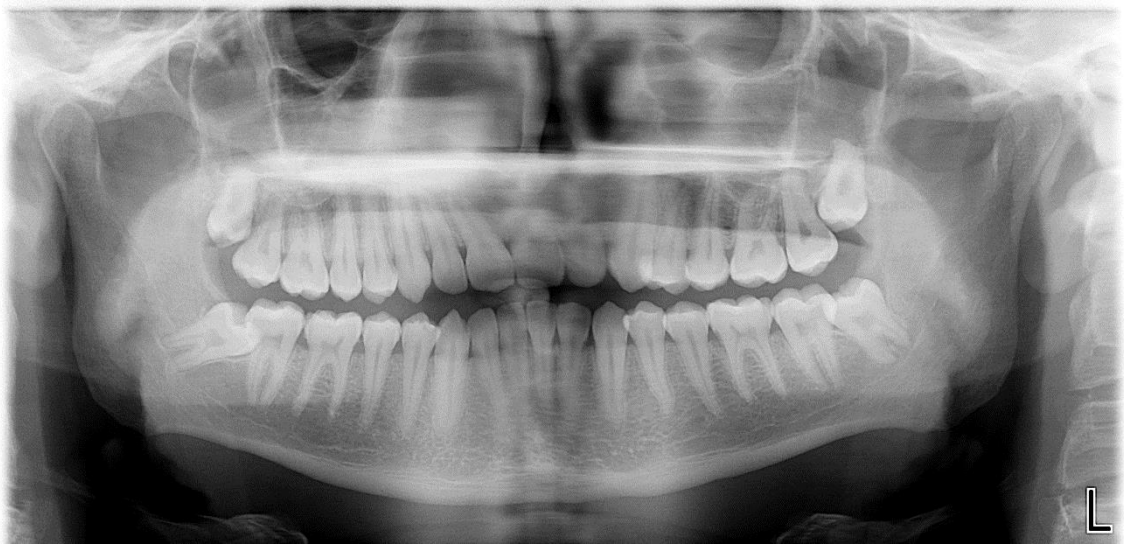


Figure 5: Patient 2 – Orthopantomography

Chitosan (Q): tooth 3.8

Pell and Gregory classification: II B

Intervention time: <30 minutes;

Suture: 3 simple points.

Control (C): Tooth 4.8 - It was performed osteotomy.

Pell and Gregory classification: II B

Intervention time: <30 minutes;

Suture: 3 simple points.

Table 2: Patient 2 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	-	-	-	-	-	-	-	-	-	-
Redness	•	•	•	•	•	•	-	•	-	•
Swelling	•	•	-	•	-	•	-	-	-	-
Loss of function	•	•	-	•	-	-	-	-	-	-
Bleeding	-	•	-	•	-	•	-	-	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Patient 3

Radiological exam: orthopantomography

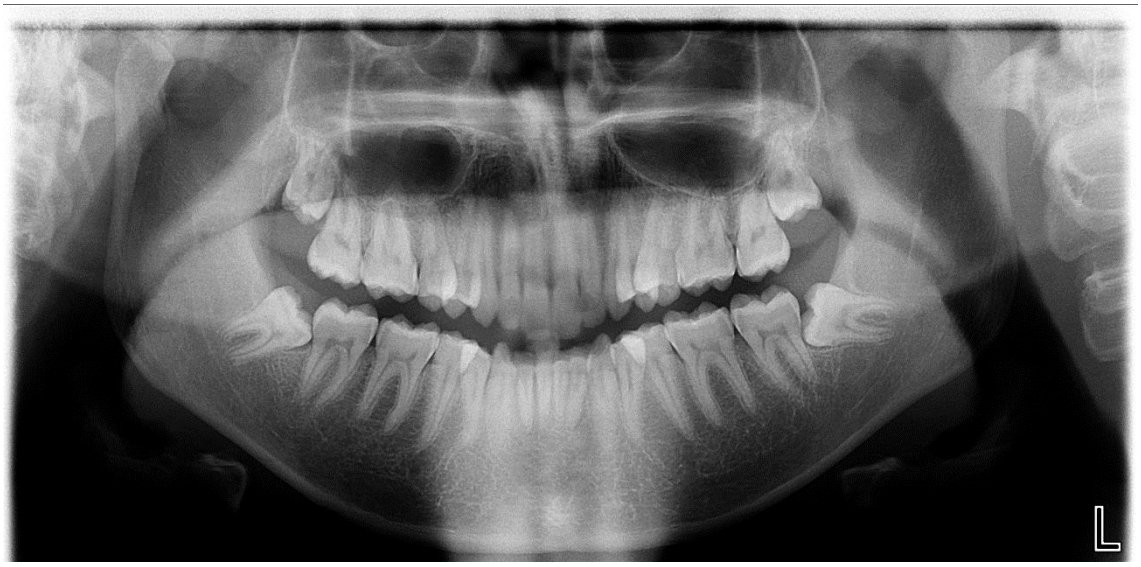


Figure 6: Patient 3 – Orthopantomography

Chitosan (Q): tooth 4.8 - It was performed osteotomy.

Pell and Gregory classification: I B

Intervention time: <30 minutes;

Suture: 3 simple points.

Control (C): Tooth 3.8

Pell and Gregory classification: II B

Intervention Time: <30 minutes;

Suture: 3 simple points

Table 3: Patient 3 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	-	-	-	-	-	-	-	-	-	-
Redness	•	•	-	•	-	•	-	•	-	-
Swelling	-	-	-	-		-	-	-	-	-
Loss of function	-	-	-	-	-	-	-	-	-	-
Bleeding	-	•	-	•	-	-	-	-	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Patient 4

Radiological exam: orthopantomography

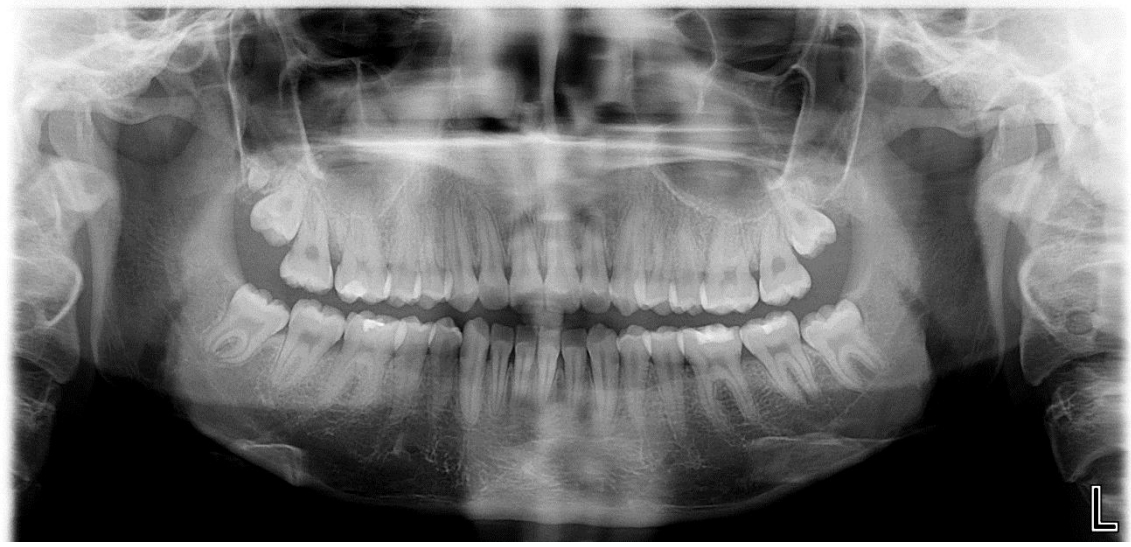


Figure 7: Patient 4 – Orthopantomography

Chitosan (Q): tooth 4.8

Pell and Gregory classification: I B;

Intervention Time: <30 minutes;

Suture: 4 simple points.

Control (C): Tooth 3.8

Pell and Gregory classification: I B;

Intervention Time: <30 minutes;

Suture: 3 simple points.

Table 4: Patient 4 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	-	•	-	•	-	•	-	-	-	-
Redness	•	•	•	•	•	•	-	•	-	•
Swelling	•	•	-	•		•		•		
Loss of function	-	•	-	•	-	•	-	•	-	-
Bleeding	-	•	-	-	-	-	-	-	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Patient 5:

Radiological exam: orthopantomography

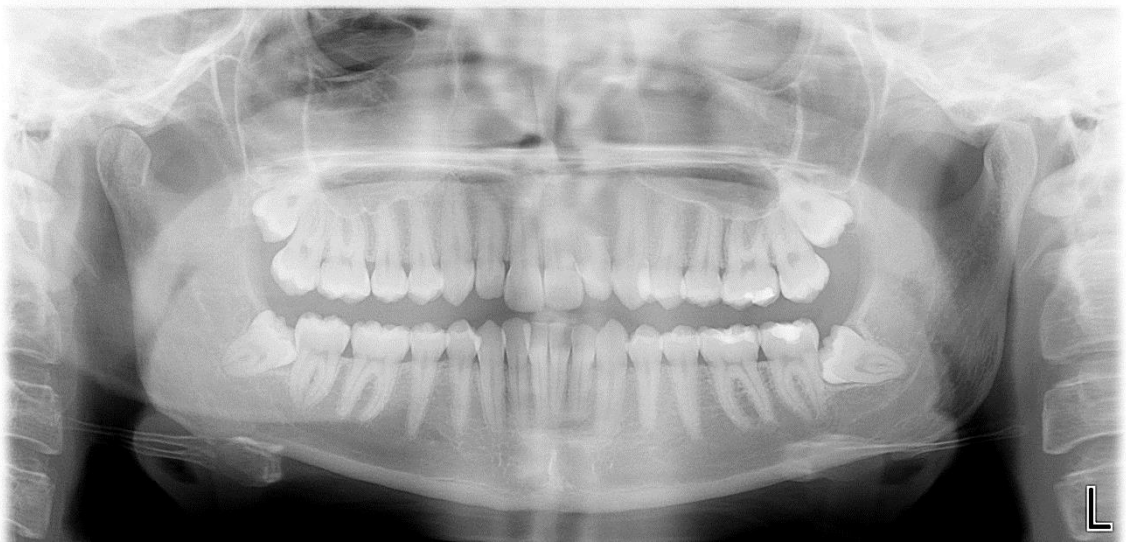


Figure 8: Patient 5 – Orthopantomography

Chitosan (Q): tooth 3.8 - It was performed osteotomy

Pell and Gregory classification: II B;

Intervention Time: 30 - 60 minutes.

Suture: 3 simple points.

Control (C): tooth 4.8

Pell and Gregory classification: I B;

Intervention Time: <30 minutes.

Suture: 3 simple points.

Table 5: Patient 5 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	•	-	-	-	-	-	-	-	-	-
Redness	•	•	•	•	-	•	-	-	-	-
Swelling	•	•	•	•	•					
Loss of function	•	•	•	-	•	-	-	-	-	-
Bleeding	-	•	-	-	-	-	-	-	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Patient 6:

Radiological exam: orthopantomography

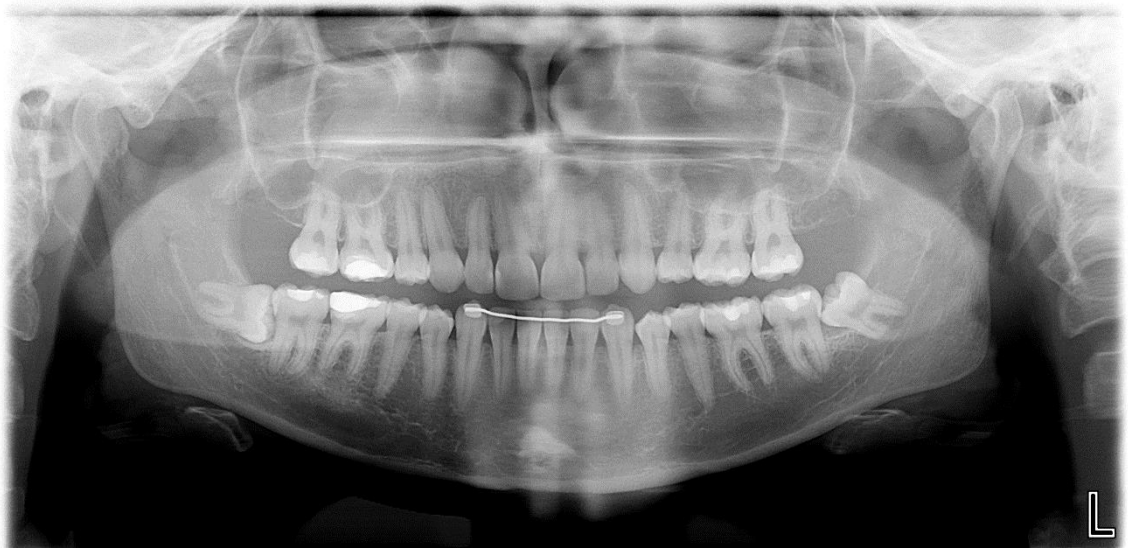


Figure 9: Patient 6 – Orthopantomography

Chitosan (Q): tooth 3.8

- Pell and Gregory classification: I B
- Intervention Time: <30 minutes;
- Suture: 3 simple points.

Control (C): tooth 4.8 - It was performed osteotomy;

- Pell and Gregory classification: II B
- Intervention Time: 30 - 60 minutes;
- Suture: 3 simple points.

Table 6: Patient 6 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	-	•	-	•	-	-	-	-	-	-
Redness	•	•	-	•	-	•	-	-	-	-
Swelling	•	•	•	•	-	-	-	-		
Loss of function	-	•		•		•		•		-
Bleeding	-	-	-	-	-	-	-	-	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Patient 7

Radiological exam: orthopantomography

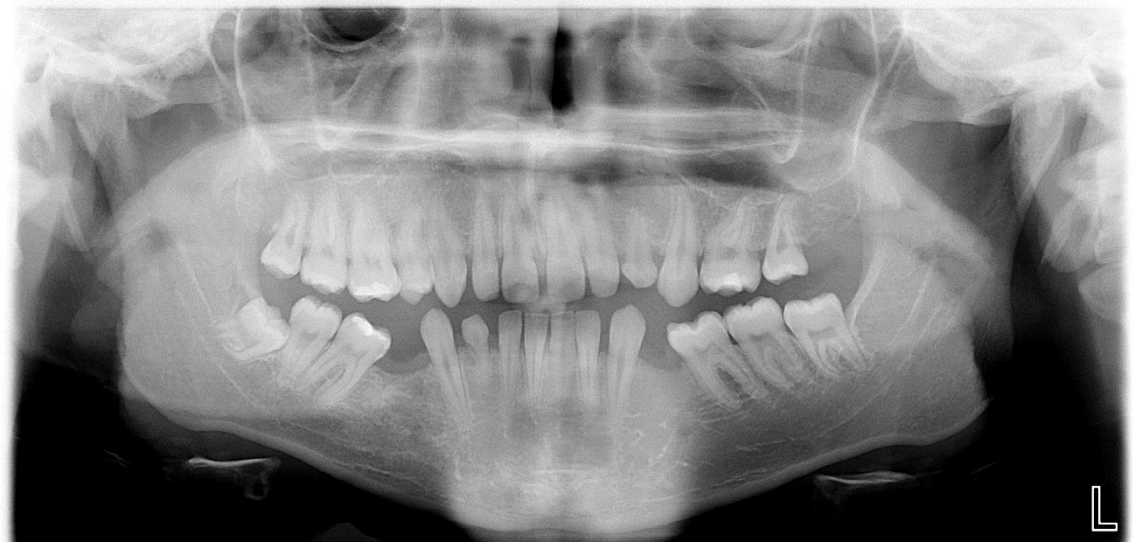


Figure 10: Patient 7 – Orthopantomography

Chitosan (Q): 4.8

Pell and Gregory classification: II B

Intervention Time: <30 minutes;

Suture: 3 simple points.

Control (C): 3.8

Pell and Gregory classification; I B;

Intervention Time: <30 minutes;

Suture: 3 simple points.

Table 7: Patient 7 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	-	-	-	-	-	-	-	-	-	-
Redness	•	•	•	•	-	•	-	•		
Swelling	-	-	-	-	-	-	-	-	-	-
Loss of function	-	-	-	-	-	-	-	-	-	-
Bleeding	-	•	-	•	-	-	-	-	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Patient 8

Radiological exam: orthopantomography

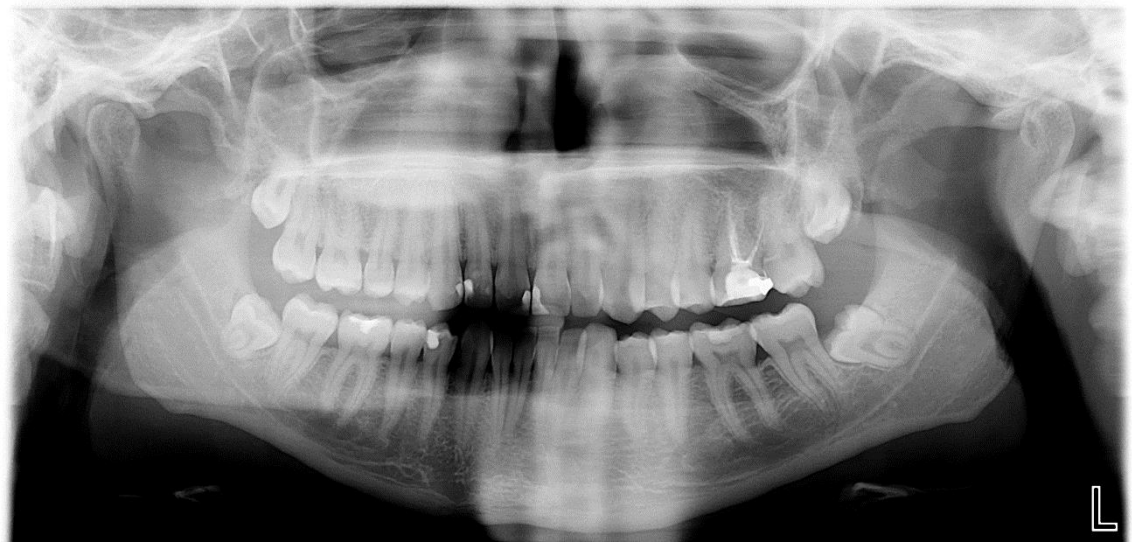


Figure 11: Patient 8 – Orthopantomography

Chitosan (Q): tooth 4.8

Pell and Gregory classification: II B;

Intervention Time: <30 minutes;

Suture: 3 simple points.

Control (C): tooth 3.8

Pell and Gregory classification: I B;

Intervention Time: <30 minutes;

Suture: 3 simple points.

Table 8: Patient 8 - Postoperative evaluation

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain	-	•	-	-	-	-	-	-	-	-
Redness	•	•	•	•	•	•	-	•	-	-
Swelling	-	•	-	•	-	-	-		-	
Loss of function	•	•	-	•	-	•	-	-	-	-
Bleeding	-	•	-	•	-	•	-	•	-	-
	Q	C	Q	C	Q	C	Q	C	Q	C

Table 9: All results of the postoperative evaluation

Patients / Variables	Follow up	1		2		3		4		5		6		7		8	
		Q	C	Q	C	Q	C	Q	C	Q	C	Q	C	Q	C	Q	C
Pain	24h	-	•	-	-	-	-	-	•	•	-	-	•	-	-	-	•
	48h	-	•	-	-	-	-	-	•	-	-	-	•	-	-	-	-
	72h	-	-	-	-	-	-	-	•	-	-	-	-	-	-	-	-
	1 week	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2 Weeks	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Redness	24h	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	48h	-	•	•	•	-	•	•	•	•	•	-	•	•	•	•	•
	72h	-	•	•	•	-	•	•	•	-	•	-	•	-	•	•	•
	1 week	-	-	-	•	-	•	-	•	-	-	-	-	-	•	-	•
	2 Weeks	-	-	-	•	-	-	-	•	-	-	-	-	-	-	-	-
Swelling	24h	-	•	•	•	-	-	•	•	•	•	•	•	-	-	-	•
	48h	-	•	-	•	-	-	-	•	•	•	•	•	-	-	-	•
	72h	-	•	-	•	-	-	-	•	•	-	-	-	-	-	-	-
	1 week	-	-	-	-	-	-	-	•	-	-	-	-	-	-	-	-
	2 Weeks	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of function	24h	-	•	•	•	-	-	-	•	•	•	-	•	-	-	•	•
	48h	-	•	-	•	-	-	-	•	•	-	-	•	-	-	-	•
	72h	-	•	-	-	-	-	-	•	•	-	-	•	-	-	-	•
	1 week	-	-	-	-	-	-	-	•	-	-	-	•	-	-	-	-
	2 Weeks	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bleeding	24h	-	•	-	•	-	•	-	•	-	•	-	-	-	•	-	•
	48h	-	•	-	•	-	•	-	-	-	-	-	-	-	•	-	•
	72h	-	-	-	•	-	-	-	-	-	-	-	-	-	-	-	•
	1 week	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	•
	2 Weeks	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.1. A case example of follow up

Follow up: 24h

Chitosan

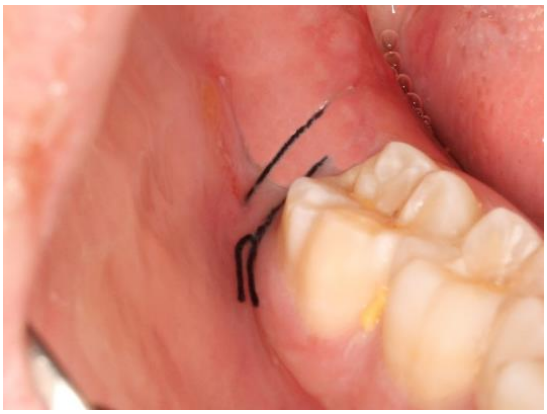


Figure 12: Chitosan, day 1

Control

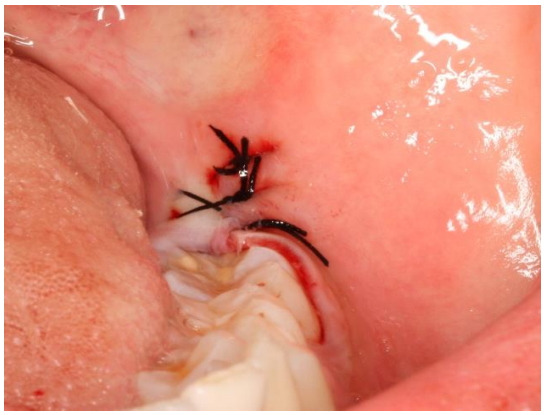


Figure 13: Control, day 1

Follow up: 48h

Chitosan

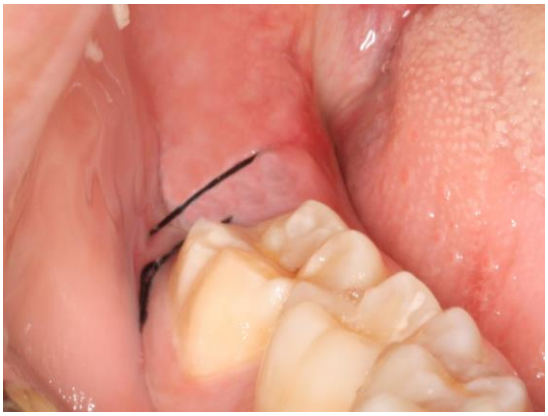


Figure 14: Chitosan, day 2

Control

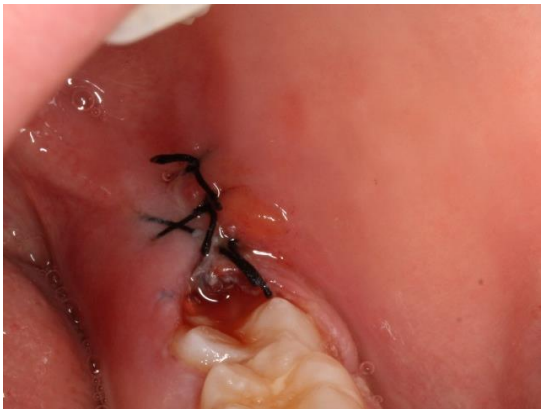


Figure 15: Control, day 2

Follow up: 72h

Chitosan

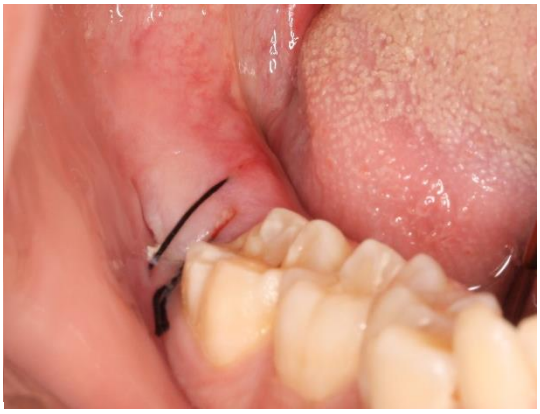


Figure 16: Chitosan, day 3

Control

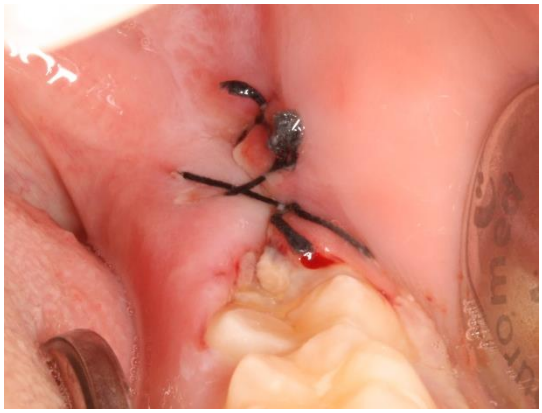


Figure 17: Control, day 3

Follow up: 1 week

Chitosan

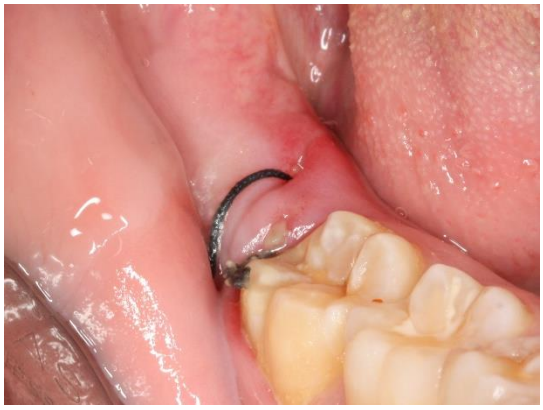


Figure 18: Chitosan, 1 week

Control

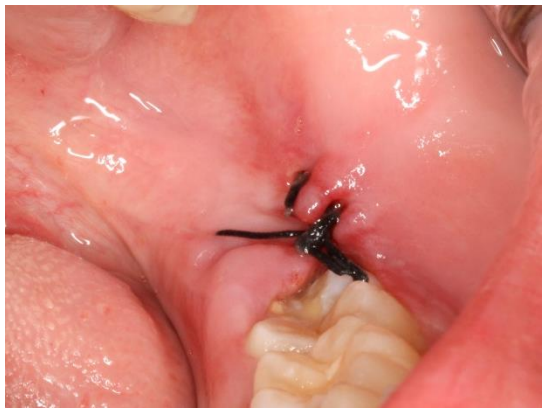


Figure 19: Control,1 week

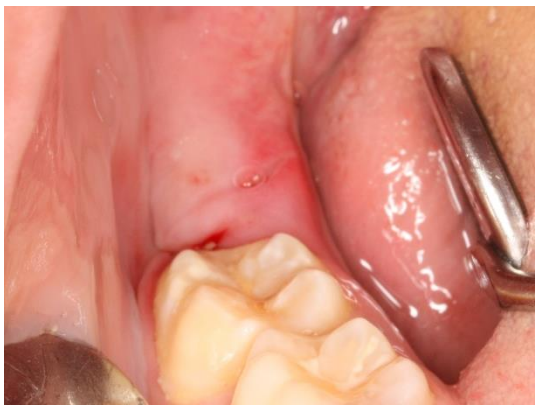


Figure 20: Chitosan, after removing stitches

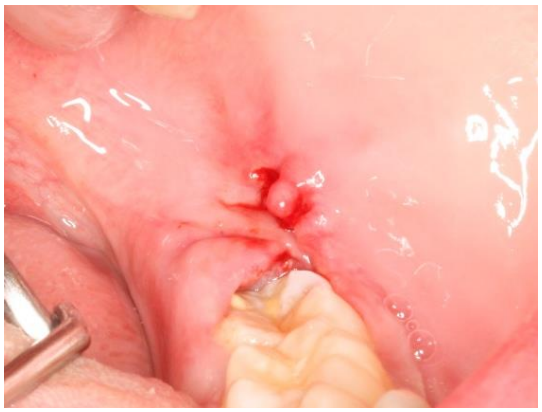


Figure 21: Control, after removing stitches

Follow up: 2 weeks

Chitosan

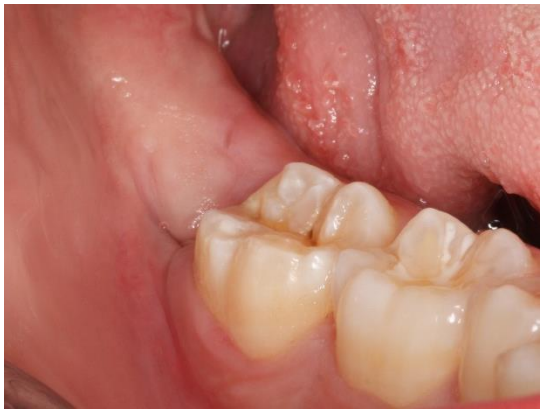


Figure 22: Chitosan, 2 weeks

Control

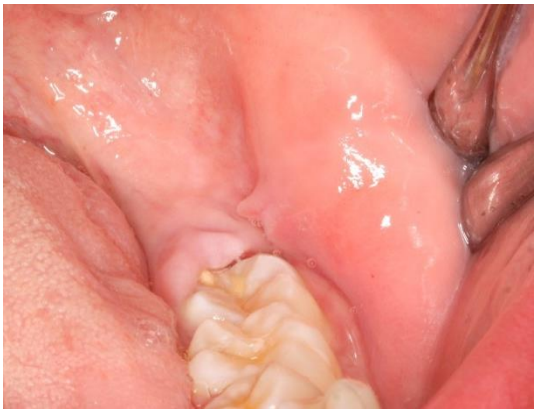


Figure 23: Control, 2 weeks

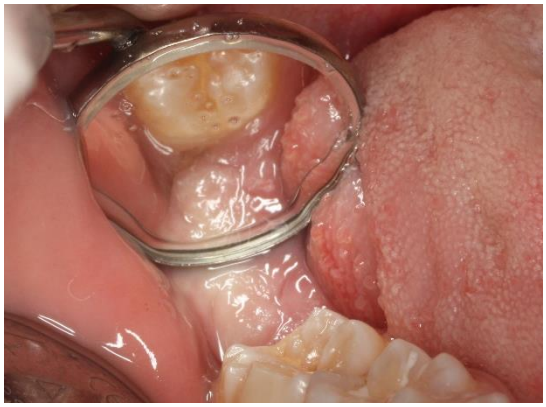


Figure 24: Chitosan, 2 weeks

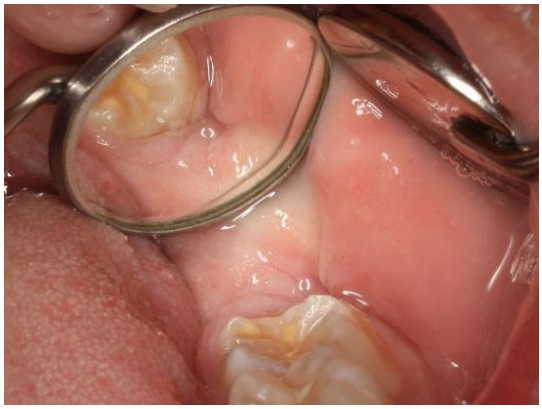


Figure 25: Control, 2 weeks

Table 10: Pain in Chitosan vs Control

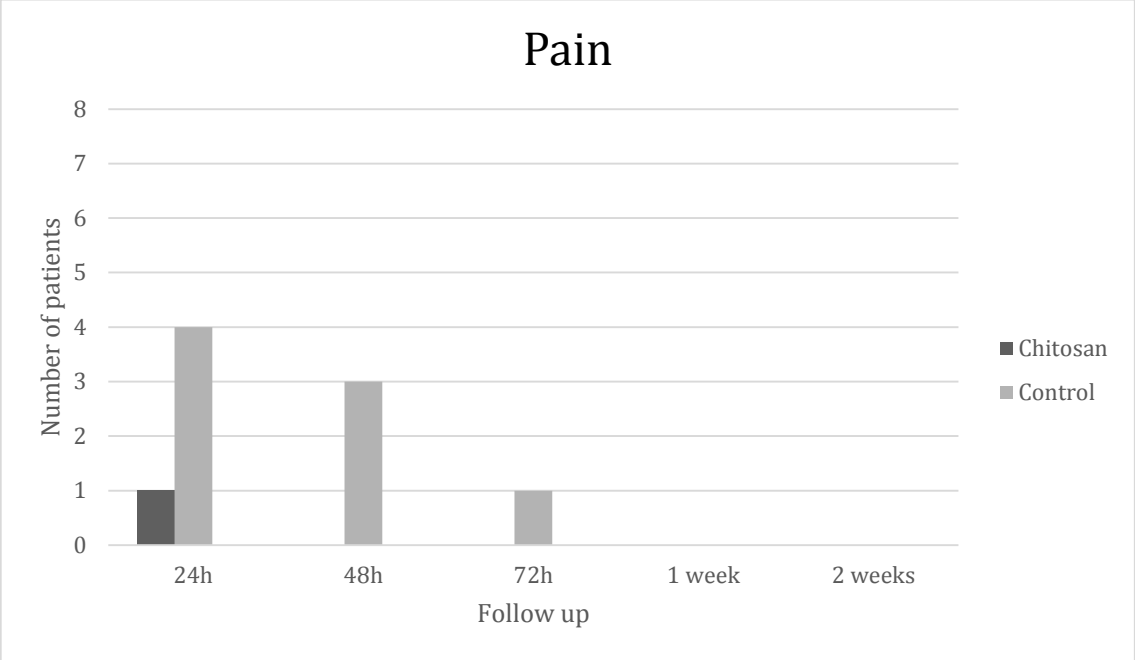


Table 11: Redness in Chitosan vs Control

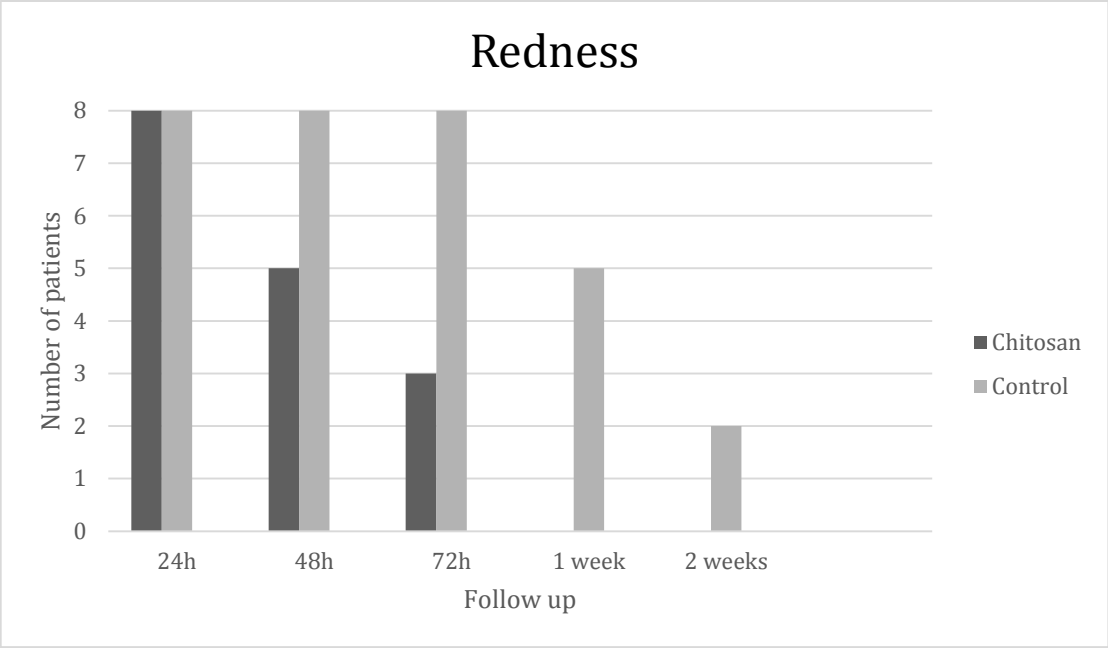


Table 12: Swelling in Chitosan vs Control

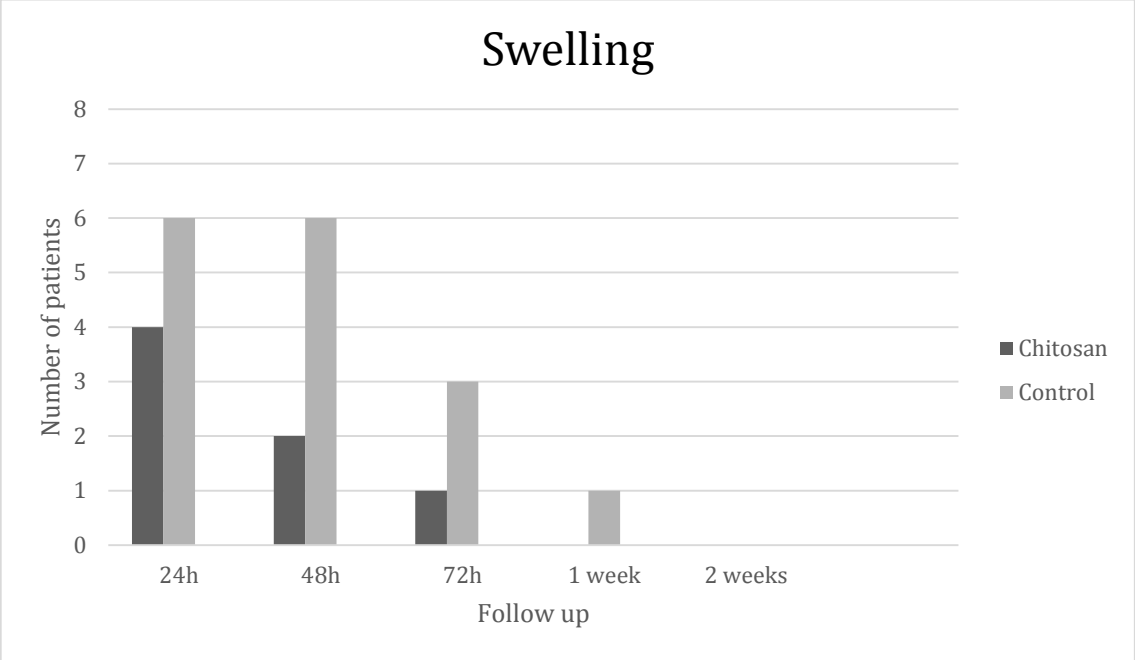


Table 13: Loss of function in Chitosan vs Control

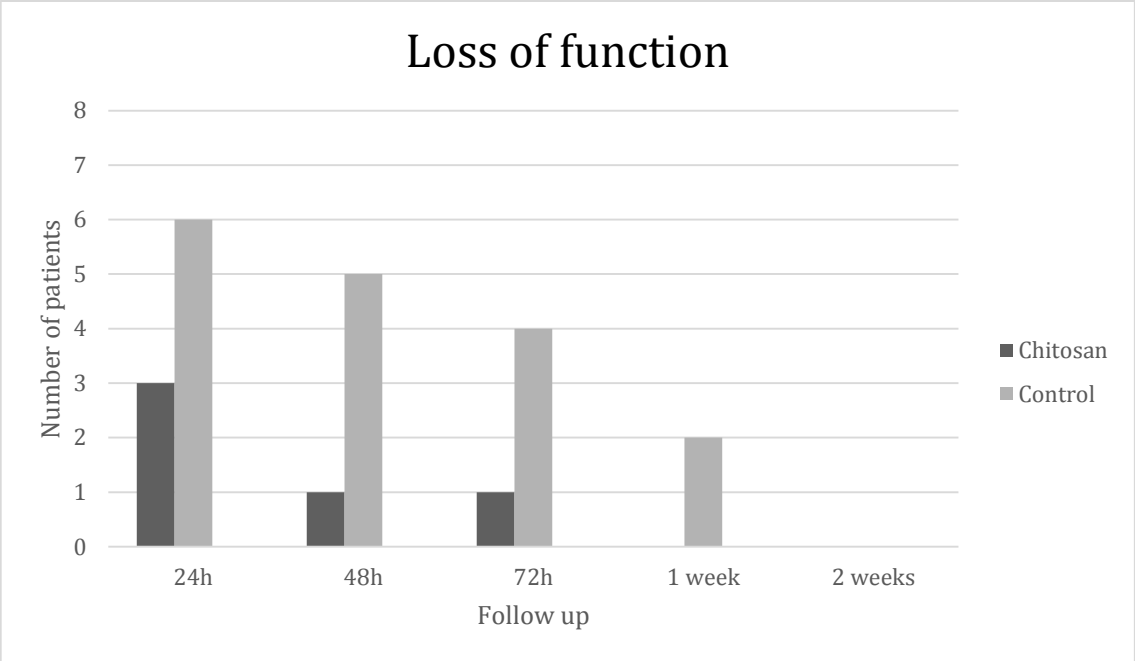
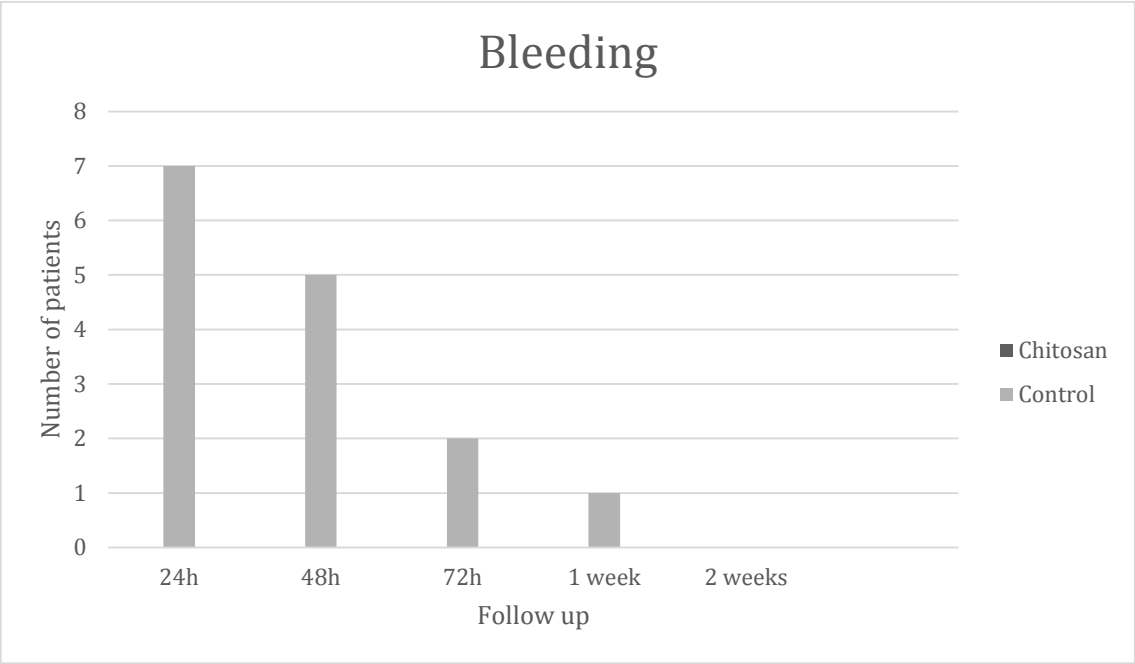


Table 14: Bleeding in Chitosan vs Control



DISCUSSION

5. DISCUSSION

The ideal biomaterial for wound healing needs to be biocompatible, ensure that the wound remains moist with exudates, but not macerated, free of infection, guarantee uniform cell distribution, maintain cell viability and phenotype and should induce migration and proliferation of epithelial cells, fibroblasts and endothelial cells as well as the synthesis of extracellular matrix components for wound repair[9].

Chitosan is a linear copolymer derived from crustacean's exoskeletons such as shrimp and crab that has been largely investigated due to its favourable proprieties in medical areas. The positive charge of chitosan is soluble in a low pH environment and it has an important capacity to promote the wound healing process due to their biocompatible, biodegradable, hemostatic and antibacterial abilities. All these factors make chitosan hydrogel a highly desirable product[4,8,40].

In this pilot study, a total of 8 patients received surgical extraction of impacted or submucous third molar by the same surgeon with the aim of evaluating the effects of chitosan in the healing process of the oral mucosa as compared with a contralateral tooth extraction which didn't use chitosan.

When the data of redness (Table 11) was analysed we found out that 24 hours after each surgery all patients had redness in the surgical area. The differences begin to emerge in the next days of follow up. In the cases control all patients showed redness at 48h and 72h. One week after surgeries the percentage of patients with redness decreased to 62.5% and in the second week to 25%. In surgeries where chitosan was used it was observed a progressive decrease in the percentage of patients with redness: after 48h and 72h the percentage was 62.5% and 37.5%, respectively. One week later there wasn't redness in the surgical area. After 2 weeks of follow up the visual result of healing of the oral tissues was similar in both cases.

This can be explained by the ability of chitosan to play an important role in the early phase of wound healing and increase angiogenesis. Chitosan attracts and activates neutrophils and macrophages and enhances the expression of cytokines and growth factors[4,6]. It also facilitates the wound healing by stimulating granulation tissue formation or re-epithelialization[6].

To what regards the swelling, pain and loss of function (table 10, 12, 13), we also verified a slight decrease in cases where the chitosan was used. It should be noted that in most cases where swelling and loss of function have occured, both corresponded to the cases in which osteotomy has been performed, even if performed in a minimally invasive way. The increase of surgical intervention time was found to lead to increase pain, swelling and trismus (which correspond to loss of function) following lower third molar surgery[14].

A possible initial slightly raised pain scores in sites with chitosan could be explained by the presence of acid acetic until it is fully dissolved in oral fluids[31].

The hydrophilic surface of chitosan promotes cell adhesion and proliferation[34]. Platelets adhere and aggregate on chitosan which promote hemostasis. This supports the previous finding that chitosan reduces the inflammatory response, improving the inflammation stage and consequently proliferation will increase and enhanced collagen deposition will occur during remodelling which accelerate wound healing[41]. Chitosan improves the tensile strength of wound by speeding up the fibroblastic synthesis of collagen in the first few days of wound healing[42]. Because of its bioadhesive properties, it's expected that chitosan remains for prolonged time on the application site[25].

In vitro assays showed that chitosan can stimulate the proliferation of human periodontal ligament cells and recruit vascular tissue growth which provide considerable evidence that chitosan helps the regeneration of periodontal cells[18]. Silva et al assessed whether chitosan is able to stimulate cell proliferation and if it collaborates with other growth factors such as PDGF to exert is mitogenic effect. They observed that chitosan stimulates cell viability and promotes cell proliferation in human gingival fibroblasts[3]. Moreover, periodontal ligament in the presence of chitosan presented more regular pattern and a denser fibre arrangement than those in the surgical control group[18].

Histological observations in wound healing experiments using a mouse model showed that wounds treated with chitosan hydrogel have an advanced granulation tissue formation and epithelialization compared to the control. The

application of chitosan hydrogel onto an open wound induces significant wound contraction and accelerates the wound closure and healing[43].

The role of chitosan in wound healing was supported by studies that had shown that chitosan amino groups are recognised by the immune system which induce migration of inflammatory cells and fibroblasts to the wound area, being activated to produce multiple cytokines. Fibroblasts are stimulated by chitosan molecules to secrete IL-8 and other cytokines, which in turn could induce angiogenesis, fibrosis and epithelialization[28,43].

Duygu B. et al evaluate the effect of chitosan gel comparing with its combination with collagen membrane and with flap alone on periodontal regeneration applied into the intraosseous lesions. They conclude that all treatment modalities provide improvements in clinical measurements of tissue regeneration. They also observed no inflammatory reaction following the application of chitosan in gel form[25].

The antimicrobial activity of chitosan also promotes the repair of damaged tissue preventing infection of the wound[6,41,42]. Chitosan increases permeability of the inner and outer membranes and ultimately disrupt the bacterial cell membranes, releasing their contents. Chitosan provides an antibacterial barrier against a wide range of Gram-positive and Gram-negative organisms, including such as methicillin-resistant *Staphylococcus*, mancomycin-resistant *enterococcus* and *Acinetobacter baumannii*[31].

Chitosan also has the ability to impair the colonization of the tooth surface by *Streptococcus mutans* which prevents a possible infection after surgery[25].

Chun Xu et al studied chitosan membranes in animal models and they conclude that they are cheaper and due to their property of bacteriostatisis it may reduce the bacteria contamination and benefit periodontal tissue regeneration[18].

Chitosan was also used in veterinary medicine because of its ability to accelerate the healing process and due to their antibacterial property. In vivo and in vitro studies have indicated that enzymatic degradation of chitosan oligomers origin a wound environment that produces a stimulatory effect on macrophages[35].

The hemostatic activity is an important phase in the early treatment of an injury[6]. In our experimental study, on the cases control 7 of the 8 patients

(87.5%) bled on the following day after surgery, 5 patients (62.5%) continued to bleed past 48 hours, 2 patients (25%) bled at 72h and only 1 patient (12.5%) was still bleeding one week after surgery. This didn't happen in cases where the chitosan was used. None of these patients had bleeding in the days following surgery, allowing us to report a peculiar ability of chitosan in hemostasis.

This happened due to the interaction of chitosan with blood cells. The outer membranes of erythrocytes and platelets are negatively charged and get attracted to the positively charged reactive amino groups of chitosan leading to platelet activation and thrombus formation[28,32].

This agrees with Y.Okamoto et al that conclude that chitin and chitosan enhance blood coagulation, and this phenomenon was more effective in chitosan than chitin. In their experimental studies they conclude that chitosan reduces blood coagulation time not only due to a physical effect of chitosan, but also related to their chemical structure, particularly amino groups that was important in platelets aggregation[44]. Electrophoretic and Western blot analysis of red blood cell surface proteins demonstrated that chitosan microfibers were bound to band three of the red blood cells and this interaction leads to the activation of the intrinsic coagulation cascade[45].

Intravenous dosing (50mg/kg/day) of chitosan in rabbits showed some deaths (but no effects at 4.5mg/km/day) and oral dosing at up to 800mg/kg/day in this species exhibited to be well tolerated which is probably due to blood cell aggregation[28].

A common approach to managing patients with oral anticoagulant therapy is to suspend the medication for 3 to 4 days before surgery, which exposes the patients to a higher risk of thromboembolism, myocardial infarction, and cardiovascular accidents. The use of chitosan minimises this risk by allowing the patient to continue his antiplatelet medication, but he still needs the postoperative evaluation and management of a patient's INR status[31].

HemCon® is a FDA approved chitosan dressing, that was manufactured from chitosan and molds to form a highly electropositive sponge-like material that bids to negatively charged red blood cells and in result rapidly form a viscous clot that seals the wound site and facilitate hemostasis. The use of this product in extraction sites (including patients taking oral anticoagulant

therapy) was evaluated and hemostasis was achieved in less than 1 minute and the control wounds in 9.54 minutes[21,46].

Tejraj P. Kale et al evaluate the effectiveness of the same product in controlling post-extraction bleeding compared to conventional methods in patients receiving oral antiplatelet therapy. The time to hemostasis was shorter in the case of chitosan product than using the conventional methods. The self-adhesive nature of HemCon® is caused by electrostatic attraction of red blood cells to the chitosan which it forms a dense viscous mass that provides adhesion and it seals the wound site. It allows the body form a clot naturally and activate its coagulation pathway effectively, initially forming organised platelets[31]. Moreover, it was found an improvement in the postoperative healing with minimal complications comparing the control site. It may be attributed a release of growth factor from human platelets stimulated by chitosan exposure and the antibacterial proprieties of chitosan. The investigation involved the application of a HemCon® in a full-thickness excisional wound in mice that had been infected with pathogenic bacteria such as *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Staphylococcus aureus* showed that chitosan not only kill the bacteria but also stimulate wound healing[47].

Sertac A. et al evaluate the effects other product made from chitosan in warfarin-treated rats and a great success in controlling bleeding intraoperatively and postoperatively was observed. It interacts directly with red blood cells and platelets to form a cross-linked barrier clot, independent of native factors. It also increases the tissue factor activity, the major initiator of the extrinsic coagulation cascade which is involved in all phases of the host response to wounding, implying a likely central role for the tissue factor in wound healing. It was also observed an insufficient resorption of chitosan granules and they recommend to remove them carefully and totally from the wound to avoid epithelization troubles and gain success in early-stage wound healing[33].

Other studies involving other abilities of chitosan such as osteogenic activity showed a tissue and cell formation during wound healing and augments new bone growth as well as facilitate hemostasis[48]. Furthermore, chitosan induces

more amount of new cementum and bone with a densely arranged cementoblasts and osteoblasts along the new bone surface[18].

Another important aspect is the postoperative care that patients had. Not all have the same care as, for example, to make the medication after surgery, put the ice packs in a controlled manner and for several periods of time. All this can influence these factors in the postoperative period and we can't control that.

CONCLUSION

6. CONCLUSION

Patients satisfaction is a key to compliance and the business success of the dental clinic[38]. In this pilot study, through an split mouth design, we evaluate the influence of chitosan in the healing process of the oral mucosa.

After analysing of the results, it may be concluded that the chitosan influences the healing process. Some classic signs of inflammation were observed in a short period of time where chitosan acts compared to the control. Literature reports that chitosan favours the early phase of healing process by attraction and activation of neutrophils and macrophages, cytokine production using macrophages and fibroblasts and angiogenesis stimulation[4]. It also favours the granulation tissue formation and the epithelialization[43].

Hemostatic effect and antimicrobial activity also play an important role in wound healing. Bioadhesive ability of chitosan hydrogel allows that it maintains an intimate contact with oral mucosa[10]. This propriety together with the primary intention wound closure performed by surgeon its permit that chitosan remains for prolonged time on the application site.

The hemostatic ability of chitosan was observed in our study. According to the literature the chitosan can be used in patients who are take oral anticoagulant medication allowing the patient continues the medication prior to surgery[31]. The studies also conclude that chitosan allow a regular deposition of collagen fibres leading more regular pattern and a denser fibre arrangement [6,18].

These unique biologic proprieties and the inexpensive cost of chitosan are important for the practice in dentistry because it eliminates any additional material necessity, such as barrier membranes and grafts in regenerative dentistry, which will enable the surgeon to spend less time in operation and offer more economical treatment option for the patient [25,29].

In a further analysis it would be important to analyse the data more specifically using for example biomarkers of inflammation.

Future studies should be conducted, taking into account not only the influence of chitosan in the soft tissues but also in the regeneration of hard tissues. Other studies should be conducted by associating chitosan with other drugs in order to check whether there is the possibility to influence and enhance the healing process.

After this study it was concluded that chitosan could be essential in a postoperative quality of patients, allowing a rapid healing of the wound, with less complications.

6.1. Limitations of study

In this study a semi-quantitative evaluation of the variable in study was realized.

Although patients were blinded the surgeon and the analyser knew what the control cases were and where chitosan was used.

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7. REFERENCES

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APENDIX

8. APENDIX

UNIVERSIDADE CATÓLICA PORTUGUESA
VISEU

UNIVERSIDADE CATÓLICA PORTUGUESA | INSTITUTO DE CIÊNCIAS DA SAÚDE

DECLARAÇÃO DE CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO

PARA PARTICIPAÇÃO EM INVESTIGAÇÃO

de acordo com a Declaração de Helsínquia e a Convenção de Oviedo

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorreto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Título do estudo: **The effects of chitosan in the healing process of the oral mucosa**

Enquadramento: Investigação de âmbito académico a efetuar na Clínica Universitária da Universidade Católica Portuguesa, tendo como responsável o Professor Doutor Rui Amaral Mendes, docente da Universidade Católica Portuguesa, e o aluno do 5ºAno do Mestrado Integrado em Medicina Dentária, Gonçalo João Pereira de Jesus.

Explicação do estudo: O estudo a realizar é um *split-mouth design* que envolve a aplicação de um gel de quitosano no alvéolo após a extração de um dente incluso/submucoso no qual será posteriormente comparado o seu processo de cicatrização relativamente à extração do dente contralateral, onde não será aplicado o gel anteriormente mencionado. O controlo será realizado fotograficamente e através do exame intra-oral nas 24h, 48h, 72h, 1 semana e 2 semanas após o procedimento cirúrgico. Será também utilizada a Escala Visual Analógica para avaliação e monitorização da dor.

Condições: Este estudo não envolve procedimentos que não se enquadrem na prática clínica normal nem pretende testar novos produtos ou medicamentos.

A participação neste estudo é totalmente voluntária, não acarretando quaisquer custos, podendo retirar o seu consentimento em qualquer etapa do estudo, sem necessidade de facultar explicações aos seus responsáveis e com total a ausência de prejuízos, assistenciais ou outros, caso não queira participar.
Ao decidir participar pode efetuar todas as questões que considerar necessárias para o seu esclarecimento ou facultar informações aos responsáveis do estudo em qualquer etapa do mesmo.

Confidencialidade e anonimato: Os dados recolhidos para o presente estudo são de uso exclusivo do investigador e tratados de modo a garantir a sua confidencialidade. A informação recolhida será tratada com a máxima confidencialidade promovendo o seu anonimato. A análise dos dados recolhidos será efectuada em ambiente que garanta a privacidade dos mesmos, sendo os mesmos utilizados exclusivamente pelos investigadores envolvidos no projecto.

Assinatura/s dos responsáveis pelo projecto:

O Aluno:_____

O Docente/Orientador:_____

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela/s pessoa/s que acima assina/m. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pelo/a investigador/a.

Nome: _____

Assinatura: _____

Viseu, ___ /___ /_____

ESTE DOCUMENTO É COMPOSTO DE 1 PÁGINA E FEITO EM DUPLICADO: UMA VIA PARA O INVESTIGADOR, OUTRA PARA A PESSOA QUE CONSENTE

Área Disciplinar de Cirurgia Oral

Registo do Acto Cirúrgico e Follow-up

UNIVERSIDADE CATÓLICA PORTUGUESA | INSTITUTO DE CIÊNCIAS DA SAÚDE

Nome do paciente:_____M ☐ F ☐Código_____Idade__

Nome do aluno_____Ano do aluno_____Turma do aluno_____

Data___/___/_____Motivo da consulta:_____

Estado geral do paciente: Saudável ☐ Patologia(s) associada(s)☐
Qual(ais)?_____

Dente(s) extraído(s)_____Erupcionado ☐ Incluso ☐ Semi-incluso ☐
Fragmento radicular ☐ Angle e Pell & Gregory se aplicável_____

Motivo da exodontia: Cárie ☐ Periodontal ☐ Ortodôntico☐Outro_____

Anestesia: Anestubos utilizados____com vasoconstrictor____sem vasoconstrictor____

Grau de dificuldade 1 2 3 4 5 Retalho☐ Osteotomia ☐ Secção☐ Ajuda docente ☐

Fio de sutura: Reabsorvível☐ Não reabsorvível☐ Monofilamento ☐ Multifilamento☐

Tempo da intervenção: ≤30m ☐ 31 a 60m ☐ 61 a 89m ☐ ≥90m ☐

Terapêutica prescrita

Analgésicos, e/ou anti-inflamatórios (com posologia)_____

Antibacteriano (com posologia)_____

_____

Outros (com posologia)_____

_____

CONTROLO PÓS-OPERATÓRIO

Paciente compareceu☐ Data___/___/_____ Paciente faltou ☐

Paciente cumpriu a terapêutica prescrita? Sim ☐ Não ☐ Porquê?_____

Ferida op.: Bordos unidos ☐ Formação de tecido cicatricial☐ Bordos separados ☐

Complicações referidas pelo paciente: Dor ☐ Hemorragia ☐ Edema☐ Trismo ☐

Complicações no controlo : Edema ☐ Infecção ☐ Alveolite ☐ Outra:_____

Sutura: Apresenta o mesmo nº de pontos ? Sim ☐ Não ☐ Quantos se perderam?____

Post operative control table

Follow up / Signs of inflammation	24h		48h		72h		1 week		2 weeks	
Pain										
Redness										
Swelling										
Loss of function										
Bleeding										
	Q	C	Q	C	Q	C	Q	C	Q	C

Q: Chitosan

C: Control

ATTACHMENT

9. ATTACHMENT

Analog Visual Scale: Scale used to assess pain in patients[49].