UNIVERSIDADE CATÓLICA PORTUGUESA

CENTRO REGIONAL DAS BEIRAS

DEPARTAMENTO DE CIÊNCIAS DE SAÚDE

ADVANCES IN PERIODONTAL REGENERATION USING RESORBABLE MEMBRANES

Dissertação apresentada à Universidade Católica Portuguesa

Para obtenção do grau de Mestre em

Medicina Dentária

Por

Catarina Marques Ribeiro Maçarico

Viseu, 2013

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Sob orientação de Professora Doutora Ana Leite Oliveira

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Abstract

The periodontal regeneration involves the formation of new cementum, new periodontal ligament and often alveolar bone that were previously lost. The discovery that stem cells present in the periodontal ligament which have the ability to populate the bone defect and induce formation of new cementoblasts, odontoblasts and fibroblasts and allowed to re-think the use of membranes to isolate these epithelial cells and gingival connective tissue to promote guided regeneration.

This monograph aims at identifying, analyze and discuss the potential for new strategies in guided tissue regeneration using resorbable membranes when comparing with more conventional approaches. It also aims at answering several questions, such as: what are the membranes already being explored in the clinical phase and which are the onesbeing developed in the preclinical phase, as well as what are the results obtained using different types of membranes for each specific case.

A bibliographic search of the literature was made taking into account research studies of materials in clinical phase of development and still in the pre-clinical phase (*in vitro* studies and *in vivo*), published in the past 5 years.

Although there are already several reviews about this subject, it is important to address in a systematic way the most recent advances in order to understand which solutions are being more effective to provide the adequate treatment for periodontal conditions. The main aim is to determine the cases where resorbable membranes can be used considering the type of periodontal defects, and the most suitable membranes according to each specific case, and how to combine regenerative therapies as for example, cellular therapies, use of certain bioactive molecules, just to name a few…

Keywoords:

Periodontal; Regeneration; Membrane; Resorbable; Biodegradable.

Resumo

A regeneração periodontal pressupõe a formação de novo cemento radicular, novo ligamento periodontal e muitas vezes também de novo osso alveolar que foram previamente perdidos. A descoberta de que as células indiferenciadas presentes no ligamento periodontal têm capacidade de popular o defeito ósseo e induzir a formação de novos cementoblastos, novos fibroblastos e novos odontoblastos permitiu pensar na utilização de membranas para isolar estas células do epitélio e do conjuntivo da gengiva no sentido de promover a regeneração tecidular guiada.

Esta monografia tem como objectivo identificar, analisar e discutir o potencial de novas estratégias em regeneração tecidular guiada utilizando membranas reabsorvíveis comparando-as com abordagens mais convencionais. Pretende-se também dar resposta a várias questões, tais como: quais as membranas mais usadas em fase clínica e quais as que estão a ser sujeitas a estudos pré-clínicos, bem como quais as mais adequadas para cada caso específico.

A pesquisa bibiliográfica do estado da arte teve em consideração estudos de investigação de produtos em fase clínica de desenvolvimento e de novas metodologias ainda em fase pré-clinica (estudos *in-vitro* e in-vivo), estudos estes publicados nos últimos 5 anos.

Existem já alguns trabalhos elaborados nesta área, não obstante será importante abordar o tema de uma forma sistemática por forma a compreender quais as soluções para promover a regeneração periodontal propostas mais recentemente. Pretende-se determinar quais os casos em que podem ser usadas membranas reabsorvíveis tendo em conta o tipo de defeitos periodontais a corrigir, quais as membranas mais aconselháveis para cada caso e como combinar outras terapias regenerativas, como por exemplo terapias celulares, a utilização de determinadas moléculas bioactivas, apenas para citar alguns…

Index

List of figures

Chapter 4: Discussion

List of tables

Chapter 7: Annexes

List of abbreviations

AAO	Anodized aluminum oxides
ACB	Autogenous cortical bone
AM	Amniotic membrane
BDX	Bone derived xenogenic bone graft
BG group	Group treated with bioactive glass
BG-NPS	Bioactive glass nanoparticles
BHC	Hydroxyapatite/collagen bocks
Bio-Gide	Membrane of swine origin
BM group	Group treated with bioabsorbable membrane
C	Chitosan
CAF	Membrane of calcium alginate
CAL	Clinical level of insertion
CQ	Cissus quadrangularis
DFDBA	Demineralized freeze-dried bone allograft
e-PTFE	Membrane of expanded polytetrafluoroethylene
FGM	Functionally graded membrane
G	Gelatin
GA	Incorporating glutaraldehyde

GBR	Guided bone regeneration
GTR	Guided tissue regeneration
HA	hydroxyapatite
HA-PLGA	Poly(lactic-co-glycolic acid) with grafted hyaluronic acid
HCG	Membrane multicomponent hydroxyapatite-gelatin-chitosan
HGF	Human gingival fibroblasts
hMSCs	Human mesenchymal stem or stromal cells
HOB	Human osteoblasts
IPPF	Technique immersed with immersion-precipitation
MEPEG	Methoxypoly ethyleneglycol in the membrane of PLGA
MPM	Modified perforated collagen membrane
NaOH	Sodium hydroxide
nAP	Nano-apatite
OCM	Oxidized cellulose membrane
OM	Occlusive barrier membrane
PAC	Collagen polymionic
PCL	Poly(caprolactone)

PCL	Poly(E-caprolactone)
PCNA	Proliferating cell nuclear antigen
PDL	Periodontal ligament
PDLF	Fibroblasts of the periodontal ligament
PDLLA/BG	Poly(D-L-lactic acid) and bioglass
PDMS	Membrane of polydimethylsiloxane
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLA-PGA	Polylactic-polyglicolic acid
PLGA	Copolymers of lactic acid polymers and glycolic acid
PLLAXMWNTs/H	Poly(L-lactic acid) and multilayers of nanotubes of carbon
PRF	Fibrin rich in platelets
PRP	Plasma rich in platelets
PTFE	Membrane of polytetrafluorethylene
T	Tetracycline hydrochloride

Chapter 1: Introduction

1.Introduction

1.1. Periodontal Disease: available therapies

The periodontium is composed of gum, periodontal ligament, cementum and alveolar bone. It forms a biological and functional tissue which can be subject of morphological changes as a consequence of age or functional modifications of the oral environment (Figure 1) [2].

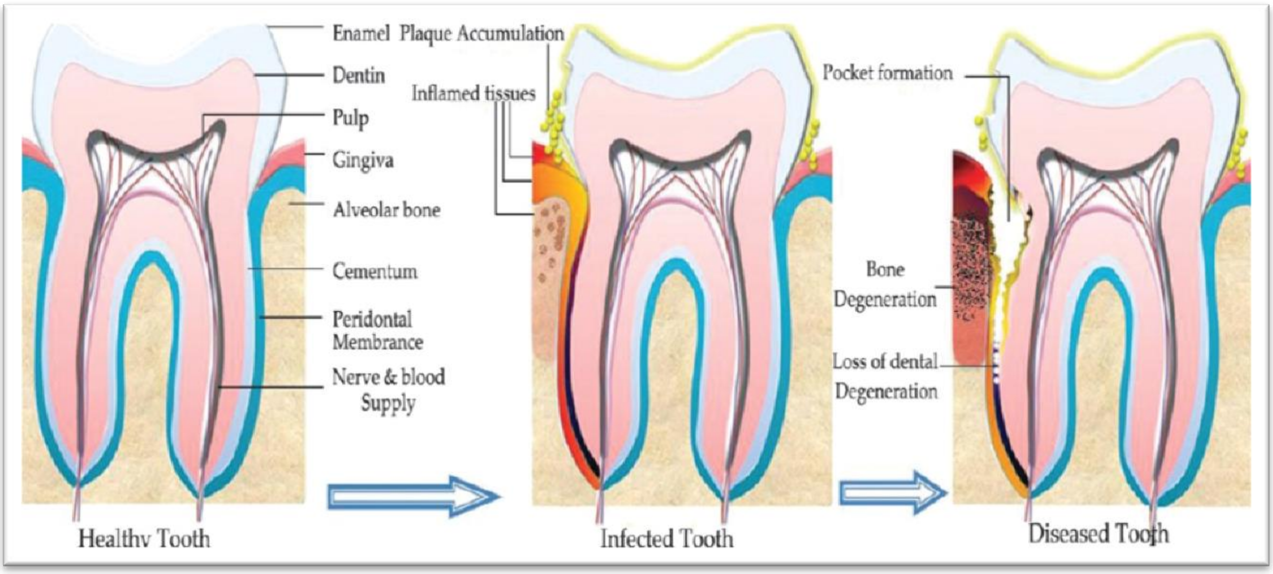


Figure 1. Periodontal disease [2].

Changes affecting the periodontium depend on the level of accumulation of the oral biofilm, being classified into three general categories: periodontal health, gingivitis and periodontitis.Periodontitis is a pathology characterized by the immuno-inflammatory progressive destruction of tooth supporting structures. With the progression of the disease, these tissues are gradually destroyed, resulting in a loss of adhesion between the tooth and the supporting structures. The definitive diagnosis is determined by the assessment of periodontal probing depth and evaluating the bone crest level, after performing an intraoral radiograph (figure 2) [3].

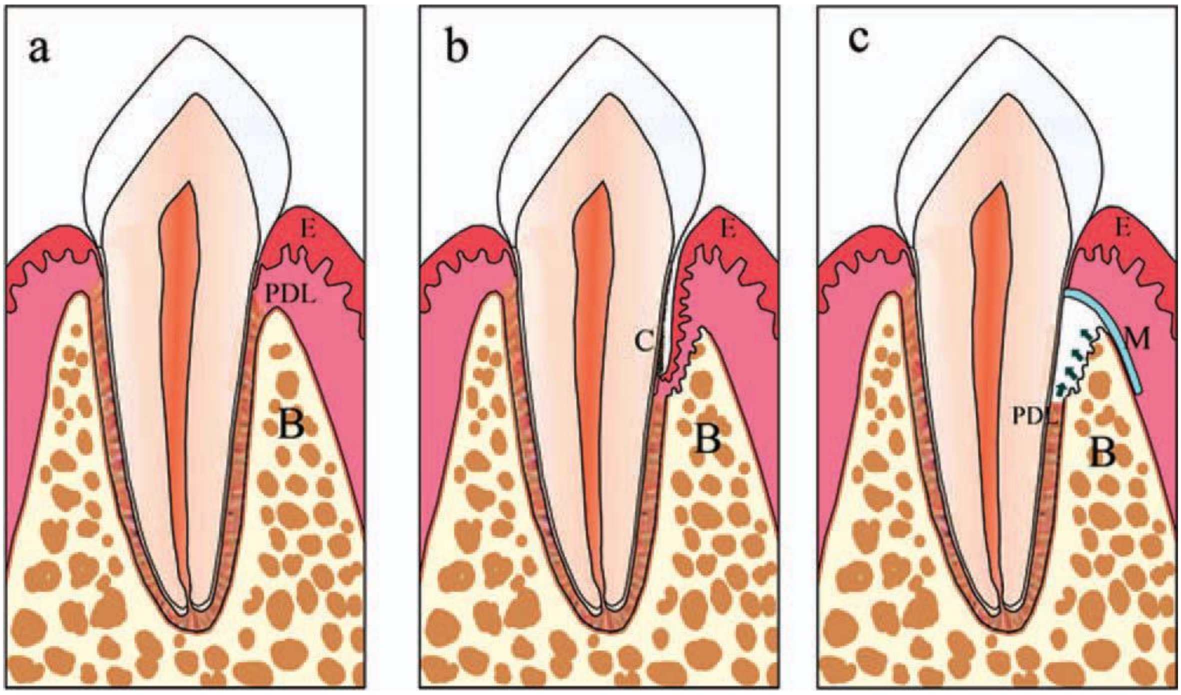


Figure 2. Drawing illustrating the normal periodontal tissue (a), periodontal tissue changes in periodontitis (b), and the principle of GTR (c). In (b), disconnection of periodontal ligament fiber attachment, bone loss, and detachment of the epithelium can be observed. In (c), the process of regeneration after GTR is shown. The membranes prevent the epithelium and gingival connective tissue from contacting the root surface during healing and allow cells from the periodontal ligament to repopulate the previously periodontitis-involved root surface and promote the regeneration of alveolar bone. B, alveolar bone; PDL, periodontal ligament; E, epithelium; C, calculus and plaque; M, membranes. [3].

An objective of the treatment is to reduce periodontal probing depth in order to stop the continuous evolution and progression of periodontal disease. However in cases of advanced periodontitis there is already a very high periodontal destruction, with the need to perform surgery [4]. This option has major disadvantages such as the process of gingival recession, which leads to an unfavorable cosmetic result. Thus, it is quite advantageous to choose periodontal regenerative strategies to restore the anatomy and function of the periodontal structures lost or damaged. Regeneration can be defined as the reconstruction of lost or damaged tissue, restoring architecture, anatomy, form and function [4], however there are other viable therapies for the treatment of periodontitis [5].

During the last decades, several regenerative surgical modalities have been suggested and examined for regeneration of periodontal tissues, alveolar bone, cementum, periodontal ligament and gun. These treatments encompassed the use of a wide variety of surgical approaches: membranes, a number of bone grafts and other osteocondutive materials or mixture of exogenous proteins, growth factors and gene recombinant technology, technology-based cells. Among these approaches, the periodontal tissue regeneration had considerable success after use of GTR / GBR. However, the results vary depending on the patient's age, size of the defect and the effects of lifestyle [5].

The gingival recession is the result of apical migration of the gingival margin, which leads to the exposure of the surface of the root which it is another problem that affects millions of people worldwide. The gingival recession is considered as a multifactorial condition, in that periodontal disease, trauma, sex and smoking are factors that may be correlated primarily with adults, regardless of oral hygiene. To treat cases of gingival recession traditional surgical procedures using autologous tissue transplants (the palate region), however, collagen membranes began to replace this procedure, avoiding many surgical consequences, so as to significantly reduce the pain and morbidity associated [5].

Other treatment modalities have been suggested to regenerate periodontal tissue damage in case of both: periodontitis and gingival recession [5].

Different strategies for periodontal therapy aimed at reducing and / or eliminating the inflamed tissues caused by bacteria, correct anatomical defects or problems due to illness, and new periodontal tissue regeneration. Develop new cementum with periodontal ligament fibers attached to the alveolar bone is the main goal of periodontal regeneration. Two surgical techniques have been increasingly used to restore / regenerate the periodontal tissues, including guided tissue regeneration (GTR) and guided bone regeneration (GBR) [5].

The use of a membrane at the interface of epithelial tissue, and the periodontal ligament and alveolar bone to promote regeneration of periodontal tissue is called GTR. A membrane acts as a barrier when placed in the surgical site, because it prevents the migration of epithelial and connective tissue into the defect. Progenitor cells located in the remaining periodontal ligament, alveolar bone or blood are then able to recolonize the area of the root and differentiate, forming a new unit periodontal support with the formation of new bone, periodontal ligament and cementum. Another important application of the concept is related to the restoration of disabled alveoli (for example, a local extraction and impaired alveolar) for subsequent implant placement, this process has been guided bone regeneration (GBR) [5].

1.2. The use of membranes in Guided Tissue Regeneration

Regenerative Medicine is a field that can provide promising alternatives in situations where the disease is in an advanced state and conventional treatments, although able to block its progression, are uncapable to regenerate damaged structures[6]. In this sense the use of membranes in surgical procedures aiming at periodontal guided tissue regeneration is already a very common clinical approach. This technique involves placing a barrier: a biocompatible - resorbable or non-resorbable membrane is surgically placed so as to cover and protect the bone defect, due to pathological progression [5].

As a strategy to isolate the periodontal defect, usesbio-resorbable and non-resorbable membranes which act as a physical barrier to prevent the invasion of cells. This technique is referred to as guided tissue regeneration (GTR) and guided bone regeneration (GBR). These membranes used in GTR / GBR must present: biocompatibility to allow integration with the host tissue without inducing inflammatory responses, a correct degradation profilethat coincides with the formation of new tissue, adequate mechanical and physical properties which allow their placement *in vivo* and strength sufficient to sustain the membrane avoiding collapse [7].

GTR membranes in GBR / are divided into two groups: non-resorbable and resorbable, according to the characteristics of degradation. The non-resorbable membranes are also known as conventional membranes.These membranes have many structural, mechanical and bio-functional limitations. Membranes of polytetrafluoroethylene and polytetrafluoroethylene reinforced with titanium membranes are examples of commercially available products. Polytetrafluoroethylene membranes are inert and biocompatible, act as a barrier, providing space for tissue regeneration and enable the integration of tissue [7].

The disadvantages of non-resorbable membranes such as polytetrafluoroethylene is in the fact that it requires a second surgery for removal. Collagen membranes, although resorbable also have disadvantages, such as insufficient mechanical properties, and unpredictable degradation profiles.[7]

Studies show that the enhancement of polytetrafluoroethylene membranes with titanium led to a higher regenerative capacity when compared to traditional expanded polytetrafluoroethylene membrane [7]. One of the disadvantages of non-resorbable membranes is the need to perform additional surgery for its removal, which means not only additional pain and discomfort, but also a higher economic cost. In order to eliminate the second surgical procedure, resorbable membranes have been developed. Among the resorbable membranes there are the natural and synthetic. Most synthetic polymers and resorbable membranes on the market for periodontal regeneration are based on polyesters, for example poly (glycolic acid) (PGA), poly (lactic acid) (PLA), poly (caprolactone) (PCL) and copolymers there [7].

The resorbable membranes need to be biocompatible, biodegradable and easily manipulated as compared to the clinically non-resorbable membranes. The degradation of the resorbable membranes is important, insofar as these membranes must operate at least 4-6 weeks to allow successful periodontal regeneration system [7].

Generally, the biodegradation of the membranes of poly (glycolic acid) and poly (lactic acid) involves the cleavage of PGA and PLA in pyruvic acid and lactic acid, respectively, which are common end products digestion of carbohydrates [7].

1.3. Resorbable membranes: possibilities and limitations

The resorbable and non-resorbable membranes currently used have limitations at the structural, mechanical and biofunctional level, and the ideal membrane for use in regenerative periodontal therapy is still far from being developed [8,9,10,11,12,13]. The use of bio-absorbable membranes is particularly interesting because, besides being able to eliminate the need for a second surgical intervention for their removal, presents new opportunities, for example, the ability to create dynamic systems for controlled release of molecules that can help modulating tissue regeneration [5].

Thus, the study of a new generation of biologically active membranes able to mimic the extracellular matrix interface becomes a very important subject [7]. Several interesting discoveries were made during the last five years.

The present review aims at identifying, analyzing and discussing the potential for new strategies in guided tissue regeneration using resorbable membranes as compared to more conventional approaches. A systematic review is presented considering the most important advances in periodontal regeneration. Furthermore, we intend to answer several questions, such as: what approaches are being used in the clinical and preclinical phases, as well as what are the results obtained using different types of membranes for each particular stage of the periodontal condition.

Chapter 2: Materials and Methods

2. Materials and methods

We conducted a bibliographic search in the literature, analyzing research studies of membrane-based products in clinical phase of development and new methodologies still in the pre-clinical phase (*in vitro* and *in vivo* studies), all published in the last 5 years.

The search engines used were PubMed and Science Direct. An initial search was performed with the following combination: periodontal AND regeneration AND membrane. All items included in the study were published in the last 5 years. Book chapters, review articles, conference proceedings and case reports were excluded.

Since this monograph intends to evaluate the published studies that have been done in the preclinical phase and the membranes that have been tested in clinical trials, the keywords *in vivo, in vitro* and "patient" were also used.

The results were grouped into research studies of products in a clinical phase of development or new approaches still in pre-clinical stage (*in vitro* and *in vivo*). A comparative analysis will be performed in terms of effectiveness in the results targeting periodontal regeneration.

The study focus only on studies regarding periodontal regeneration using resorbable membranes in the pre-clinical and clinical stages and comparing these with more conventional approaches, with the aim of identifying the most suitable for each type of pathology related to the periodontium.

Chapter 3: Results

3. Results

The research yielded sixty-one references, twenty-seven of which did not meet the inclusion criteria. This study encloses thirty-four articles, where clinical trials are grouped into ten articles, eight articles refer to *in vivo* and sixteen refer to *in vitro* studies.

3.1. *Clinically* tested resorbable membranes

The guided periodontal regeneration recurring to the use of membranes for periodontal regeneration has advanced in the latest years, being clear that new challenges and possibilities are being considered.

From the present bibliographic search, according to the inclusion criteria, several materials and their combination are being tested clinically nowadays. These include: demineralized freeze-dried bone allograft (DFDBA) [10] and bone derived xenogenic bone graft (BDX, Bio-Oss) with amniotic membrane (AM) [10], for treatment of defects of furcation grade II in humans [10]; oxidized cellulose membrane (OCM) together with hydroxyapatite (HA) and cissus quadrangularis (CQ) and comparing with the regular bone cicatrization [6]; membrane of chitosan evaluating its effects in the periodontal regeneration[14]; syntetic and reabsorbable biopolymers for the stimulation of bone regeneration, such as, copolymerized polylact, polyglicolic acid and polyglactin [15]; membranes of fibrin [16,17,4]; modified perforated membranes of collagen; traditional cell occlusive barrier membrane[18]; reabsorbable membrane of collagen from swine origin (Bio-Gide) associated with plasma rich in platelets (PRP) [11]; membranes of titanium [8]; membrane of expanded polytetrafluoroethylene (e-PTFE) and absorbable membrane of polylactic acid in association with autogenous cortical bone (ACB), in the regenerative treatment of intra-bony defects [9,18].

A study was done, in 2007, by Toygar and co-workers [8] having the aim of comparing the titanium membrane with the e-PTFE membrane and to explore the effect of bacterial contamination in the growth of the membrane. Seven titanium membranes and eight e-PTFE membranes were exposed between four and six weeks. There were no significant differences between the groups for the bacterial plaque and gingival index. The probing depth and the clinical level of insertion (CAL) were reduced in both groups when compared to the beginning, however, those differences were not statistically significant. The gain of CAL amongst the groups was statistically different in 3, 6, 9, 12 and 24 months and the gain of CAL was significantly higher in titanium membranes. The titanium membrane is equivalent to the e-PTFE membrane of GTR in the treatment of periodontal defects (table 1) [7].

Natural-based polymers such as chitosan and their combinations with other polymers or osteoinductive species such as demineralized bone matrix constitute an interesting approach for the periodontal regeneration.

In 2008, a chitosan membrane was clinically tested by Boynuegri *et. al* [14] to evaluate its effects in the periodontal regeneration. For the study there were used 4 groups, one group with chitosan gel, another group treated with chitosan gel and demineralize bone matrix, a third group with chitosan gel and collagen membrane and finally a control group treated only with retail. The clinical data did not show significant differences between the groups. However, the radiograph data revealed that except for the control group, all the other groups have shown statistically a significant raise in the bone growth, when compared with chitosan alone (table 1).

The combination of Polylactic – polylactic acids (fisiograft) with polylactic-polyglicolic acids (PLA-PLGA) (Vicryl Mesh) was used for treatments of intra-bony defects [9]. In 2009, several reabsorbable syntetic biopolymers were applied in a study by Chhabra *et al*. [9]: copolymerized polylactic, polyglycolic acid and polyglactin. A group was treated with polylactic-polyglycolic acids (fisiograft) while the other group was treated with polylactic-polyglycolic acid (PLA-PGA) together polyglactin acid (vicryl Mesh). Both groups have shown statistically a significant reduction in the pocket depth and gain in clinical attachment level and a linear bone growth. Both treatment modalities are beneficial to the treatment of infra-bony defects (table 1) [9].

In the same year, a study was done by Shaila [10] having per aim to test the combination of DFDBA (demineralized freeze dried bone allograft) with amniotic membrane (AM) and BDX (Bio-OSS) (bovine derived xenogenic bone graft) with amniotic membrane (AM), for the treatment of grade II furcation defects in humans. The results were positive except in one patient treated with BDX+AM. After 9 months, patients treated with DFDBA+AM had a reduction in pocket depht (PD) and a gain in attachment level, while the patients treated with BDX+AM obtained lower values in the reduction of the probing level and in the gain of the attachment level. The group treated with BDX+AM obtained better results regarding the gain in bone. However, the statistical analysis does not reveal big differences between the 2 materials (table 1) [10].

Besides these two studies done in 2009, there was also another work where a totally different strategy was used. The biological effects of fibrin rich in platelets (PRF) in cells periodontally related were studied. The obtained results refer that PRF models a specific proliferation for each type of cell, stimulates the proliferation of osteoblasts, cells of periodontal ligament and growth factors. These specific actions for each type of cell may be beneficial for periodontal regeneration [17]. The use of fibrin rich in platelets (PRF) as the single graft material seems to be a effective modality in the regenerative treatment of intra-bony defects (table 1) [17].

A reabsorbable collagen membrane of swine origin (Bio-Gide) was investiged regarding the clinical effects when combined with plasma rich in platelets (PRP) in the bone regeneration after extraction of the bottom 3rd molar in inclusion mesio-horizontal, in comparison with the use of PRP alone. Although the obtained results in the evaluation of PRP with the reabsorbable membrane and PRP alone are similar, from a histological point of view, the association of PRP with the membrane Bio-Gide have shown signs of bone maturation (table 1) [11,17].

A clinical application of autogenous cortical bone (ACB) with GTR and without GTR was tested in humans, being observed a clinical and radiograph improvement. The combination of GTR with ACB did not present any additional benefit in the treatment of intra-bony defects [19]. In 2010, a clinical essay has been done by Keles et al [19] having as major aim to evaluate the additional benefits of the use of GTR with autogenous cortical bone (ACB) vs autogenous cortical bone without GTR, in the regenerative treatment of periodontal intra-bony defects. With the results it was concluded that there was a significant gain in the

CALs and in the alveolar bone by radiograph analysis and a reduction on the pockets depth in the patients treated with ACB and GTR. However, the differences between the treatments are not statistically significant. Both in group treated with ACB alone as in the group treated with ACB+GTR, it was observed a clinical and radiograph improvement after 6 months. The combination of GTR with ACB did not present any additional benefit in the treatment of periodontal intra-bony defects (table 1) [19].

The clinical application of fibrin rich in platelets for the treatment of periodontal intra-bony defects was done in 2010 there were obtained reductions in the probing depth and gain at the insertion level in a study by Tsai and co-workers [16]. From a clinical and radiographic point of view, the use of PRP as the single material of graft seems to be an effective modality in the regenerative treatment of periodontal intra-bony defects (figure 3 and 4) (table 1) [16].

Figure 3. Fibrin membrane. The platelet-rich fibrin clot was gently pressed between two layers of sterile dry gauze to form a membrane. [17].

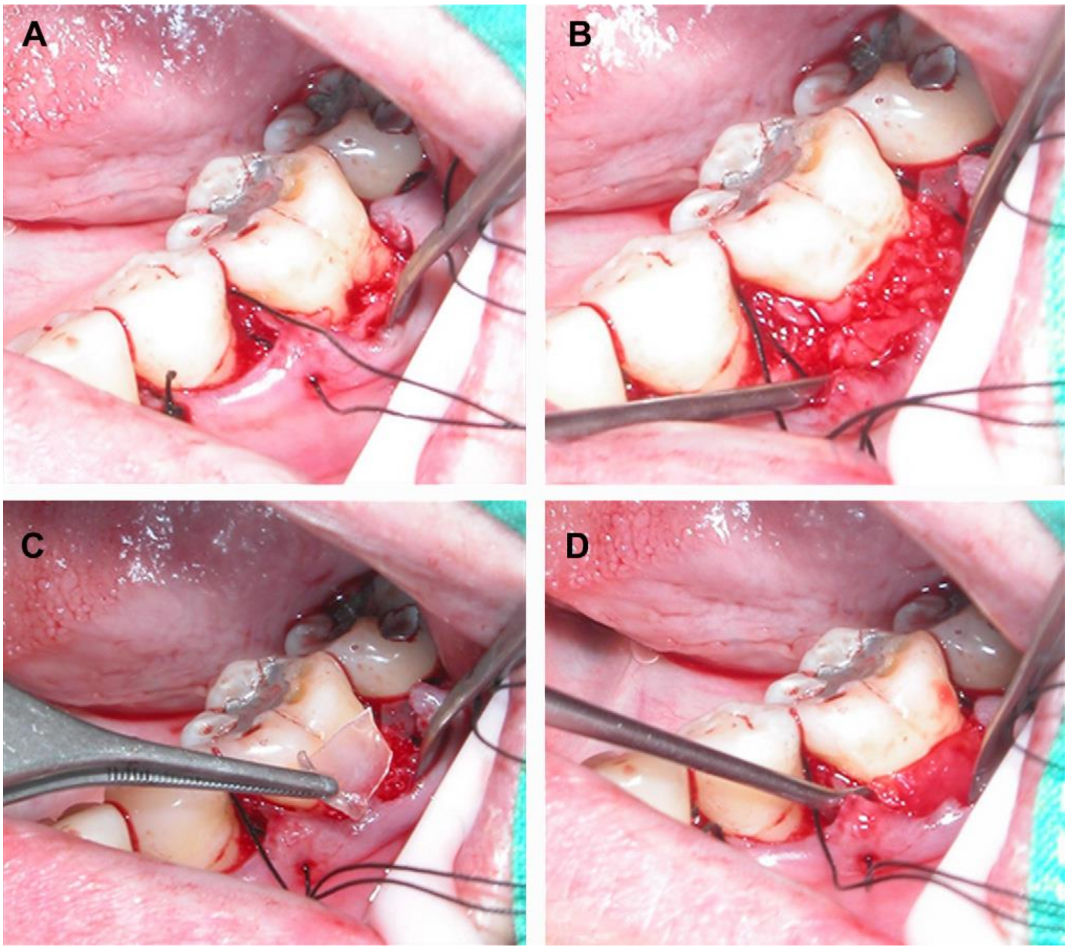


Figure 4. Clinical application of platelet-rich fibrin (PRF) on the buccal aspect of #36. (A) Minced PRF was applied to the defect walls and root surfaces. (B) Minced PRF was tightly packed in the furcation area. (C) PRF membrane was trimmed to cover the osseous defects. (D) PRF membrane was adapted over the grafted defect and above the cementoenamel junction [16].

In 2012, through clinical essays it was determined by Gamal et al [18] if the exclusion of gingival connective tissue and periosteum containing stem cells had positive or negative effect in the periodontal regeneration comparing to the use of modified perforated collagen membrane (MPM) with the traditional cell occlusive barrier membrane (OM). In the observation after 6 and 9 months, MPM has shown a significant increase in the reduction of probing and gain of CAL comparing with OM control group. The level of bone crest was higher in group MPM. This study demonstrated an improvement in the clinical results by the time of use of modified perforated collagen membrane comparing with cell occlusive barrier membranes in the processes of guided tissue regeneration (GTR). These results may be affected with the penetration of gingival connective tissue containing stem cells and periosteal cells and its differentiation in components for attachment apparatus [18]. The modified perforated collagen membrane (MPM) was clinically applied together with the occlusive bovine collagen membranes

(OM), the study has shown improvement in the clinical results by the time of the use of the modified perforated collagen membrane comparing with cell ocllusive barrier membranes in the processes of guided tissue regeneration (GTR) (table 1)[18]. In the same year, it was done a study by Lal et al [6] with the aim of clinically evaluating osteogenic potencial of hydroxyapatite (HA), *cissus quadrangularis* (CQ) and oxidized cellulose membrane (OCM). A group was treated with hydroxyapatite, a second group with *cissus quadrangularis*, a third group with oxidized cellulose membrane and for last a group of control was trated by debridement alone. There was a big reduction in the pocket depth in all groups, but however this reduction was higher in the group treated with hydroxyapatite. The gain at the level of attachment was higher in the groups treated with hydroxyapatite. Both hydroxyapatite membranes as OCM membranes have shown a good reduction in the pocket defect, a gain at the level of attachment and in the filling of bone defect. A deeper study shall be realized through a combination of HA and OCM in the treatment of periodontal bone defects with growth factors and stem cells [6]. The membranes of hydroxyapatite, cissus quadrangularis and oxidized cellulose membrane presented good results in the reduction of the pocket defect, in the gain of level of attachment and in the filling of bone defects. However, a deeper study shall be performed with the combination of membranes of hydroxyapatite and oxidized cellulose membrane in the treatment of periodontal bone defects with growth factors and stem cells (table 1) [6].

3.2. Results of the studies "*in vivo*"

At the *in vivo* studies level there were tested in animals, mainly in the the dog and mouse, six types of membranes, allowing to be used as membranes to promote the growth of cells of the periodontal ligament.

When considering the preclinical stage of research and development the *in vivo* studies are of great importance. Several studies were conducted over the last years an the following membranes were evaluated: poly (L-lactic acid) with multilayers of nanotubes of carbon and hydroxyapatite [12]; collagen polymionic (PAC) incorporating glutaraldehyde (GA) [20]; hydroxyapatite/collagen blocks (BHC) [4]; cross-linked collagen for extending the time of degradation[21]; semi-rigid PLGA (polylactide-co-glycolide acid)/CAP (calcium phosphate) [22] (figure 5); the cell-seeded biphasic scaffolds [23]; poly(lactic-co-glycolic acid) with grafted hyaluronic acid (HA-PLGA)[24]; membrane of calcium alginate [25] ;

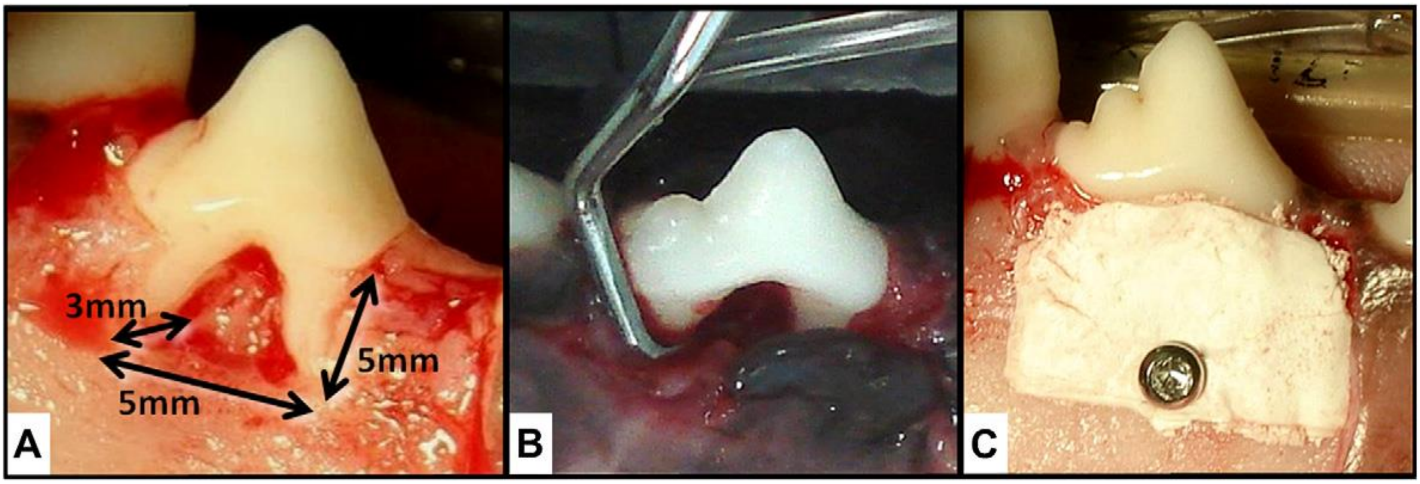


Figure 5. Sequence of *in vivo* procedures. (A) Shows the 5 mm _ 5 mm_ 3 mm class II furcation defect partially exposing the roots. (B) Scaling and root planing was performed after periodontal disease induction by filling the defect with an impression materialfor 21 days. (C) Fourteen days later, the PLGA þ CaP bilayered biomaterial was placed covering the defect of the treated group [22]

In 2007 a new membrane was produced by Mei *et al.* to be used in guided tissue regeneration by electrospinning, being constituted by poly(L-lactic acid), multilayers of nanotubes of carbon and hydroxyapatite (PLLAXMWNTs/HA). Cytological researches revealed that the membrane PLLA/MWNTs/HA improves the adhesion and proliferation of cells of the gingival epithelium. Histological exams proved that PDLCs attached to membranes function well alive. This new type of membrane presents excellent dual biological functions and satisfies the requirements of the techniques of GTR, although it presents a structure of monolayers. Comparing with other membranes of GTR commercially available or under research, this membrane may simplify the production process, reducing the production cost and avoiding possible errors in the clinical application. Furthermore, it does not need to be removed after surgery (table 2)[12].

A study with the objective of characterizing the effects of the biological membrane of calcium alginate (CAF) on the regeneration of bone tissue using the jaw level defects in rabbits was conducted in 2008. The results showed regeneration of defects on the edges. The membrane calcium alginate was compared with the bioabsorbable collagen membrane and came up to the conclusion that the membrane calcium alginate allows for greater bone growth compared with the collagen membrane (table 2) [25].

In 2009 Park *et al.* established a new protocol for the synthesis and controlled degradation of poly(lactic-co-glycolic acid) and grafted hyaluronic acid (HA-PLGA), being developed with success for applications as periodontal membranes. Four different samples, membrane OSSIXTM, film of PLGA and HA-PLGA/PLGA were evaluated as membranes for regeneration of periodontal bone defects of critical size in mouses calvaria SD. Histological and analysis of histomorphometric revealed that the HA-PLGA/PLGA membrane resulted in a bone regeneration more effective comparing with other samples, with an area of regenerated bone of 63,1% that covers the areas of bone defect [24].

In 2010, Veríssimo *et al.*[20] studied the effect of glutaraldehyde (GA) in membranes of colagen polyanionic (PAC) through an histological evaluation of biodegradation and biocompatibility in membranes implanted in mouse. The glutaraldehyde membranes presented a reduced inflammatory answer leading to the conclusion that glutaraldehyde prevents the degradation of the membrane. These membranes are thus an attractive option when the production of new bone tissue depends on the extended presence of a mechanical barrier (table 2).

In the same year Lee *et al.* a studied the ability of non-chemical crosslinking of porcine derived collagn nanofibrous membrane. This study was done in intrabony defects of beagle dogs. For all parameters that evaluate the bone regeneration, the experimental group showed significantly superior results as compared to the control group. On the other hand, for the evaluation parameters of periodontal tissue regeneration, including migration of the junctional epithelium and new cementum were no significant differences between the 2 groups. Within the limitations of this study, this collagen membrane enhances bone healing in intra-bony defect. However, no influence of this membrane in periodontal tissue regeneration could be observed in this study [26]

In 2011, was developed a semi-rigid PLGA (polyglactide-co-glycolide acid)/CAP (calcium phosphate) by Reis *et al.* [22] being tested alive in class II furcation defects in dogs. The treated animals with this membrane had a significant raise in the bone volume and thickness of trabecular bone. The use of PLGA/CAP has advantages in the periodontal regeneration, in the way that, doesn't collapse in the defect and has the capacity of retaining the blood clot in the place. The results are promising for the periodontal regeneration in comparison with the flexible membranes traditionally used (table 2).

In the same year, Jung *et al.* studied in order to elucidate the effect of a block of hydroxyapatite / collagen (BHC) in intraosseous defect in dogs (figure 6), the results showed that the application of BHC alone without a membrane intraosseous periodontal defect produced results unconscious. Thus, the use of a membrane is recommended to improve the stability of the implanted material and condense it (table 2) [4].

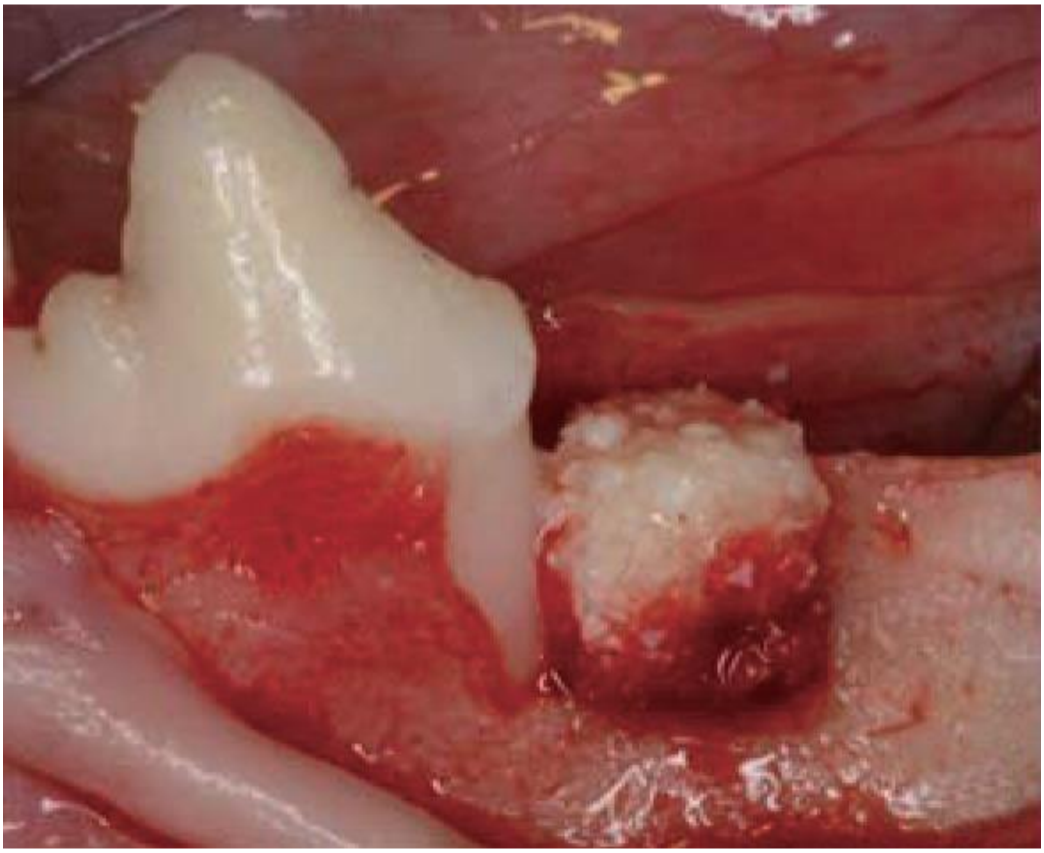


Figure 6. Clinical photograph showing the bovine hydroxyapatite/collagen block graft site [4].

In order to simultaneously achieve the regeneration of the periodontal ligament and alveolar bone an *in vitro* strategy was performed in 2012, by combining cultures of osteoblasts in bone compartment and the placement of multiple sheets periodontal ligament cell in electrospum membrane. *In vitro* data showed that osteoblasts have formed a mineralized matrix of bone compartment after 21 days in culture. The cell sheets biphasic scaffolds were placed in a block of dentin and implanted for 8 weeks in athymic rat subcutaneous models. The scaffolds exhibited good integration and deployment in subcutaneous bags, no significant reactions or infections. This study demonstrated that the combination of several sheets of periodontal ligament cells and biphasic scaffold enables simultaneous delivery of cells required for *in vivo* regeneration of alveolar bone, cementum and periodontal ligament [23].

3.3. Results of the studies *"in vitro"*

"In vitro" studies belong to the pre-clinical phase of a study, it is a very important step, as it serves to make an initial selection of materials in terms of biocompatibility to be applied later *in vivo* studies and clinical trials.

In the present sample 26 types of membranes were developed and evaluated *in vitro.* A membrane composed of HA, chitosan (C) and gelatin (G), was developed by Hunter, *et al.* [27], being their interactions studied with human mesenchymal stem or stromal cells (hMSCs).

Membrane of nano-carbonated hydroxyapatite/collagen/poly (lactic-co-glycolic) was also target of a biodegradation study.

Conventional membranes (C), membranes impregnated with tetracycline hydrochloride (T) and membranes impregnated with doxycycline were analyzed *in vitro* in order to assess the capacity and the time of inhibition of bacterial growth (table 3) [27].

The aim of making a multicomponent membrane is based on improving the characteristics of the membrane itself, or by combining different materials in a membrane can be camouflaged and they often improve disadvantages could hinder the regeneration process of the periodontal. Increasingly has been studying multicomponent membranes in that it is a lifeline to certain problems sometimes appear within the periodontal regeneration.

In a study by Kasaj *et al.* [28] commercially available reasorbable and non-reabsorbable membranes were placed in cultures of primary human gingival fibroblasts (HGF), fibroblasts of the periodontal ligament (PDLF) and human osteoblasts (HOB) in order to evaluate the biological effect. The studied membranes were the following: TutoDent, Resodont and BioGide and non-reabsorbable membranes of polytetrafluoroethylene: ACE, cytoplast and TelGem-FD (tabela 3)[28].

In the studies by Bottino [1] a biodegradable membrane was made with chitosan and polycaprolactone.

In study by Wang [29] a novel functionally graded membrane (FGM) was fabricated and a membrane of polydimethylsiloxane (PDMS) was fabricated with anodized aluminum oxides (AAO).

Asymmetric membranes of chitosan were developed [30]. A membrane with the combination of chitosan (C) and bioactive glass nanoparticles (BG-NPS) was produced [30].

The plasticized membrane poly(lactic-co-glycolic acid) (PLGA) was tested to be used in GTR (table 3) [31]. The biocompatibility of membrane of poly(vinildeno-trifluoroethylene)/barium titanate was also studied *in vitro* [31].

Three types of fibrous membranes based in nano-apatite (nAp) and poly (E-Caprolactone) (PCL) were made by electrospinning and a membrane of chitosan was produced by the technique of immersion-precipitation by Yang (table 3) [30].

Biodegradable GTR membranes possess only a limited clinical efficacy, since they have no biological effects on cellular proliferation or differentiation. Furthermore, significant variability in the surgical outcomes of GTR procedures has been observed frequently due to bacterial colonization of the membrane following its placement into the periodontal pocket, which may limit the proliferation of the regenerating cells like osteoblasts. Thus it becomes very important to the incorporation of drugs in membranes [32].

The controlled release of drugs was tested in the membranes poly (lactic-co-glycolic acid) (PLGA) [32]. The membrane poly (vinylidene-fluoride-trifluoreothylene)/barium titanate was evaluated regarding the biocompatibility in the guided periodontal regeneration [33]. A membrane of poly (D-L-lactic acid) with Bioglass was composed and tested [13].

In 2006, Hong et al [30] developed a new type of GTR using the technique of immersion-precipitation with a natural biodegradable membrane of chitosan.

The membrane presents an asymmetric grading-porous structure including a dense skin layer, a transition region and a porous spongy layer. The results indicated that the porous of the chitosan membrane has an excellent biocompatibility and biodegradability, which allows it to be used as a membrane to prevent the apical migration of epithelial cells of the gum and promotes the growth of cells of the periodontal ligament in the periodontal therapy (table 3)[30].

In the same year, new alternatives were proposed for guided periodontal regeneration as alternatives for the more conventional techniques. The treatment of periodontal recessions may be performed by the technique of guided periodontal regeneration using reabsorbable as well as non-reabsorbable membranes. There are several advantages in the use of absorbable membranes when comparing with the non-reabsorbable, such as: absence of a second surgical act and elimination of damages to the neoformed tissues [7]. Currently used collagen membranes present similar results to the technique of gingival conjunctive tissue graft, however, further studies shall be performed regarding its regenerative efficiency [34].

In the same year in which amniotic membrane was studied, the proliferative activity of gingival epithelium was compared using proliferating cell nuclear antigen (PCNA) with a marker of the cellular proliferation after surgical treatments with graft of bioactive glass and bioabsorbable membrane [28]. After 12 months of surgical treatment, the number of inflammatory cells has a significant decrease. The expression of proliferating cell nuclear antigen (PCNA) had a significant raise. There were no significant differences between the PCNA of the 2 groups at the beginning, however after 12 months from treatment there was a bigger raise in the group treated with bioactive glass (BG Group) when compared with the group treated with bioabsorbable membrane (BM Group) (table 3) [35].

The biodegradation of membranes of nano-carbonated hydroxyapatite/collagen/poly (lactic-co-glycolic acid) used in GTR was studied *in vitro*, by Liao [36]. Till the 4 weeks the membrane didn't suffer alterations, however, between the 8-12 weeks the membrane has become into powder. The degradation rate of the membrane is appropriated for the practice of periodontal regeneration. Furthermore, the new formation of minerals on the surface of the membrane is a big advantage for the bone regeneration *in vivo* [36].

In 2008 chitosan membranes were studied *in vitro* in the asymmetric form in order to verify the advantages in this new structure. The obtained results have shown us that the asymmetric structure was advantageous by the time of the use of this membrane for the controlled release of drugs over different periods of time. Moreover, the asymmetric chitosan membrane has good biocompatibility, tissue integration and osteoconduction (table 3) [37].Studies by Yang *et al.*[38] in 2008, were done in order to develop a biodegradable membrane that might be also used in the bone regeneration. Three types of fibrous membranes based in nano-apatite (nAp) and poly (E-caprolact) (PCL) were made by electrospinning. A study made with osteoblastic cells in vitro has shown that great part of membranes supported the proliferation of osteoblasts, but the presence of nano-apatite in the membranes allowed to induce a the bioactive behaviour [36].

Earlier studies [39] have proved that cells of periodontal pathogenic bacteria, such as, porphyromonas gingivalis may degrade the membranes used in the periodontal regeneration. To prove this fact, in 2008, studies *in vitro* were made using fragments of bacteria P.gingivalis associated to proteases, having concluded that proteases are responsible for the *in vitro* degradation of the collagen membrane. Furthermore, in this study it was also evaluated the inhibiting enzymatic factor of the antibacterial agents and used antibiotics in the periodontal therapy. The presented data have shown that although all tested collagen membranes are prone á lise by oral bacterial proteases, cross-linked membranes are more resistant to proteolysis. However, it was demonstrated that therapeutic concentrations of antibacterial agents and antibiotics, such as clorohexidine, cetylpyridiniumchloride, minocycline and doxycycline inhibit the enzymatic collapse of membranes, while metronidazole had not such effect. These results suggest that the presence of cells and extracellular vesicles of p.gingivalis in the neighborhood of membranes used for periodontal regeneration, may modify the physical structure and biological properties of membranes. However, by using collagen membranes as antibacterial agents the proteolytic process can be inhibited [39].

The biological effect of several bioabsorbable and non-reabsorbable membranes was evaluated in cultures of primary human gingival fibroblasts (HGF), fibroblasts of the periodontal ligament (PDLF) and human osteoblasts

(HOB) of cells *in vitro* [7]. Thus, there were evaluated three bioabsorbable collagen membranes (TutoDent, Resodont and BioGide) and three non-reabsorbable membranes of polytetrafluoroethylene (PTFE) (ACE, Cytoplast and TelGem-FD). The results have shown that from the six tested membranes, TutoDent and Resodont presented a higher proliferation rate of HGF (table 3)[28].

Polydimethylsiloxane (PDMS) was produced with anodized aluminum oxide (AAO) allowing for the production of a nano-PDMS. The cultures of cells in nano-PDMS have a higher proliferation rate, lower apoptosis rate and higher metabolic activity when comparing with PDMS (table 3) [29].

In 2010 it was made a study to evaluate if plasticized poly (lactic-co-glycolic acid) (PLGA) had the capacity of being used as a membrane in GTR and as a system for controlled release of antibiotics, tetracyclines and anti-inflammatories. Cells of the periodontal ligament were used to evaluate the effect of the material and the release of drugs in the cellular morphology. The addition of methoxypoly ethyleneglycol in the membranes of PLGA (MEPEG) has caused an increase in the release rates of both drugs (table 3) [31].

In 2010, a novel functionally graded membrane (FGM) was designed using electrospinning to produce a sequential multilayered construct. This membrane consists of a core layer and two functional surfaces, with which it is in contact with the bone comprising nano-hydroxyapatite and which contacts with the epithelium contains metronidazole. The incorporation of n-HA (nano-hydroxyapatite) raises the osteoconductive behavior and the incorporation of metronidazole fights the pathogenic periodontal, becoming the membranes a promise for solving the inconvenient of current membranes (figure 7) (table 3) [1].

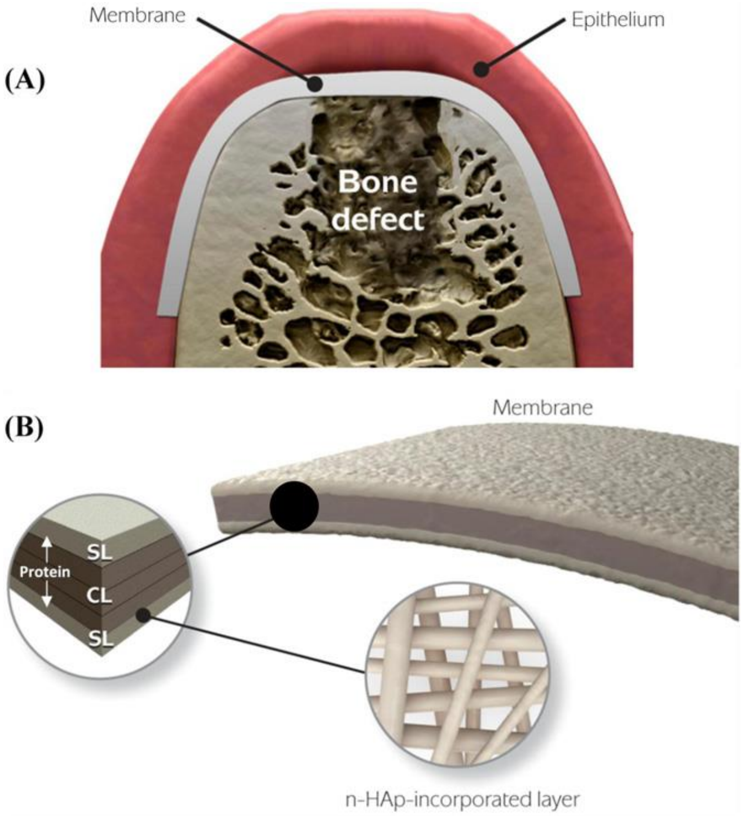


Figure 7. Schematic illustration of the spatially designed and functionally graded periodontal membrane developed by *Bottino et al.* [1]. (A) Membrane placed in a guided bone regeneration scenario. (B) Details of the core layer (CL) and the functional surface layers (SLs) interfacing bone (n-HAp) and epithelial (MET) tissues. Note the chemical composition step-wise grading from the CL to SLs, i.e., polymer content decreased and protein content increased.

In 2011, Chakraborti and co-workers [32] proposed the controlled realease of tetracycline and alondronate in a membrane of poly(lactic-co-glycoid acid) (PLGA) in order to allow an appropriate support for the differentiation and proliferation of osteoblasts. Tetracyclines are released more quickly in comparison with the alendronate. Both drugs simultaneously released were biocompatible with osteoblasts. After five weeks of incubation, it was observed the formation of bone nodules (table 3) [32].

In 2012, studies were done to describe the development and the characterization of membrane composed of poly(D-L-lactic acid) and Bioglass (PDLLA/BG), working with the hypothesis that the presence of microparticles of Bioglass could improve the structural and osteoconductive performance of the membrane of pure PDLLA [13]. This stimulates the cellular differentiation, mineralization, production of extracellular matrix and calcium nodules, what suggests that there is a positive effect by the time of addition of bioactive microparticles in the matrix of PDLLA, indicating that these membranes may have potential to be used in the therapy of guided tissue regeneration (table 3) [13].

Chitosan, gelatin and hydroxyapatite have been studied to be used as bone scaffold, by the fact that they have chemical similarities with some of the natural bone components [27]. Hydroxyapatite-chitosan-gelatin (HCG) Membrane is a biodegradable membrane in which the high expression of proteins and genes bone markers allows for a progression in the osteogenic differentiation of the human mesenchymal stem or stromal cells (hMSCS) in the absence of chemical induction. The results have shown that the membrane hydroxyapatite-chitosan-gelatin (HCG) has sufficient mechanical and structural properties to be functional. Moreover, when tested in human mesinchymal stem or stromal cells (hMSC) it was verified that the absorbed proteins of the extracellular matrix are efficiently immobilized in the HCG membrane and promote the osteogenic differentiation of hMSC (table 3).

A study *in vitro* based in the combination of chitosan membrane (C) with bioactive glass nanoparticles (BG-NPs), come to results that this combination allows for a reduction of its mechanic potential [35]. Nevertheless, the bioactivity increases. Besides the *in vitro* analysis, it was also done the *"in vivo*" analysis sing periodontal ligament cells and from human bone marrow for biological tests. The obtained results from the *"in vivo"* studies were promising in the way that it was verified that the combination of chitosan membrane with bioactive glass nanoparticles promoted metabolic activity and mineralization of cells of the periodontal ligament and marrow (table 3) [35].

A different strategy of other proposals was presented during the study amniotic membrane, since it involves the use of a biological material. One of the disadvantages of having a biological material is that this is not so controlled in terms of composition and variability. Also, since cells contain the entire handling under sterile conditions will be complex [40].

The amniotic membrane was studied to verify if it would be a possible candidate to the guided periodontal regeneration (GTR). The amniotic membrane has pluripotent cellular elements and a structure semipermeable, it is an immunotolerant structure. The fact that it has pluripotent stem cells that have the capacity of transdifferentiation in other cells, makes this membrane a great candidate for GTR. Other big advantage of this membrane is the fact that it induces an excellent biomechanical revascularization by not only maintaining the structural and anatomical configuration of regenerated tissues, but also contributing to the enhancement of healing through reduction and providing a rich source of stem cells. It has been demonstrated that amniotic membrane enhances gingival wound healing properties and reduce scarring [40].

In figure 8 is presented a graphic representation the number of articles were found about conventional membranes and biodegradable membranes.

Given the research conducted were incorporated into 34 articles, including 26 articles refer to biodegradable membranes.

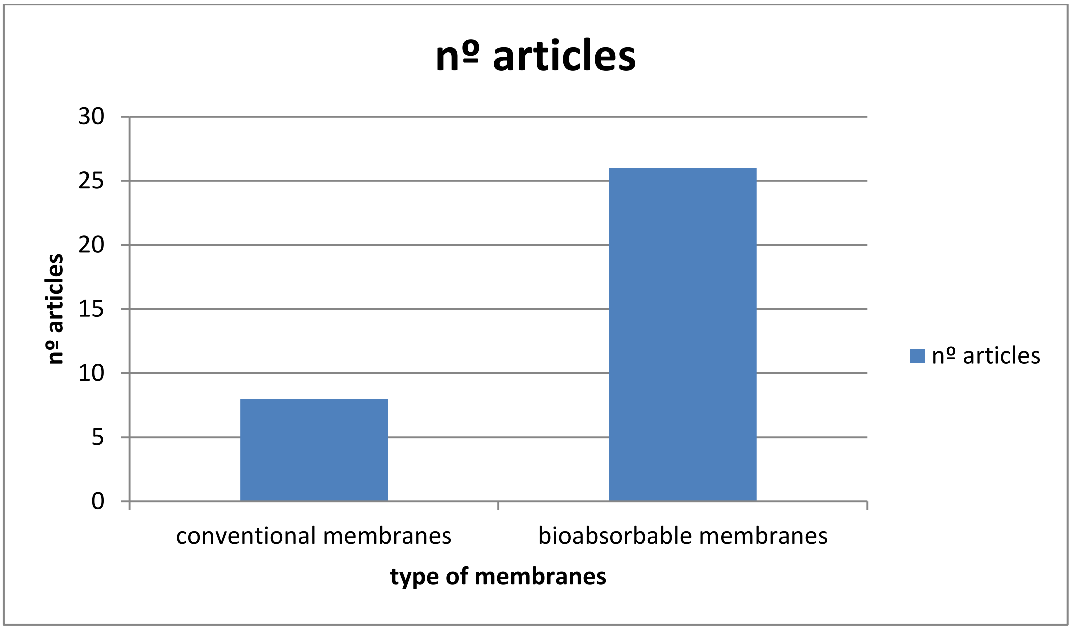


Figure 8. Comparison between the number of articles found on bioabsorbable membranes and conventional membranes.

In figure 9 the number of articles used in this review in terms of *in vitro studies*, *in vivo* studies and clinical trials are compared.

Studies *in vitro* showed to be most used for the development of new membranes 16 having been found studies *in vitro*. The clinical trials have been growing over the years, of which 10 were found.

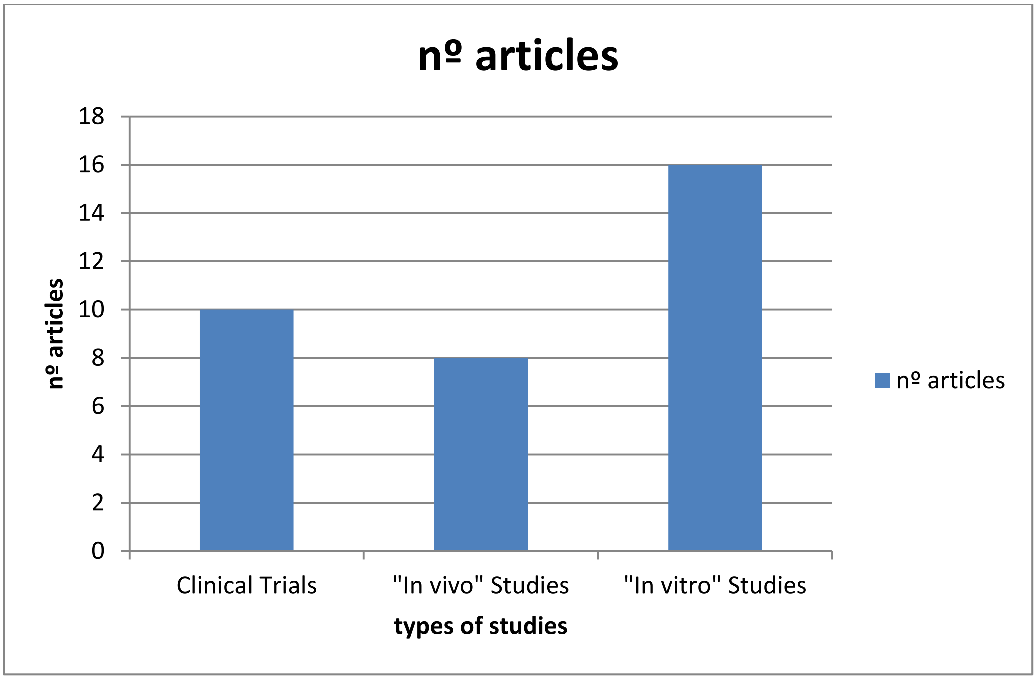


Figure 9. Graphical comparison of the total number of articles found on *clinical trials*, studies *in vivo* and *in vitro*.

Throughout these 5 years by analysis of the Figure 9 and 10, it can be understood that an increased importance has been giving to resorbable membranes and many new studies have been developed. These studies can be found in the tables presented as Annexes. However these are only a few studies, since many of them are not well specified in the articles.

In figure 10 is shown a schematic representation of biomaterials that have been most studied over these 5 years. The region of the graph associated to others includes materials/strategies where only 1 article was retrieved form our search, this means that or the study is less relevant or is still in a initial stage of research.

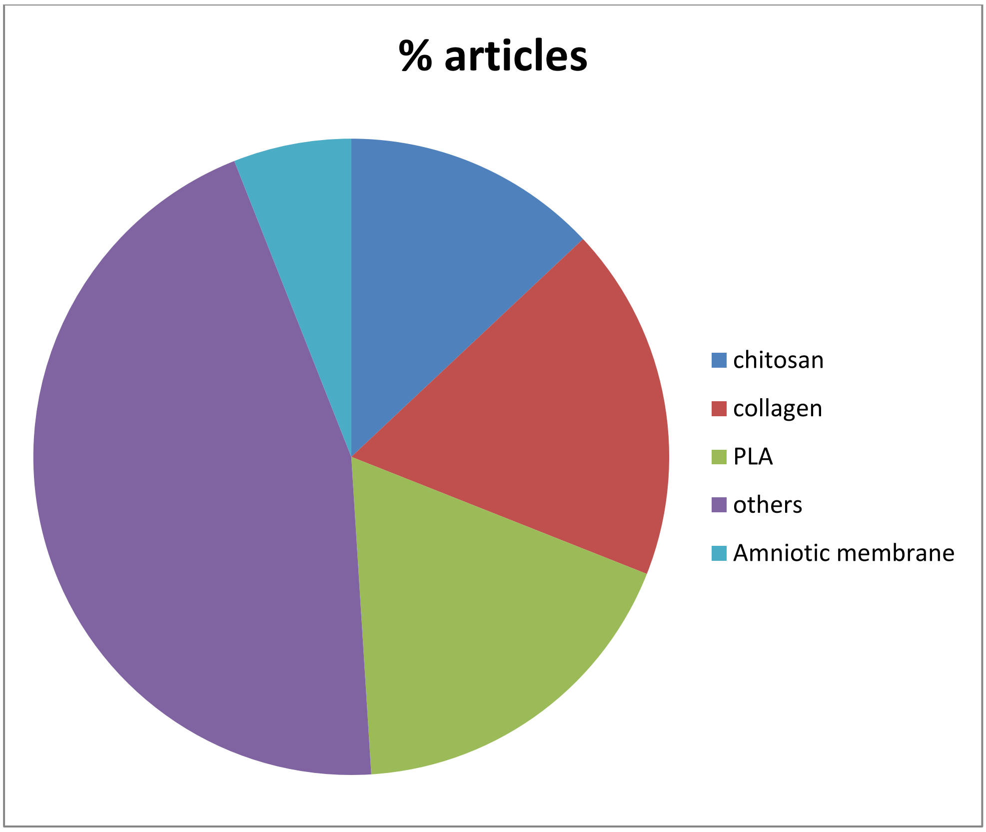
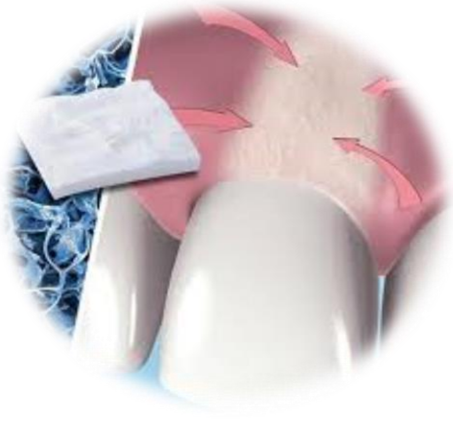


Figure 10. Graphical representation that systematizes the number of articles found for each type of materials for prodicing the GTR membranes. The region of the graph associated to others includes materials/strategies where only 1 paper was retrieved form our search.

In all the research carried out with the intention we were able to identify the membranes that had more emphasis at the level of periodontal regeneration. By this analysis collagen was identified as the most studied material for membrane production, followed by chitosan.



Chapter 4: Discussion

4. Discussion

The present thesis is mainly focused on biodegradable membranes as compared to conventional membranes. Therefore, the bibliographic search was done considering the developments on both types of membranes mainly over the past five years.

The primary objective was to understand the advantages in using biodegradable membranes as compared to conventional and furthermore understand the types of results have been obtained with the use of biodegradable membranes. Throughout these last five years, many investigations have been made with the aim of using biodegradable membranes in periodontal regeneration. However the ideal membrane is still far from being developed. Another aim was to find what kind of membrane would be the most used for the regeneration of the periodontal ligament. However, most of the studies present an integrated approach considering the repair/regeneration of adjacent tissues, in situations that include intrabony defects, intrabony and furcation involvement.

Periodontal disease can cause damage to the bone level (intrabony, alveolar bone and infrabony defects) and often causes furcation involvement. The recurrence to guided tissue regeneration therapy in the involvements of furcation presents evident advantages, as demonstrated by the achieved results with the semi-rigid membranes of PLGA, amniotic membrane with DFDBA or BDX [22][10]. Regarding the resolution of intra-bony defects there were obtained using standardized block hydroxyapatite collagen (BHC), porcine-derived collagen nanofibrous membrane, collagen membrane and PRF. [4,26,16,17]

Besides furcation involvement and intrabony defects, periodontal disease can also cause alveolar bone defects and infrabony defects, among which underline, respectively, particulate equine bone mineral [41] and for the resolution of infra-bony defects modified perforated collagen membrane [18], hydroxyapatite [6], Cissus quadrangularis [6] and Oxidized cellulose membrane [6].

Resorbable and non-resorbable membranes act as a physical barrier to prevent invade epithelial tissue defect, thus favoring the regeneration of periodontal tissues. The conventional membranes have many limitations at the level of structural and mechanical properties. It is evident that the "ideal" membrane for use in periodontal regenerative therapy has yet to be developed. Based on the approach of different types of existing biomaterials, the ideal would be a biologically active membrane that mimics the extracellular matrix of the cells will colonize the area of the defect [7]. According to our results, the materials that have been most researched over the past five years were collagen and chitosan.

4.1-Collagen

Collagen is a major constituent of the extracellular matrix. Collagen membranes from human skin, bovine Achilles tendon and pig skin are an important alternative to synthetic polymers in regenerative periodontal procedures due to its excellent biocompatibility and cell affinity. However, collagen type 1 may have limitations in its use due to the high cost and low defining their commercial sources, which makes it difficult to control their degradation and their mechanical properties [7]. On the other hand, collagen membranes have shown poor mechanical performance when they begin to degrade, and moreover, there is a risk of disease transmission, since the collagen has its origin in humans or animals, as well as religious beliefs its use has to be taken into account.

The GTR collagen bioresorbable membrane is commonly used for the treatment of periodontal defects.

The use of non-absorbable membranes increases the risk of exposure of the membrane and bacterial colonization and thus may inhibit healing, this is related to the fact that these membranes need a second surgical intervention for its removal. Membranes including bioabsorbable collagen have been developed to avoid these problems during its use in GTR. Collagen used in periodontal regeneration is often processed to change the properties of the final product. The cells are able to attach to the collagen membranes regardless of their origin or mode of processing of collagen.

The advantages of the use of collagen membranes used in periodontal regeneration have to do with the fact that this material is abundantly available, non-antigenic, biodegradable, non-toxic, synergistic with bioactive components, hemostatic, formulated in different forms biodegradability can be regulated by cross-linking and is also biocompatible synthetic polymer. However collagen also has disadvantages such as the high cost of collagen type I collagen isolated variability (e.g., crosslink density, fiber size, trace impurities), hydrophilicity and swelling which leads to faster release, and also the fact of presenting a variation in the speed of the enzymatic degradation compared to hydrolytic degradation [41].

Over 20 genetically distinct forms of collagen have been identified, being type I the most abundant and investigated for biomedical applications. Native collagen is degraded within a few days [42]. To overcome these problems various cross-linking techniques have been developed. The crosslinking involves the multiplication of naturally occurring linkages between collagen and the number of crosslinking of collagen is proportional to both the rigidity of the membrane molecules as the degradation time. In addition to the effects of cross-linking the surface topography of a collagen membrane and its source can affect the properties of a GTR and GBR membrane [43].

Of the various studies along these 5 years, different methods have been developed for processing collagen membranes. In the study by Sela *et al.* collagen membranes were subjected to fluorescence technique. The collagen membranes were immersed in NaOH (pH 9) for 10 minutes, then washed with water, acetone and further incubated overnight with fluorescence isothiocyanate 0.6 mg / ml in acetone in the dark at 41 ° C. Subsequently the membranes were washed with acetone and water until the fluorescence reaches baseline levels and were used immediately or kept at 41 ° C in the dark [39]. In a study by Junge t al. a block of hydroxyapatite / collagen was obtained and fixed in 10% neutral buffered formalin for 10 days. After rinsing in sterile water block was decalcified in 5% formic acid and dehydrated in a series of ethanol solutions and paraffin [4]. Verissimo *et al.* fabricated of polyanionic collagen membrane through alternative processes of immersion [20]. The main reason for the use of collagen for biomedical applications is that collagen can form fibers that are stabilized by self-aggregation and cross-linking. Physical treatments such as ultraviolet irradiation and gammaray and hydrothermal treatment have been used effectively to introduce crosslinking in the collagen matrix [44].

Due to its excellent biocompatibility and safety the use of collagen in biomedical applications has been growing rapidly to areas of bioengineering. However, collagen has some disadvantages, such as the difficulty of ensuring an adequate supply, its poor mechanical strength and inefficiency in the management of contaminated sites [44]. Improved systems for the distribution of collagen with a precision in the control of release can be achieved by adjusting the structure of the collagen matrix or add other proteins such as elastin, fibronectin and glycosaminoglycans [43].

Applications of collagen membranes for periodontal regeneration focus more on infrabony defects, with or without addition of bone substitutes. The combination of the collagen membrane with a bone substitute can prevent the collapse of the barrier, especially in infrabony defects and can thus guarantee the maintenance of space [44].

4.2 - Chitosan

Chitosan is a natural polymer derived from chitin which is not toxic, biologically safe, has hemostatic and antimicrobial activity, and in addition is biodegradable. The use of chitosan is seen in a wide range of scientific areas, including biomedical applications, various food and chemical industries [45] It is composed of a linear polysaccharide comprising copolymers of glucosamine and N-acetyl-glucosamine (figure 11) [46], that can be prepared chemically by alkaline N-deacetylation of chitin, which is a structural component of the shells of crustaceans, the cuticles of insects and wall of some fungi and microorganisms, with the biopolymer after cellulose the most abundant in nature [45]. As the only alkali polysaccharide, chitosan and its derivatives have potent bacteriostatic effects in many pathogens. A number of studies have shown that chitosan polymer is capable of inhibiting both gram + and gram bacteria, depending on the molecular weight of chitosan [47]. Due to its interesting properties, its application in the biomedical field has been growing, including in the dentistry field [14].

One of the advantages in the use of this biodegradable polymer is the easiness of preparation, and some demonstrated stability in biological fluids. For example, in the case of controlled drug delivery using nanoparticulate polymers the nano- and microparticulate polymer remain at the site of application and the drug is released by diffusion, chemical reaction, or degradation of polymer ion exchange mechanism, depending on the polymer used and its interaction with the encapsulated drug.

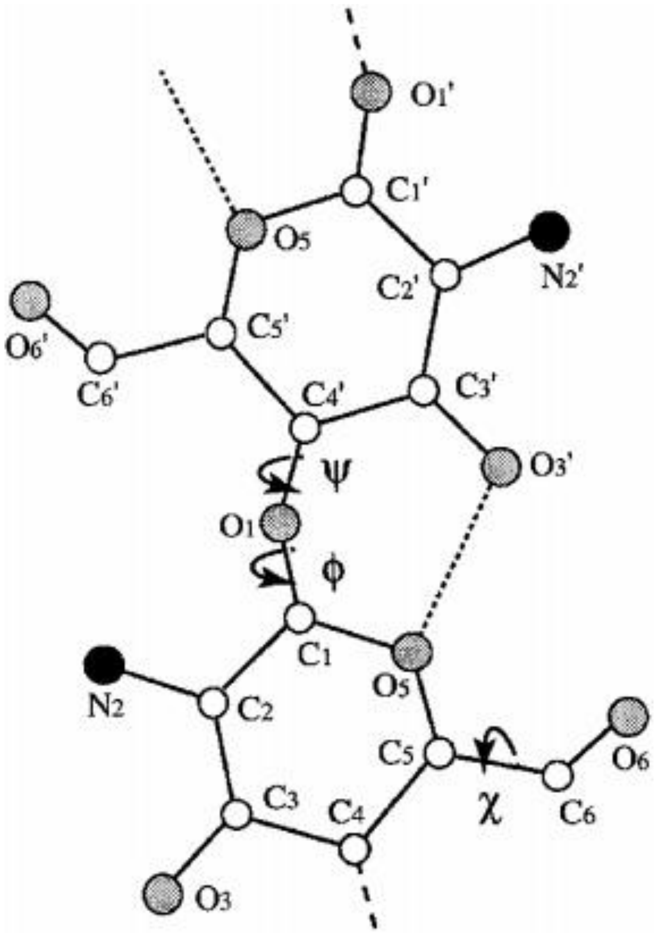


Figure 11: Chitosan chemistry [45].

Chitosan was discovered in 1859 by Royget, upon contact of chitin with a boiling solution of potassium hydroxide. The production of chitosan occurs industrially by alkaline deacetylation of chitin. During the course of alkaline deacetylation, N-acetyl bonds of chitin are ruptured to form D-glucosamine having a free amino group. When the amount of D-glucosamine, or the degree of deacetylation becomes greater than 50% chitin becomes insoluble in the aqueous medium and the polymer shall be called chitosan. Another option for the industrial production of chitosan is the deacetylation through microbiological using specific enzymes or microorganisms [46].

Chitosan is insoluble in water, alkali and organic solvents, but soluble in the organic acid solution with a pH below 6 [47]. In all the studied articles on chitosan several techniques for processing were proposed. Hong *et al.* used a technique immersed (IPPF) with immersion-precipitation stage for processing chitosan membranes. In work done by Mota *et al.* chitosan membranes were obtained by dissolving 0.7% (w / v) of chitosan and 0.3% (w / v) solution of bioactive glass in a volume of 2% acetic acid. The membrane and pure chitosan was obtained by dissolving 1% (w / v) chitosan solution in 2% acetic acid by volume. After evaporation of the solvent the membranes were neutralized in a solution of 0.4% NaOH and allowed to dry at room temperature [30]. In a study by Ho *et al.* Chitosan powder was dissolved in aqueous acetic acid to form a polymer solution of 2% by weight. The solution was used to prepare chitosan membranes. Porous chitosan membranes were prepared with the so-called freeze-gelation method. The chitosan solutions of 2% by weight were placed in aluminium cans in contact with 1M NaOH solution for varying lengths of time at room temperature. At this stage the gelation has occurred in the chitosan solution coming from the interface in contact with the NaOH solution. The chitosan solution with a layer of gel was then frozen at 20 ° C, separate aluminium and immersed in an aqueous solution of ethanol so as to permit adjustment of the pH and gelation of chitosan. Dried gelled solution at room temperature to remove the liquid content and thus obtained chitosan porous asymmetric membranes [37]. Hunter *et al.* proceeded to manufacture membrane multicomponent hydroxyapatite-gelatine-chitosan (HCG). This membrane was prepared as follows: 0.4 g of HA was stirred in 100 ml of water for 30 minutes, then was incorporated into 0.5 g of chitosan and 2 ml of glacial acetic acid and was stirred overnight at room temperature. Subsequently were added 0.5 g of gelatine and the mixture was submerged in a water bath at 37 ° C until dissolution. The solution was then dried at room temperature for 96h. The dried membranes were hydrated and neutralized with sodium hydroxide, then washed with buffered saline sterilization and tri by washing in 70% ethanol. After sterilization and washing the membrane was incubated in saline for 7 days [27].

The use of chitosan membranes in patients with chronic periodontitis showed a reduction in markers of gingival inflammation due to antimicrobial properties [35]. Chitosan has been evaluated as an effective delivery system for DNA and growth factors *in vitro*, with a promise for the future. Chitosan can induce mesenchymal cell differentiation into cementoblasts. All properties of this membrane allows us to affirm that this is a promising material to be used for periodontal regeneration [45]. Chitosan scaffolds have been widely used in bone defects [3]. The chitosan membranes act as a moisturizing agent and osteoinduces the effect tissue healing.

The chitosan can be easily processed into membranes, gels, nanofibers, nanoparticles, scaffolds, sponges, and can be used as drug delivery system. Furthermore, chitosan has been shown to be useful in regulating the release of bioactive agents. The incorporation of bioactive molecules in chitosan membranes, such as growth factors may be highly beneficial to obtain more bone and cementum [46]. The mechanical properties of chitosan membranes, such as stiffness and strength can be a disadvantage of these membranes compared with other bioabsorbable membranes, and these properties may cause the collapse of the membrane in some cases [46].

Chitosan membranes are used as drug carriers, especially of active antitumor compounds. It is evident that chitosan with its low molecular weight is a useful vehicle for drugs that require administration for targeted molecules [45]. Compared with the majority of bioabsorbable membranes currently used in clinical practice, chitosan membranes are cheaper, have better curative effect tissue such as cementum and bone. The properties of bacteriostasis can reduce bacterial contamination and to benefit the regeneration of periodontal tissue [45,46].

4.3 - New resorbable systems

The development of biomaterials for tissue engineering has greatly enhanced the treatment options available for periodontal disease [48]. An attempt to overcome the problems related to the collapse of the membrane in preventing growth of epithelial defect area are being investigated. The combination of membrane graft materials is considered to be an interesting strategy. For instances, chitosan membranes when combined with other biomaterials can acquire greater mechanical strength and a surface-modified morphology, which provides the best results during use, this has been proven in two studies, 2012, *Mota et al.* [35] conjugated chitosan membrane with bioactive glass and the *Hunter et al*.[27] conjugated membrane nanoparticles with gelatin and chitosan hydroxyapatite so as to form a biodegradable film with improved properties. Hydroxyapatite commonly used as bone filling material and coating material showed good miscibility in a wide range of compositions with chitosan. The membranes composed of chitosan and hydroxyapatite present a high tensile strength and flexibility [45,27,35]

To further improve the cytocompatibility of chitosan with osteoblast mixed polycations some researchers developed composite membranes for GBR. It has been shown that osteoblasts embedded in chitosan membranes favor the organization of the cytoskeleton, cell adhesion was significantly higher and differentiation was also higher [47].

The mechanical properties of pure chitosan membranes prepared by the conventional methods can not effectively ensure the function of deleting unwanted tissue and thus preventing the application of chitosan at once, however, present a considerable improvement in the preparation of technical and methods overcome this obstacle. For example, the mixing of chitosan with other materials, such as hydroxyapatite for forming a electrospinning nanofiber membrane structure, being a promising way to manufacture these membranes. Electrospinning is a process by which a polymer solution or melt can be spun into smaller diameter fibers using a high potential electric field. This generic description is appropriate as it covers a wide range of fibers with submicron diameters that are normally produced by electrospinning [45].

Although this method needs further exploration, a number of studies have demonstrated the important strengthening and satisfying the mechanical properties of electrospined chitosan membranes and suitable degradation rates for clinical applications in GTR/GBR [45].

Recent studies have been made of collagen membranes to improve their properties. in 2012 *Gamal et al.* conducted a study in order to verify whether the perforated membranes and collagen modified would have better results in the treatment of intrabony defects compared to occlusive bovine collagen membranes [18]. The incorporation of growth factors in the membranes could be another improvement in periodontal regeneration. Preliminary studies have shown that chitosan membranes loaded with growth factors lead to an increased regeneration of tissues. The platelet-rich plasma has also been playing an important role in tissue healing and bone formation [47]. More research is needed to solve the current problems of chitosan membranes, as these have been widely used in clinical practice.

The properties of chitosan and the progress of the application indicates that it is an excellent candidate for periodontal regeneration.[35].

In terms of future prospects it is clear that the combination of two or more biomaterials to produce a membrane is advantageous in that this combination allows changing the time degradation of membranes and even their physical and chemical properties. In recent years, several research groups have attempted to design and develop periodontal membrane GTR / GBR fulfilling characteristics and properties through a combination of natural and synthetic polymers. The evolution of scaffolds as cell delivery, proteins and genes is being studied. More research needs to be focused on *in vivo* systems to improve the results of the distribution systems of various biomaterials, most approaches in therapy with the combination of different biomaterials should be made [44].

Recent advances in stem cell biology and regenerative medicine have presented opportunities for tissue engineering . Stem cells are the foundation cells for every organ and tissue in the body including the periodontium [39]. A stem cell has two defining characteristics : the ability of indefinite self-renewal to give rise to more stem cells and the ability to differentiate into a number of daughter cells specialized to perform a specific function.

One of the areas that have been of great interest among researchers it is the periodontal regeneration using stem cells by an individual, ie , homing of transplanted cells or another individual . These adult stem cells remain in the dormant stage in their niches until a need for differentiation of the cell to be awakened [49] There are stem cells in different organs and as such there are also dental stem cells. The dental stem cells in these years has been attracting interest because they have the potential to differentiate into different cell types to form the lost due to periodontal disease.

Human embryonic stem cells are the ones in which it is possible to keep them in an undifferentiated state *in vitro* for an indefinite period of time, however, due to ethical issues were not yet tested *in vivo*. Mesenchymal stem cells from bone marrow are the most studied because they are easily available in quantities suitable for clinical applications. For these cells to be used therapeutically a large-scale expansion is needed to produce enough quantities for its culture. However, the use of fetal bovine serum for its expansion brings certain disadvantages, such as the risk of viral transmission. Moreover, the deployment of the exogenous stem cells to regenerate the periodontal membrane can trigger the formation of tumors due to gene mutations that may be found in implanted cells. Because of these disadvantages, tissue engineering needs to develop new protocols so that these exogenous stem cells be used safely used in periodontal regeneration [49,50].

While the use of autologous cells eliminates the potential for an immune response against the donor cells by the host, it is often difficult to generate sufficient endogenous cells. Over the past years cells instructive materials have aroused great interest for periodontal regeneration since they are capable of encoding local stem cells (e.g. PDL stem cell niches) to to act positively towards a regeneration effect [50,49]. Such materials have been extensively investigated [49]. This new field of research in which the cells are activated materials to build new biological tissues was initiated in 1980 , and from that time recognized as a major strategy in the new field of tissue engineering [49]. Cell instructive materials have evolved to serve not only as carriers of inductive factors and cells , but the cells actively instruct and provide step-by - step in tissue formation [49]. In the future, these innovative biomaterials and devices promise to have a profound impact on periodontal reconstructive therapy [49]. The endogenous regeneration of periodontal tissues is the light of improvements in our understanding of wound repair [50,49].

Chapter 5: Conclusion

5.Conclusion

The periodontal regeneration is a stage in the periodontal treatment. Therefore its application depends on the type of bone defect resulting from periodontal pathology. Evidence shows clear advantages in the recurrence to therapy of GTR in the defects at periodontal level, such as involvement of furcation, bone defects and periodontal recessions.

The clinical results, in terms of gain of periodontal support, reduction of the pocket depth and recession of the gingival margin present better clinical results when treated with regenerative therapy compared with conventional methods.The clinician should be able to choose the type of treatment for adapting to a specific periodontal defect, taking into account the individual characteristics of the patient and the defect. The whole regenerative process is optimized with the choice of surgical technique and type of biomaterial for use in that specific situation.

The reabsorbable and non- reabsorbable membranes currently used have limitations at a structural, mechanic and biofunctional level. In this sense, the ideal membrane to be used in a periodontal regenerative therapy is still far from being developed, although it would be certainly a biodegradable one, avoiding the need for a second surgery for its removal.

From the analysis performed of the *in vitro* studies that have been carried over the last 5 years, it can be concluded that the collagen and chitosan and their combination with bone graft and synthetic membranes has been further enhanced and have been the subject of the most relevant studies *in vitro*. Chitosan has been the subject of studies *in vitro* and in combination with gelatin hydroyapatite, in order to prove whether actually have chemical similarities with those of natural bone. This combination of materials has been evaluated as bone scaffold and its interaction with human mesenchymal stem or stromal cells (hMSCs) allowed it to function as a biodegradable membrane for bone regeneration [27]. In addition to this study, the chitosan was also evaluated in conjunction with bioactive glass nanoparticles. After these tests it can be concluded that chitosan together with bioactive glass nanoparticles allows for the regeneration of periodontal bone [35].

The field tissue regeneration has undergone major developments, the membranes were previously studied alone, however with the advance of research has been found that it is possible to improve the properties of membranes by combining them with other materials, synthetic bone or by changing forms and dimensions. As a consequence, in the herein presented pre-clinical studies there is an increasing interest in biodegradable membranes mainly of natural origin, with special emphasis for chitosan and collagen, in conjugation with other polymers, or ceramics or bioactive factors (multicomponent membranes) for improving their final properties in a GTR approach. Also more studies must be done within the area of periodontal regeneration using instructive materials for cell homing as this strategy is advantageous over the traditional TE approach. An endogenous regeneration approach could be utilized to optimize the conceptually useful principles of GTR (selective cell population, space maintenance, wound stabilization) through the use of scaffolds that can deliver instructive cues towards an effective periodontal regeneration.

Chapter 6: References

6. References

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Chapter 7: Annexes

7. Annexes

Table 1. Membranes being clinically evaluated for periodontal regeneration

References	Membrane type	Aim of the study	Patient information	Local of the defect/time of follow-up	Methods	Results
Toygar *et al*. (2007)	Membrane of titanium and membrane of e-PTFE	This study aimed at evaluating the clinical results of the titanium membrane and comparing these results with the clinical results of e-PTFE membrane and to investigate the effect of bacterial contamination on the growth of membranes	Periodontally involved teeth	16 membranes of titanium and 16 membranes of e-PTFE were surgically placed adjacents to periodontally involved teeth, of those membranes 7 of titanium and 8 of e-PTFE were exposed during 4-6 weeks. There were no differences between the groups for the bacterial plaque and gingival index.		The depth-gauge and the clinical level of insertion (CAL) were reduced in both groups, when compared with the begining, however, those differences were not statistically significant. The gains of CAL amongst the groups were statistically different in 3,6,9,12 and 24 months (p < 0,05) and the gain of CAL was significantly bigger in membranes of titanium. There was a significant fall in the bleeding at the depth-gauge of the basis line in both groups. This study has demonstrated that the titanium membrane is equivalent to membranes of e-PTFE of GTR in the treatment of periodontal defects.

Boyneugri *et al.* (2008)	Chitosan	To evaluate the effects of chitosan in the periodontal regeneration.	20 patients with periodontitis.	It was made a clinical and radiographic evaluation after 3 and 6 months of the surgery.	The 20 patients were divided into 4 groups: Group A – chitosan gel; Group B – chitosan gel + demineralize bone matrix Group C – chitosan gel + membrane of collagen Group D – snips only (control group)	The clinical data didn't present big differences in the 4 groups. However, the radiographic data revealed that, except the control group, all other groups have statistically shown a significant increase in the bone growth, when compared with chitosan alone, or in combination with demineralized bone matrix/collagen membrane. The combination of chitosan both with collagen membrane as with demineralized bone matrix Is a promise to the periodontal regeneration.
Chhabra *et al.* (2009)	POlylactic-polyglycolic acids (fisiograft); Polyglactic-polyglycolic acids (PLA-PGA) together with polyglactin acid (vicryl Mesh)	To evaluate the differences of the results obtained using the membrane of polylactic-polyglycolic acids alone or in combination with polyglactin acid	40 patients with 2 or 3 walls of intraosseous defects.	The local of the study was in intraosseous defects. There were evaluated the following clinical parameters: depth of the pocket and attachment level. Besides that, it was done a pre-surgery radiographic evaluation , at 12 and 24 months after surgery.	The 40 patients were divided into 2 groups: Group A: patients treated with polylactic-polyglycolic acids (fisiograft); Group B: patients treated with polylactic-polyglycolic acids (PLA-PGA) together with polyglactin acid (vicryl Mesh)	Both groups statistically show a significant reduction in the depth of the pocket and gain in clinical attachment level and a linear bone growth. Both treatment modalities are beneficial for the treatment of intraosseous defects.

Salmmartino *et al.* (2009)	Plasma rich in platelets (PRP) in combination with swine reabsorbable collagen membrane (Bio-Gide)	The aim was to investigate the clinical effects of a swine reabsorbable collagen membrane (Bio-Gide) associated with PRP in the bone regeneration after extraçtion of the lower 3rd molar in inclusion mesio-horizontal , in comparison with the use of PRP alone.	The criteria of inclusion were the presence of a distal pocket of the mandibular 2nd molar.	Evaluation of periodontal defects on the distal root of the 2nd molar triggered by the extraction of 3rd molars deep inclusives	The periodontal pocket of the mandibular 2nd molar should present a depth of sondagem ≥ 7,5mm. This 2nd molar had to present a post-extraction defect with the vestibular cortical bone and lingual intact.	Although the obtained results in the evaluation of PRP without the reabsorbable membrane and PRP alone are similar, from a histological point of view, the association of PRP with the Bio-Gide membrane has shown signs of bone maturation, but not a higher degree of bone regeneration. The junction of PRP with the reabsorbable collagen membrane (Bio-Gide) raises the bone maturation, however does not provide a bigger degree of bone regeneration.
Tsai *et al.* (2009)	Fibrin rich in platelets (PRF)	To investigate the biological effects of PRF in gingival human fibroblasts (GFs), cells of the periodontal ligament (PDL), epithelial cells and osteoblasts.	10 voluntary patients.	Data was evaluated in a period of 3 days.	In the 10 patients there were made blood collections.	PRF doesn't interfere with the cellular feasibility of related periodontal cells. PRF stimulated the proliferation of osteoblasts, cells of the periodontal ligament and growth factors, during a period of 3 days. Fibrin rich in platelets (PRF) models a specific proliferation for each type of cell. Those specific actions for each type of cell may be beneficial for the periodontal regeneration.
Kothiwale *et al.* (2009)	Amniotic membrane	To test the combinatio of these membranes in the treatment of furcation defects grade II in humans.	10 patients with chronic periodontitis with furcation defect grade II bilateral	Treatment of furcation defects of grade II in humans	10 patients with chronic periodontitis and with furcation defect grade II bilateral were treated using DFDBA with AM	The results were positive excepto on one patient treated with BDX + AM. After 9 months, the patients treated with DFDBA+AM showed pocket depht (PD) reduction of 4.7mm±0,58, and relative

					(experience A). 10 patients with chronic periodontitis and with furcation defect grade II bilateral were treated using BDX with AM (experience B). Both groups were tested at 6 and 9 months, clinically and radiographically.	attachment level gain of 4.8mm + 0,32, while the patients treated with BDX+AM showed a PD reduction of 4.4mm ± 0,27, and AL gain of 5.1mm ± 0,09. Osseous measurements showed bone fill of 2.1mm ± 0,36 for the DFDBA+Am group and 2,43mm ± 0,38 for the BDX + AM group. Percentage gain in bone was 76,3% for the DFDBA+AM group and 79,6% for the BDX+AM group. The statistical analysis reveals that there are no statistical differences amongst the 2 materials.
Keles *et al.* (2010)	Autogenous bone cortex (ACB) with GTR VS autogenous bone cortex without GTR	The aim was to evaluate the additional benefits of the use of GTR with autogenous bone cortex (ACB) VS autogenous bone cortex without GTR, in the regenerative treatment of intra-bone defects	12 patients with chronic periodontitis (5 men and 7 women), aged between 45.3 ± 4,6 anos.	Intra-bone defects evaluated before the surgery and after 6 months of the surgery.	The patients presented a pocket depth of ≥6mm (PPDs) and were divided into 2 groups: Group 1 – combination of ACB with GTR (absorbable membrane of polyglactic acid); Group 2: ACB alone, without GTR.The evaluated parameters were: the pocket depth, clinical attachment levels (CALs) and radiographic alveolar bone height.	There was a significant gain in the CALs and in the alveolar bone by radiographic analysis and a reduction of the depth of pockets in the patients treated with ACB and GTR (4.25 ± 1.06, 5.50 ± 2.24 e 4.58 ± 1.08) and in the patients treated with ACB grafting only (4.25 ± 1.06, 5.92 ± 2.24 and 4.58 ± 1.08. The differences between treatments are not statistically significant. Both in group treated with ACB + GTR and in the group treated with ACB alone a clinical improvement was radiographically observed after 6 months. The combination of GTR with ACB didn’t present any additional benefit in the treatment of periodontal intra-bony defects.

Chang *et al*. (2011)	Fibrin rich in platelets (PRF)	To present the clinical and radiographic changes of a patient with periodontal intra-bony defects treated with PRF.	38 patients of female sex with intra-bony defects on teeth 36 and 27.	There were studied intra-bony defects, being that there was a radiographic evaluation in the beginning and at 3 and 6 months after surgery.	Teeth 36 and 27 with intra-bony defects were filled with PRF in 38 patients of feminine sex.	The results have shown that the application of PRF as the single material used for intra-bony defects, exhibit reduction of periodontal pockets and gain of the clinical insertion after 3 and 6 months. Comparing the radiographs after 6 months the bone density presented na increase of 1,6 to the tooth 27 and of 1,3 to the tooth 36 in comparison with the pre-surgery radiographs. From a radiographic and clinical point of view at 6 months after the surgery, the use of PRF as the single material of graft seems an effective modality in the regenerative treatment of periodontal intra-bony defects.
Lal *et al*. (2012)	Hydroxyapatite (HA); Cissus Quadrangularis (CQ); Oxidized Cellulose membrane (COM);	The aim of this study was to clinically evaluate the potential osteogenic of hydroxyapatite, cissus quadrangularis and oxidized cellulose membrane	20 patients with periodontitis with age between the 20 and the 40 years old, that presented deep periodontal pockets, defect probing >5mm, vertical bone defect observed radiographically and with 2 or 3 walls involved when the surgical exposure.	The studied locals were the infra-bony defects; Periodontal pockets and the attachment level were evaluated regularly in the months after surgery.	The infra-bony defects were divided into 4 groups, each group comprising 5 defects: Group 1 –control group; Group 2 – group treated with HA; Group 3 – group treated with CQ; Group 4 – treated with OCM;	There was a gradual reduction in the pocket depth in all groups, but however this reduction was higher in the group treated with HA. The gain at the attachment level was higher in the group treated with HA, being of 3,2mm after 6 months. Both the hydroxyapatite membrane and the oxidized cellulose membrane have presented good results in the reduction of the pocket defect, in the gain of the attachment level and in the fill of the bone defect. However, a deeper study shall be realized through a combination of HA and OCM in the treatment of periodontal bone defects with growing factors and stem cells.

Gamal *et al.* (2012)	Modified perforated collagen membrane (MPM) and occlusive barrier membrane (OM)	To determine if the exclusion of gingival and periosteum connective tissue containing stem cells has a positive or a negative effect in the periodontal regeneration comparing to the use of modified perforated collagen membrane with the traditional cell occlusive barrier membrane.	20 patients non smokers with advanced chronic periodontitis	The study was made in intraosseous defects. There were made evaluations at 6 and 9 months after therapy, in order to evaluate the quantitative alterations in the defect, at plaque index level, probing depth (PD), clinical attachment level (CAL) and level of bone crest.	The 20 patients were divided into 2 groups: The group treated with occlusive bovine collagen membranes (OM control group, 10 sites); The group treated with modified perforated collagen membrane (MPM test group, 10 sites)	In the observation after 6 and 9 months, MPM has shown a significant increase in the reduction of probing and gain CAL comparing with OM control group. At the level of defect base level there was no significant difference amongst the 2 groups in the observation periods of 6 and 9 months. The level of bone crest washigher in the MPM group. The post-surgery difference amongst the 2 groups was of 2mm and 1,7mm at 6 and 9 months, respectively, in favor of group treated with MPM. The present study demonstrates improvement in the clinical results when the utilization of modified perforated collagen membrane comparing with cell occlusive barrier membranes in the processes of guided tissue regeneration (GTR).

Table 2: Membranes being tested for its *in vivo* biofunctionality in periodontal regeneration

Reference	Membrane type	Aim of the study	Studied animal/Implementation time	Defect types	Methods	Results
Jung *et al.* (2011)	Bloc of Hydroxyapatite/collagen (BHC)	To elucidate the effect of a bloc of hydroxyapatite/collagen (BHC) in an intra-bony defect in dogs.	Study made in dogs of beagle breed being the time of implementation 8 weeks	Intra-bony defect	A periodontal intra-bony defect (4mm width and 5mm depth), was prepared bilaterally in mesial of 4^{th} bottom PM in 5 dogs of beagle race. After thorough root planning the bloc of BHC wasp placed in a local, the contralateral area of the defect didn't receive any defect, serving as control. The histological analysis of the areas where the membrane was placed was realized after 8 weeks.	Two of five samples of the experimental group were well cicatrized, without dissipation of the membrane. The histological analysis revealed excellent regeneration of the periodontal tissues. However the majority of the grafted materials were deplaced in the other 3 samples, resting only a small portion of graft. The average of the obtained values did not differ significantly between the experimental and the control. The application of BHC alone without membrane in a periodontal intra-bony defect produced inconsistent results both regarding the periodontal regeneration as well as regarding the substantivity of the graft materials. Thus, the use of a barrier membrane is recommended to improve the stability of the grafted material and condense it.

He *et al.* (2008)	membrane of calcium alginate	characterizing the biological effect of the new membrane on the regeneration of bone tissue	rabbits	defects within the mandibles of rabbits	was created a defect 5mm diameter at the corner of the jaw 45 adult rabbits, bilaterally	Quantitative analysis revealed a greater precentagem newly generated bone in the defects treated with calcium alginate membranes than in defects treated with collagen membrane conventional. CAF allows a greater bone growth compared to bioabsorbable collagen membrane. This study of jaw bone defects suggest that reultados with CAF are much more promising perspective in histology and histomorphometry.

Mei *et al.* (2007)	Poly (L-lactic acid) with multilayers of nanotubes of carbon and hydroxyapatite (PLLAXMWNTs/HA)	In this study it was developed a new type of membrane used for guided tissue regeneration (GTR) by electrospinning	Mouses		Nanoparticles of NWTs/HA were evenly dispersed in the membranes and the characteristics of degradation were much improved. After PDLCs were sown in the membrane, this membrane was implanted in the muscle of the paw of little mouses immunodeficients.	Cytological researches revealed that the membrane PLLA/MWNTs/HA improves the accession and proliferation of periodontal ligament cells (PDLCs) in 30% and inhibits the accession and proliferation of cells of gingival epithelium in 30% also, in comparison with the control group. Histological exams proved that PDLCs attached in membranes perform well in life. This new type of membrane satisfies the requirements of GTR techniques, despite of presenting a structure of monolayer. Comparing with other GTR membranes GTR on sale or under research , This membrane may simplify the producing process, reduce the producing cost and void possible errors in the clinical application. Moreover, it doesn't need to be removed surgery. This membrane has a high potential, may being used in the guided tissue regeneration (GTR) and tissue engineering.

Veríssimo *et al.* (2010)	To study the effect of glutaraldehyde (GA) in polyanionic collagen membranes (PAC), through a histological evaluation of the tissue answer (biocompatibility) and evaluating the biodegradation of subcutaneous membranes implanted in mouses				There were studied 6 different samples: PAC, PAC 25, PAC 75, PAC 25 cross-linking with GA and PAC 75 cross-linking by GA. After 1, 7, 15 and 30 days there were evaluated: inflammatory infiltrate, cytokine dosage, thickness of capsule fibrosis, metalloproteinase immunohistochemistry and membrane biodegradation.	The inflammatory answer was more intense in the membranes without cross-linking, while the fibrosis capsules has become higher in the cross-linking membranes after 30 days. The membrane without cross-linking suffered intense biodegradation while the membranes with cross-linking remained intact after 30 days. The cross-linking with GA reduces the inflammatory answer and prevents the degradation of membranes, all over the course of the observation period. These membranes are thus an attractive option when the production of new bone tissue depends of the prolonged presence of a mechanic barrier.

Reis *et al.* (2011)	Semi-rigid PLGA (polyglactide-co-glycoide acid)/CAP (calcium phosphate)	To develop the production of a bilayer biomaterial, made to promote the periodontal regeneration.		The animal used for the study was the dog. Grade II furcation defects	It was developed the semi-rigid PLGA (polyglactide-co-glycolide acid)/CAP (calcium phosphate). This membrane was tested in grade II furcation defects in dogs.	The cicatrization ocurred without complications, the bone volume and the thickness of the trabecular bone had a significant raise in the treated group. The results are rewarding for the periodontal regeneration in comparison with the flexible membranes traditionally used.

Table 3: Membranes being proposed for periodontal regeneration in early stage of development/evaluation

References	Membrane type	Aim of the study	Cells Types	Conditions/Duration of the experiment	Results
Owen *et al.* (2010)	Plasticized poly (lactic-co-glycolic acid) (PLGA)	To evaluate if plasticized poly (lactic-co-glycolic acid) (PLGA) has the ability for being used as a membrane in GTR. The material was also explored as a system of controlled release of antibiotic, tetracycline and anti-inflammatory drugs.	Cells of the periodontal ligament	Films produced of PLGA with methoxypoly /ethyleneglycol (MePEG) or diblocks copolymer [poly (D,L – lactic acid)-block-methoxypoly (ethgleneglycol) were loaded with tetracyclines. The drug release was evaluated using liquid chromatography of high efficiency (HPLC).	The addition of MePEG or diblock caused an increase on the concentration of the rates of release of both drugs. The morphology of the cells of the periodontal ligament is not affected by the inclusion of tetracycline. Plasticized PLGA films present desired characteristics for possible use in membranes of GTR.
Hong *et al.* (2006)	Chitosan (natural biodegradable membrane)	To produce a new type of membrane using na immersion technique with phase of immersion-precipitation (IPPI) with a natural bioabsorbable membrane polymer-chitosan.		The concentration of chitosan solution and the solvent evaporation time were two key-factors that had a significant effect In the porosity, the average size of pores, water absorption rate and the mechanic properties of the porous membrane.	The porous membrane not only degrades as also maintains the integrity of the structure for 5-6 weeks in the enzymatic solution that may attend to the required guided tissue regeneration. The results indicate that the porous of chitosan membrane have excellent biocompatibility and biodegradation, which allows it to be used as a membrane to prevent the apical migration of epithelial cells of gum and promotes the growth of cells of the periodontal ligament in the periodontal therapy.

Liao *et al.* (2006)	Nano-carbonated hydroxyapatite / collagen / poly (lactic-co-glycolic acid).	To explore *in vitro* the biodegradation of membranes used in GTR. Nano-carbonated hydroxyapatite / collagen / poly (lactic-co-glycolic acid).	*In vitro* degradation speciems of Nano-carbonated hydroxyapatite / collagen / poly (lactic-co-glycolic acid) were immersed into artificial saliva solution at 37°C for 1,2,4,8 and 12 weeks. PLGA membrane was used for control of degradation behavior. The concentration of calcium in the residual solution was evaluated.		Till 4 weeks the membrane didn't suffer any alterations. Between 8-12 weeks the membrane has turned into powder. The loss of weight of membranes raised continuously, with a reduction of mass of 23,1% after 4 weeks and 88% after 12 weeks. The calcium concentration in the residual solution has shown a significant raise after 4 weeks, what refers to the degradation of the nano-carbonated hydroxyapatite. The membrane degradation is appropriated for the practice of periodontal regeneration. Furthermore, the new formation of minerals on the membrane surface is a big advantage at the bone regeneration level *in vitro*.

Cetinkaya *et al.* (2006)	Bioactive glass and bioabsorbable membrane	The aim of this study was to compare the proliferative activity of the gingival epithelium using proliferating cell nuclear antigen (PCNA) with a marker of the cellular proliferation after surgical treatments with graft of bioactive glass and bioabsorbable membrane.	Intraosseous defects and cells of the gingival epithelium	20 intraosseous defects were treated with bioactive glass (BG group) or bioabsorbable membrane (BM group). There were made biopsis to the gum before the surgical treatment and after 12 months. It was also histologically evaluated the number of inflammatory de cells.	After 12 weeks of surgical treatment the number of inflammatory cells had a significant decrease. The expression of PCNA had a significant increase. There were no significant differences on the PCNA of the 2 groups in the beginning, however after 12 weeks from the treatment there was a bigger increase in the BG group than in the BM group. These results suggest that the proliferation of epithelium cells is more prominent after the treatment of intraosseous defects with bioactive glass when comparing with the treatment with bioabsorbable membrane.
Ho *et al.* (2008)	Asymmetrical membranes of chitosan	To develop asymmetrical membranes of chitosan for the guided tissue regeneration (GTR)			The results had shown that the asymmetrical membranes of chitosan prepared in this study are promising for the treatment of periodontal diseases. The asymmetrical structure is advantageous when the use of this membrane for the controlled release of drugs in different time periods. A chitosan membrane had also success, in the way that it prevents the proliferation of bacteria, much significantly superior than products of GTR currently commercially sold.

kasaj *et al.* (2008)		To evaluate the biological effects of several bioabsorbable and non-bioabsorbable membranes in cultures of primary human gingival fibroblasts (HGF), fibroblasts of the l periodontal ligament (PDLF) and human osteoblasts (HOB), of cells *in vitro*.	Primary human gingival fibroblasts (HGF), fibroblasts of periodontal ligament (PDLF) and human osteoblasts (HOB)	3 collagen membranes commercially available (bioabsorbable membranes) (Tutodent (TD), Resodont (RD) and BioGide (BG)) and 3 non-reabsorbable membranes of polytetrafluoroethylene (PTFE) (ACE (AC), cytoplast (CT) and TelGem-FD (TG) were tested. The effect of membranes in HGF, PDLF as well as in HOB was evaluated recurring to Alonar Blue fluorometric proliferation after 1,2.5,4,24 and time periods of 48 hours. The structural and morphological properties of membranes were tested by scanning electron microscopy (SEM)	Results have shown that of the 6 tested membranes, TD e RD presented a higher proliferation rate of HGF. In the same way, TD, RD, e BG had significantly a higher number of cells in all the periods of time, when compared with the control group in culture of PDLF. The scanning electronic microscopy (SEM) has shown a microporous structure of all collagen membranes, a compact top surface and a porous bottom surface and the non-reabsorbable membranes (PTFE) have shown a homogeneous structure with a layer of dense symmetric skin. The results of the current study suggest that the materials of GTR membrane, only by itself, may influence the proliferation of cells in the process of periodontal tissue/bone regeneration. Among the 6 weeks analyzed, the biodegradable membranes have shown to be more appropriated to stimulate the cellular proliferation, in comparison with the non-reabsorbable membranes of PTFE.

Hunter *et al*. (2012)	Chitosan (C)-gelatin (G)-Hydroxy apatite (H) (biodegradable membrane)	To study the dynamic interactions existing between the hydroxyapatite-chitosan-gelatin membrane (HCG) and the hMSCs	Human mesenchymal stem or stromal cells (HMSCs) (osteoprogenitor cells)		The results have shown that the membrane (HCG) has sufficient mechanic and structural properties to function as membrane, furthermore, the absorbable proteins of the extracellular matrix promote the osteogenic differentiation of hMSCs.
Wang *et al*. (2009)	Polydimethylsiloxane (PDMS)	To verify if the cellular behavior may be affected by the substrate monotography	Fibroblasts of the periodontal ligament	Plydimethylsiloxane (PDMS) was produced with Anodized aluminum oxide (AAO), becoming nano -PDMS	The cultures of cells in nano-PDMS have a higher proliferation rate, lower apoptosis rate and higher metabolic activity comparing with PDMS without surface modifiers. PDMS is the appropriate material for the growth of cells of periodontal ligament. The aluminum oxide (AAO) supply a simple but effective alternative to modify the surface of PDMS desiring a nano-structure. This may be integrated in other technics of microfabrications for several applications of micro/nano fluidic.

Yang *et al.* (2008)	Nano-apatite (nAp) and poly (E-caprolactone) (PCL) were used for the production of the new membrane	To develop a bioabsorbable membrane that may be used in the bone regeneration	Osteoblastic cells	3 types of porous membranes based in nano-apatite and poly(E-caprolactone) were made by electrospinning. There were evaluated their structural, mechanic, chemical and biological properties.	A test that simulated body fluid immersion confirmed that the presence of nAp improved the bioactive behavior of the membrane. A study made with osteoblastic cells *in vitro* has shown us that great part of membranes support the proliferation, but the presence of nAp facilitates the cellular differentiation. This study demonstrated that in electrospum membrane incorporating nAp is strong, raises bioactivity and supports the proliferation and differentiation of osteoblasts. This membrane may be used as a prototype for a future development of a membrane that will be ideal for having a clinical application.
Mota *et al.* (2012)	Chitosan (C) and bioactive glass nanoparticles (BG-NPs)	To evaluate the behavior of the combination of these two membranes	Cells of the periodontal ligament and cells of the bone marrow of humans	Biological tests were made in the cells of periodontal ligament and cells of the bone marrow of humans in order to evaluate the combination of these two membranes	The combination of these membranes promote the metabolic activity of cells and the mineralization.

Bottino *et al.* (2010)	In this study it was created the novel functionally graded membrane (FGM)	To create a new membrane to be used in the periodontal regeneration.		FGM consists in a nucleous (CC), 2 superficial functional layers (SLs), interface with bone (nano-hydroxyapatite) (n-HAp) and epithelial tissue (metronidazole) (MET). Its design and production was made by sequential multilayers of electrospinning. The morphology of individual fibers and the roughness of functional layers, that contains ano-hydroxyapatite and a layer with incorporation of drugs were evaluated at microscopic level as atomic forces.	The nucleous has shown bigger force and elastic behavior comparing with FGM. The incorporation of n-HAp raises the osteoconductive behavior and the incorporation of MET fights the periodontal pathogenics. The incorporation of metronidazole and nano-hydroyapatite in membranes is a promise for the resolution of the inconvenients of currently available membranes.
Leal *et al.* (2012)	Poly (D-L-lactic acid) Bioglass (PDLLA/BG)	To describe the development and the characterization of membrane composed of poly (D-L-lactic acid) Bioglass (PDLLA/BG) with asymmetric bioactivity.	The cellular behavior in the membrabes Was evaluated using both human bone marrow stromal cells and human periodontal ligament cells	The membranes were prepared through a method of solvent evaporation that adjusted have promoted a non-uniform distribution of the inorganic component along the membrane thickness. Bioactive behavior *in vitro* and the properties of the developed biomaterials were evaluated.	The bioglass indicated being a biomaterial that exhibits asymmetric osteoconductive properties. This membrane composed of poly (D-L-lactic acid) and bioglass stimulates the celular differentiation, mineralization, production of extracelular matrix and calcium nodules, what suggests that there is a positive effect by the time of the addition of bioactive microparticulate in the matrix of PDLLA. Results indicate that membranes PDLLA/BG asymmetric may have potential to be used in the therapy of guided tissue regeneration or in orthopedic applications, with better results.

Chakraboti *et al.* (2011)	Poly(lactic-co-glycolic acid) (PLGA)	To develop the controlled release of tetracycline and alendronate in the poly membrane (lactic-co-glycolic acid) (PLGA) in order to allow an appropriate support for differentiation and proliferation of osteoblasts.			The Alendronate of release was controlled with success The tetracyclines are released more quickly in comparison with the Alendronate. The 2 drugs released simultaneously were biocompatible with osteoblasts and after 5 weeks of incubation, a significant raise of the activity of alkaline phosphatase has been observed and also the formation of bone nodules.