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Bioresorbable Collagen Membranes for Guided Bone Regeneration

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1. Introduction

Localized lack of bone volume in the jaws may be due to congenital, post-traumatic, postsurgical defects or different disease processes. Increasing the bone volume has long been an attractive field of basic and clinical research. The introduction of implant therapy, and the proven relationship between long-term prognosis of dental implants and adequate bone volume at the implant site (Lekholm *et al.* 1986), dramatically increased the interest of both clinicians and scientists in this field, making augmentation procedures an important part of contemporary implant therapy. Basically, four methods have been described to augment bone volume: a. *osteinduction*, using appropriate growth factors (Reddi 1981; Urist 1965); b. *osteoconduction*, using grafting materials that serve as scaffolds for new bone growth (Buch *et al.* 1986; Reddi *et al.* 1987); c. *distraction osteogenesis*, by which a surgically induced bone fracture enables slow controlled pulling apart of the separated bone fragments (Ilizarov 1989a,b); d. *guided bone regeneration*, which allows selective bone tissue growth into a space maintained by tissue barriers (Dahlin *et al.* 1988, 1991a; Kostopoulos & Karring 1994; Nyman & Lang 1994). Among the different methods, guided bone regeneration (GBR) is the most popular and best documented for the treatment of localized bone defects in the jaws, probably due to its relative simplicity of use while allowing the placement of endosseous implants in areas of the jaw with bony defects and/or insufficient bone volume. Highly predictable success rates can be achieved using GBR; in fact, it has been shown that success rates of implants placed at GBR treated sites and sites without bone augmentation are comparable (Hammerle *et al.* 2002).

Guided Tissue Regeneration (GTR) has been introduced into clinical dental practice over 30 years ago, soon after Melcher (1970,1976), followed by Karring *et al.* 1980 and Nyman *et al.* 1980, presented its basic principles to the professional community. The mechanisms of GBR generally followed the same principles i.e. that under certain conditions, cells that originate from tissues adjacent to an exclusively provided space are able to form their parent tissue. In order to allow exclusive repopulation of cells from desired tissues into that space, preference must be given by preventing access of cells from neighboring undesired tissues using tissue barriers, commonly referred to as membranes (Figs. 1,2).

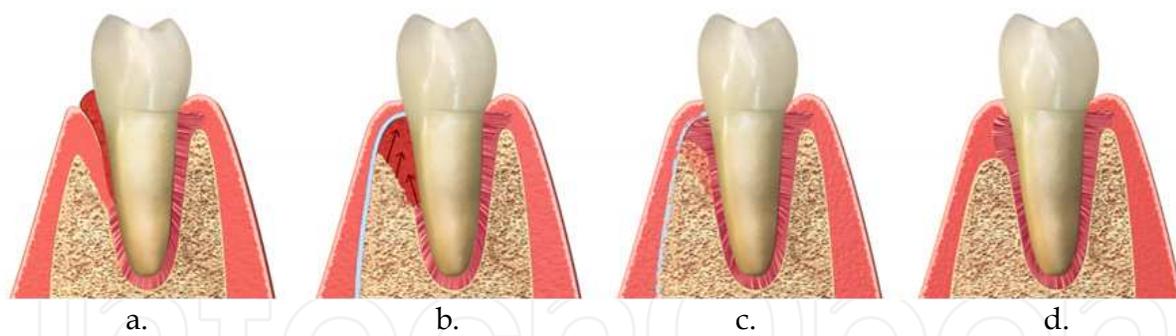


Fig. 1. Guided tissue regeneration scheme describing the use of a resorbable barrier membrane. a. vertical periodontal lesion in the mandible b. resorbable membrane is stabilized over the debrided lesion and covered by the mucosal flap c. after 3- 5 months the membrane starts to resorb; new bone, new periodontal ligament and new cementum are visible d. reestablishment of most of the periodontal attachment apparatus is completed.

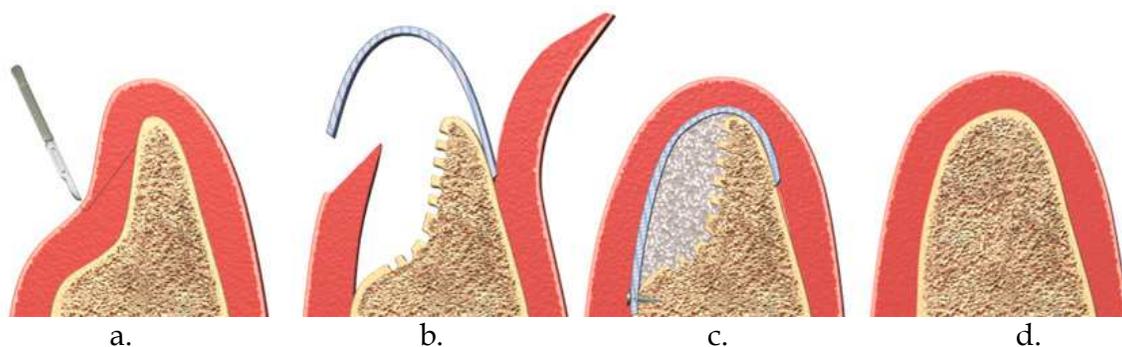


Fig. 2. Guided bone regeneration scheme describing the use of a resorbable barrier membrane. a. bony defect is diagnosed b. the defect is debrided, bone cortex is perforated, membrane supporting scaffold material and a membrane are placed. c. the membrane is stabilized and shaped to dictate the desired bone contours d. a few months later bone regeneration is observed restoring the desired shape of the jaw.

2. Tissue barriers

A wide range of tissue barrier materials has been used in experimental and clinical studies in GTR/GBR procedures, including polytetrafluoroethylene (PTFE), expanded PTFE (e-PTFE), polyglactin 910, polylactic acid, polyglycolic acid, polyorthoester, polyurethane, polyhydroxybutyrate, calcium sulfate, freeze-dried fascia lata, freeze-dried dura mater allografts, native and/or synthetic collagen, micro titanium mesh, and titanium foils. Among the requirements set for GTR/GBR devices are safety and effectiveness. Based on the strict criteria applied to cosmetic/plastic medicine, where no life threatening conditions are involved, documentation of the effectiveness of the procedures and materials should be available while at the same time adverse effects emerging from the implanted devices should be kept to a minimum. In 1994, Hardwick *et al.* formulated a list of criteria for tissue barriers used for GTR; these may also be applied for barriers used for GBR. Briefly, the main essential qualities expected are: a. biocompatibility, b. cell occlusiveness, c. integration by the host tissues, d. clinical manageability and ease to apply, e. space making ability. Since bioabsorbable and biodegradable barriers are degraded *in vivo* and are absorbed by the

body, another additional two requirements need to be fulfilled for these, i.e. that tissue reactions resulting from the resorption of the barriers and its by-products should be minimal and reversible and that the regenerative process should not be negatively affected (Gottlow 1993). There are a number of factors which are critical for the success or failure of GTR/GBR; these include membrane stability, duration of barrier function, ample blood fill of the area for regeneration, enhanced access of bone and bone marrow-derived cells to that space, and prevention of soft tissue dehiscences over the membrane.

Tissue barriers may be classified according to several criteria. For the purpose of this chapter, however, tissue barriers are divided into two main groups: non-resorbable and resorbable. Briefly, non-resorbable barriers are made of thin sheets of materials, mainly polymers. They are stable, non-degradable and biocompatible. The earliest commercial and most popular non-resorbable membranes were expanded polytetrafluoroethylene (ePTFE) membranes; these became a standard for bone regeneration shortly after GBR has been recognized as accepted dental therapy. Expanded PTFE is a polymer with high stability in biologic systems. It resists breakdown by host tissues and by microbes and does not elicit immunologic reactions (Fig. 3). The main limitation of ePTFE and other non

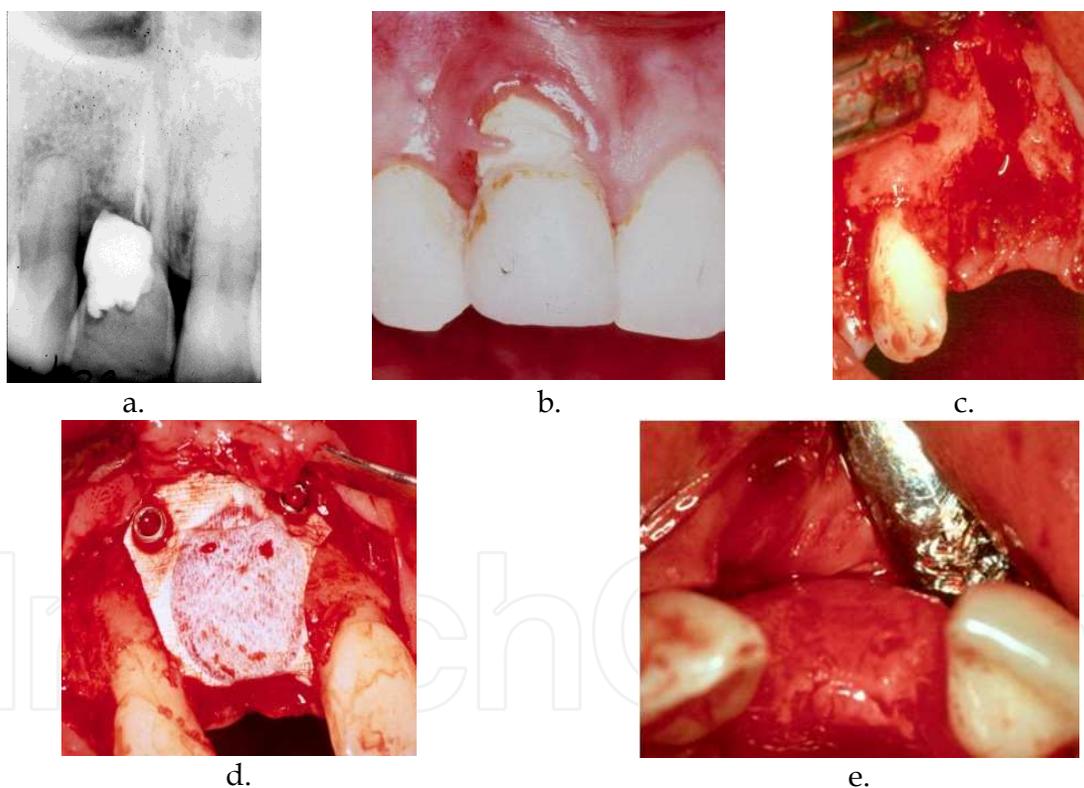


Fig. 3. Successful GBR procedure aimed for implant site development using ePTFE membrane. a. radiographic view showing secondary caries and root resorption indicate extraction of the upper right central incisor b. clinical view and examination support the radiographic findings c. extraction of the tooth reveals significant bone loss at the esthetic zone d. guided bone regeneration using e-PTFE membrane stabilized by two buccal and one palatal titanium screws, and by demineralized freeze-dried bone allograft supporting the sub-membraneous space. e. six months following the procedure the membrane is removed presenting adequate bone volume and proper contours for an implant supported fixed restoration.

resorbable membranes is the need for an additional surgery for membrane retrieval. Non-resorbable membranes have to be removed during a second surgical intervention imposing significant additional morbidity on the patient and additional risk for tissue damage.

A frequent complication associated with non-resorbable membranes is soft tissue dehiscence and membrane exposure; these may be followed by infection. Animal experiments (Gottfredsen *et al.* 1993; Kohal *et al.* 1999a) and clinical studies (Gher *et al.* 1994; Simion *et al.* 1994; Hämmerle *et al.* 1998) have shown that premature membrane exposure and wound dehiscence impair the amount of bone regeneration. Figure 4 presents the exposure and removal of an infected e-PTFE membrane.

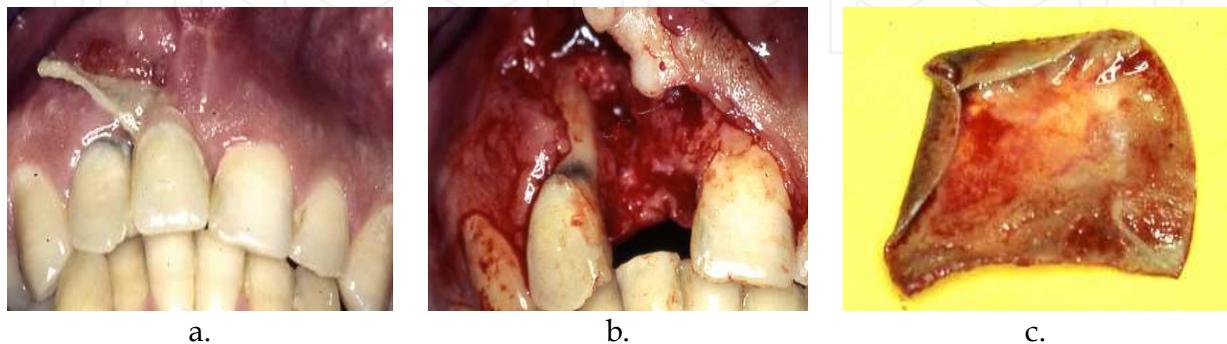


Fig. 4. Failure of a GBR procedure due to premature exposure and infection of an e-PTFE membrane three weeks following membrane placement. a. soft tissue dehiscence expose the membrane to the oral environment. The membrane is contaminated with dental plaque. b. The membrane is surgically removed and the defect debrided c. clinical view of the e-PTFE membrane after removal.

With the development of bioresorbable membranes, and the increasing evidence regarding their effectiveness, the use of non-resorbable membranes has become limited to specific indications.

3. Bioresorbable membranes

Bioresorbable membranes offer many advantages compared with non-resorbable materials. Apart from the fact that there is no need for a second surgical intervention for removal of the membrane, they present improved soft tissue healing (Lekovic *et al.* 1997, 1998; Zitzmann *et al.* 1997); the incorporation of the membranes by the host tissues and rapid resorption if exposed eliminate open microstructures prone to bacterial contamination (Zitzmann *et al.* 1997). Bioresorbable materials that may be used for the fabrication of resorbable membranes belong to the groups of natural or synthetic polymers. The best known groups of polymers used for medical purposes are *aliphatic polyesters* and *collagen*. Over the past decade membranes are made of polyglycolide, polylactide or copolymers thereof or of collagen (Hutmacher & Hürzeler 1995, Tal *et al.* 1991, 1996; Tal & Pitaru 1992; Moses *et al.* 2005, , Friedmann *et al.* 2002, von Arx *et al.* 2001,2005, Rothamel 2005). The qualities of a wide variety of bioresorbable membranes have been investigated experimentally and clinically (Lundgren *et al.* 1994; Mayfield *et al.* 1997; Simion *et al.* 1997; Zitzmann *et al.* 1997; Buser *et al.* 1999, Tal *et al.* 2008a; Tal *et al.* 2008b). Most researchers and clinicians agree that in comparison to non-resorbable membranes, sites treated with

bioresorbable membranes present a lower rate of complications, and have thus largely replaced the non-resorbable e-PTFE membranes becoming the standard for most clinical situations. In a few well designed studies which compared bioresorbable and non-resorbable membranes, (Zitzmann *et al.* 1997, 2001; Christensen *et al.* 2003) no significant difference was found between the two treatment modalities. It should be noted however, that Chiapasco *et al.* (2006), who carried out a systematic review of comparative studies between bioresorbable and non-resorbable membranes, concluded that drawing definite conclusions was impossible due to a lack of sufficient well designed studies.

The development of bioabsorbable membranes for GTR started in the late 80's, soon after the concept had been considered *accepted dental medicine*; in the early 90's however, with the emerging usage of endosseous implants and the growing need for implants site development, regenerative barriers, especially bioabsorbable membranes, received more attention. Therapeutic success, complications and failures were reported, most of which dealing with inflammatory reactions in the tissues adjacent to some bioresorbable membranes, (Sandberg *et al.* 1993; Piatelli *et al.* 1995; Aaboe *et al.* 1998; Kohal *et al.* 1999; Schliephake and Krachtl. 1997).

4. Developing collagen membranes

Among the different materials which were experimented and clinically examined for their potential application as regenerative tissue barriers, collagen appeared to be an optimal choice and was considered to meet most requirements expected from bioabsorbable membranes. Collagens are a family of proteins with a well determined triple helical configuration. Among these proteins, collagen Type I is most prevalent, constituting approximately 25% of the body's proteins and about 80% of the connective tissue proteins. Collagen Type I polymerizes to form aggregates of fibers and bundles. Collagens are continuously remodeled in the body by degradation and synthesis. Type I collagen is degraded only by a specific enzyme - collagenase, and is resistant to any non-specific proteolytic degradation. Collagen biocompatibility, biodegradability and low immunogenicity render it advantageous for extensive application in pharmaceutical or biotechnological disciplines.

Collagen is a weak antigen and most of its antigenicity resides in the telopeptides non-helical terminals of the molecule. These terminals may be removed by enzymes such as pepsin, producing atellocollagen. Atellocollagen's weak antigenicity and weak immunogenicity (Cooperman and Michaeli 1984; Schlegel *et al.* 1997). As well as its relative resistance to degradation, make collagen an optimal choice for implantable devices. Additional advantages that it possess are hemostasis, chemotaxis of periodontal ligament and gingival fibroblasts (Postlethwaite *et al.* 1978, Yaffe *et al.* 1984, Locci *et al.* 1997), easy manipulation and ability to augment tissue thickness (Tal *et al.* 1996). To the best of the author's knowledge, cross-linked medical collagen was first introduced for the purpose of guided tissue barriers in 1984 (Chu 1985, 1987). Using type I and type III dermal bovine collagen, cross linked with Glutaraldehyde (Collagen Inc. Palo Alto, CA) Becker and Tal (1984 - unpublished) have successfully examined the application of collagen membranes on class II furcation lesions in the lower mandibular molar (Fig. 5). It is noteworthy however that extreme care should be taken to avoid any remnants of amides or aldehydes in the final

products since if left behind, these may induce a severe inflammation and damage to the peripheral tissues (Fig.6). Further laboratory improvements, turned this glutaraldehyde cross-linked collagen membranes into a safe and effective device (Pfeifer et al 1989; van Swol et al 1993). The main limitation of this membrane (Periogen, Collagen Inc. Palo Alto, CA) was a relative rapid absorption time varying from 4 – 8 weeks.



Fig. 5. Clinical presentation of a class II Furcation lesion in a lower first molar tooth treated with glutaraldehyde cross-linked type I bovine collagen membrane. a. furcal defect is carefully debrided b. membrane is placed to seal the class II lesion c. clinical view 1 year after surgery d. one year re-entry presents impressive regeneration of the lesion. (Becker and Tal, 1984; unpublished).



Fig. 6. Severe inflammatory gingival reaction in a patient in whom a prototype glutaraldehyde cross-linked bovine collagen membrane was placed beneath the buccal gingiva of the upper front segment, in an attempt to treat gingival recession. The clinical assumption was that glutaraldehyde remnants left after the cross-linking process were the cause for this severe tissue reaction.

4.1 Ribose induced cross linking of medical collagen

Since the resistance of collagen fibrils towards degradation directly relates to the density of the intermolecular cross-links, various collagen cross-linking methods have been used including aldehyde fixatives and imides and treatments such as hydration and radiations. The main drawbacks of such treatments were toxicity and inability to accurately control the degree of cross-linking. In 1988 Tanaka et al. described a novel technique to cross-link collagen that is safe and clinically effective. Briefly, their invention was based on the fact that a glycated collagen-based matrix may be prepared by non-enzymatic glycosylation of native collagen fibrils to irreversibly cross-link collagen polypeptide chains. The sugars employed as cross-linking agents, especially D-ribose, are typically non-toxic and non-immunogenic. Since the degree of cross-linking correlates well with mechanical and biodegradation characteristics of the collagen matrix, the matrix can be readily bio-stabilized

as desired by monitoring the degree of cross-linking during the procedure (Fig. 7). It has been suggested that ribose sugar, practically allows an unlimited degree of cross-linking providing a collagen barrier which is extremely resistant to enzymatic degradation. Clinically, cross-linked collagen membranes retain integrity for longer periods of time (Paul *et al.* 1992).

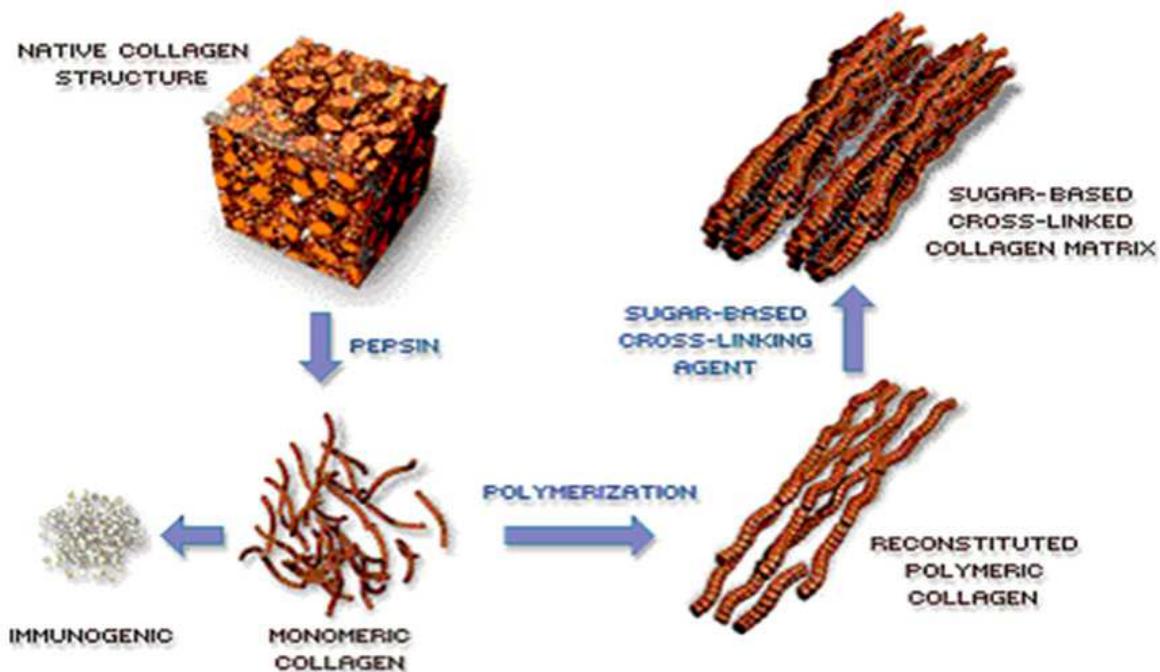


Fig. 7. Synthetic production of Enzymatic cross-linked medical collagen (modified from Colbar Life Science LTD. Ramat Hasharon, Israel).

4.2 Bilayered collagen membrane with an internal polylactide layer

Experimental investigations showed that degradation of collagen membranes may start within 4 days to 4 weeks after membrane placement (Zhao *et al.* 2000; Owens & Yukna 2001). It was therefore questionable whether rapidly degraded membranes can successfully serve as tissue barriers for GBR. In search for different techniques to delay membrane degradation, von Arx *et al.* (2002) hypothesized that combining a bilayer collagen membrane with an internal polylactide layer may prolong its barrier function, as well as enhance the mechanical properties of this hybrid membrane. They evaluated a prototype biodegradable bioresorbable collagen-polylactide hybrid membrane and compared it to ePTFE membrane placed at similar sites in the canine. Histological and histomorphometrical analysis of 2 months specimens, and percentage calculations for areas showing bone regeneration showed that ePTFE sites healed significantly better than the experimental membrane sites with 53% - 96.9% bone regeneration compared to 57% - 85% respectively. The experimental membrane induced moderate infiltration of lymphocytes and plasma cells adjacent to empty spaces corresponding to polylactide fragments. In addition, these reactions appeared to provoke subsequent resorption of newly formed bone. No such findings were seen in ePTFE sites. The authors concluded that the prototype membrane could not be recommended for clinical use.

4.3 Chemical cross-linked porcine type I and III collagens

In an ongoing search for an improved collagen membrane which combines optimal biodegradation over time, high vascularization, favorable tissue integration, and low foreign body reaction, Rothamel et al (2005) compared the biodegradation of differently cross-linked collagen membranes in rats applying the above mentioned parameters. Five commercially available and three experimental membranes (VN) were included: (1) BioGide (BG) (non-cross-linked porcine type I and III collagens), (2) BioMend (BM), (3) BioMendExtend (BME) (glutaraldehyde cross-linked bovine type I collagen), (4) Ossix (OS) (enzymatic-cross-linked bovine type I collagen), (5) TutoDent (TD) (non-cross-linked bovine type I collagen, and (6-8) VN(1-3) (chemical cross-linked porcine type I and III collagens). Specimens were randomly allocated in unconnected subcutaneous pouches on the back of Wistar rats. Histological examination and histometrical evaluation of explanted specimens representing 2, 4, 8, 16, and 24 weeks showed that the highest vascularization and tissue integration was noted for BG followed by BM, BME, VN and TD, while OS exhibited none. Subsequently, biodegradation of BG, BM, BME and VN was faster than TD. Ossix showed only a minute amount of superficial biodegradation 24 weeks following implantation. Biodegradation of TD, BM, BME, and VN was associated with the presence of inflammatory cells. Within the limits of this study, it was concluded that cross-linking of bovine and porcine-derived collagen types I and III was associated with prolonged biodegradation, decreased tissue integration and vascularization, and in case of TD, BM, BME and VN foreign body reactions (Fig 8).

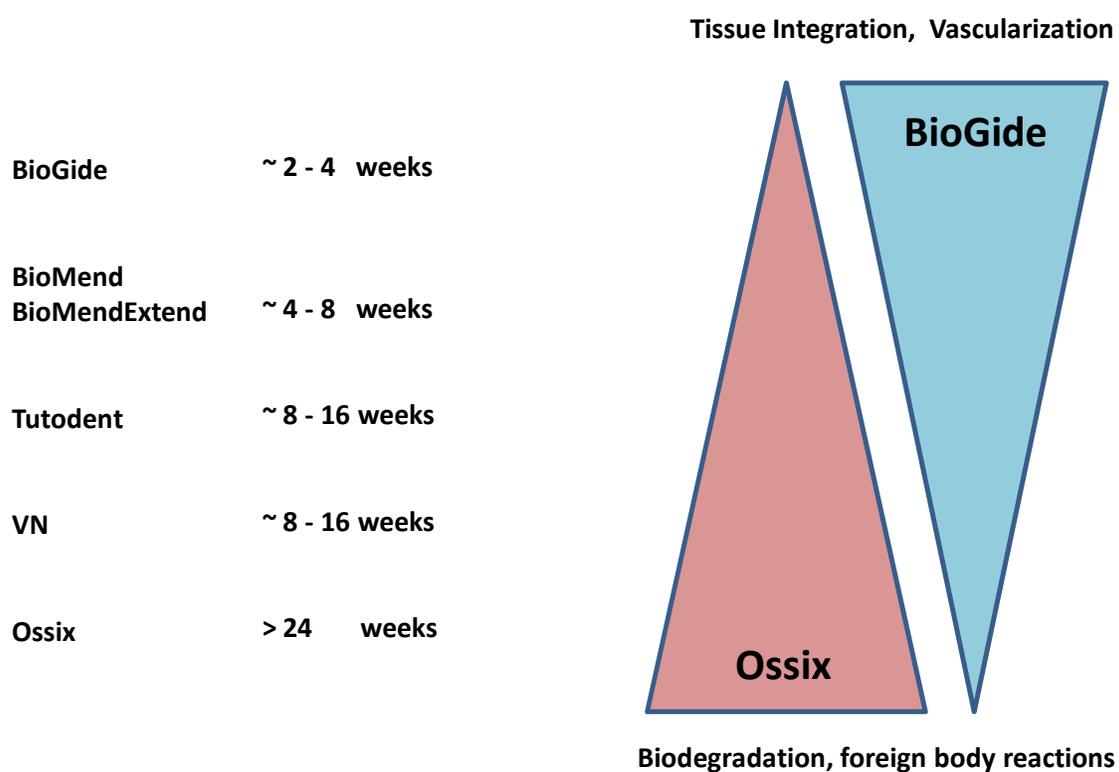


Fig. 8. Prolonged biodegradation seems to be associated with tissue integration, vascularization, foreign body reactions and frequency of tissue dehiscence. Partially modified from Rothamel et.al.(2005).

5. Collagen barrier membranes exposed to the oral environment by spontaneous mucosal dehiscence

Most commercially available collagen barriers have extensively been investigated in both animal models, (Pfeifer *et al.* 1989; Tal *et al.* 1991, 1996; Hyder *et al.* 1992; Tal & Pitaru 1992; Crigger *et al.* 1996) and human studies (Van Swol *et al.* 1993; Al Arrayed *et al.* 1995; Zitzmann *et al.* 1997; Tal 1998, 2004) presenting comparable clinical results to non-resorbable membranes (Cortellini *et al.* 1996; Caffesse *et al.* 1997). It is generally agreed that collagen membranes show a lower incidence of spontaneous exposure to the oral environment compared with non-resorbable membranes, and unlike non-resorbable membranes, soft tissue healing following exposure of collagen membranes involves no infection (Friedmann *et al.* 2002; Moses *et al.* 2005). In a comparative study between prematurely exposed non-resorbable membranes (ePTFE), non-cross-linked collagen membranes (BioGide) and cross-linked collagen membranes (Ossix), the latter were claimed to be superior, and capable of supporting healing (Moses *et al.* 2005). This finding was partially explained by the authors' impression that in prematurely exposed cases, the cross-linked collagen membranes has the capacity to withstand bacterial collagenolytic degradation while facilitating soft tissue healing over the exposed membranes (Moses *et al.* 2005). In an attempt to better understand collagen barrier bio-durability and integrity in sites treated with cross-linked and non-cross-linked collagen barrier membranes, Tal *et al.* (2008a) have clinically and histologically investigated GBR treated sites showing spontaneous mucosal perforations over barrier membranes in humans. In 52 patients, bony defects were grafted with xenograft and covered with collagen resorbable barrier membranes: 26 with cross-linked collagen membranes (Ossix™) and 26 with non-cross-linked collagen membranes (BioGide®) (Fig.9). Post-surgical spontaneous mucosal perforations and membrane exposures were recorded. Surgical sites were monitored for 6 months before implant placement.

During implant placement procedure, full thickness soft tissue discs, 3 mm in diameter, were punched out from 18 Ossix™ and 18 BioGuide® sites for histologic examination. Of the 52 sites, 33 were intact and 19 (36.5%) were associated with spontaneous membrane exposure: 13 (50%) were in Ossix™ sites and 6 (23.1%) in BioGuide® sites ($p < 0.05$). Perforation sites clinically healed within 2-4 weeks over both CL and NCL membranes. Histologically, Ossix membranes were intact in all 9 non-perforated sites, but in the perforated Ossix™ sites, membranes were interrupted in 5 and undetected in four. BioGuide® membranes were undetected in all 18 specimens examined. In 3 non-perforated Ossix membrane sites, ossification was associated with or within the membrane. It was concluded that Ossix™ membranes were more resistant to tissue degradation than BioGuide®. At non-perforated sites, Ossix™ membranes maintained integrity during the 6 months study period of time while BioGuide® membranes disintegrated (Fig.10).

Neither CLM (Ossix™) nor NCLM (BioGuide®) were resistant to degradation when exposed to the oral environment. Ossix™ membranes were associated with a significantly higher incidence of tissue perforations compared with BioGuide® membranes (Fig. 11).

There is no satisfactory explanation for the increased incidence of spontaneous exposure incidence at CLM treated sites compared with NCLM sites. Based on the observation that

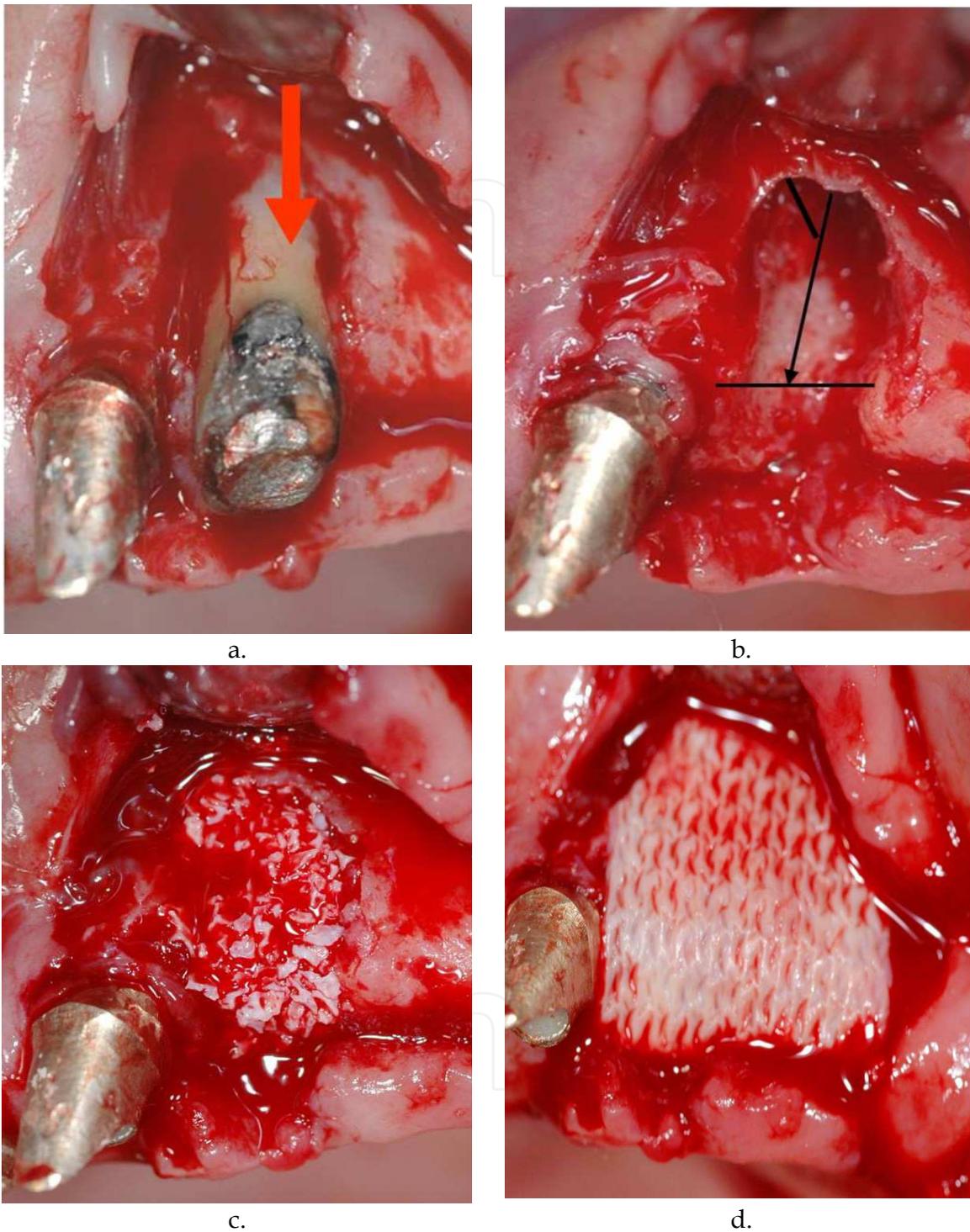


Fig. 9. Clinical view of a ridge preservation procedure a. severe bone loss and complete resorption of the buccal plate of the alveolus associated with a longitudinal root fracture. b. the fresh extraction socket is debrided and measured c. the socket and bony defect are grafted with xenograft d. the grafted socket and defect are covered with a cross-linked collagen resorbable membrane.

the degree of cross-linking is negatively correlated with the attachment and proliferation of PDL fibroblasts and human osteoblast-like cells observed in in-vitro cultures (Rothamel *et al.* 2004), the tendency of the CLM to split from the adjacent connective tissue may be due to lack of attachment between the two, and lack of membrane vascularization in the early healing phase. This phenomenon has been described by Rothamel *et al.* (2005) who examined the biodegradation of differently cross-linked collagen membranes in the rat and found that the Ossix™ membrane exhibited only slight superficial vascularization and minimal surface resorption after 24 weeks compared with Bio-Gide® - a native dermal porcine collagen which showed early vascularization and over 80% resorption after 8 weeks. It should be stressed that in their animal study Rothamel *et al.* (2005) made complete tissue closure in extra-oral subcutaneous pouches, while in our human study (Tal *et al.* 2008a) a significant number of sites were spontaneously exposed to the oral environment. However, bone growth patterns shown in this study, included membrane ossification beneath the membrane leaflets and new bone growth adherent to the membrane (Figs. 12,13), suggesting high bio-compatibility between the membrane and osteoblasts (Rothamel *et al.* 2004). Although this study was limited to evaluation of membrane bio-durability rather than sub-membraneous bone regeneration, it is noteworthy that a recent histological evaluation of changes during ossification and cellular events at GBR sites, has shown that in the rat maxillae, NCLM (BioGide®) associated with defect-derived bone had integrated to such a degree that it was difficult to distinguish the membrane-derived new bone from that generated in the cavity (Taguchi *et al.* 2005). Based on these observations, these authors suggested that NCLM participated in the osteogenic differentiation.

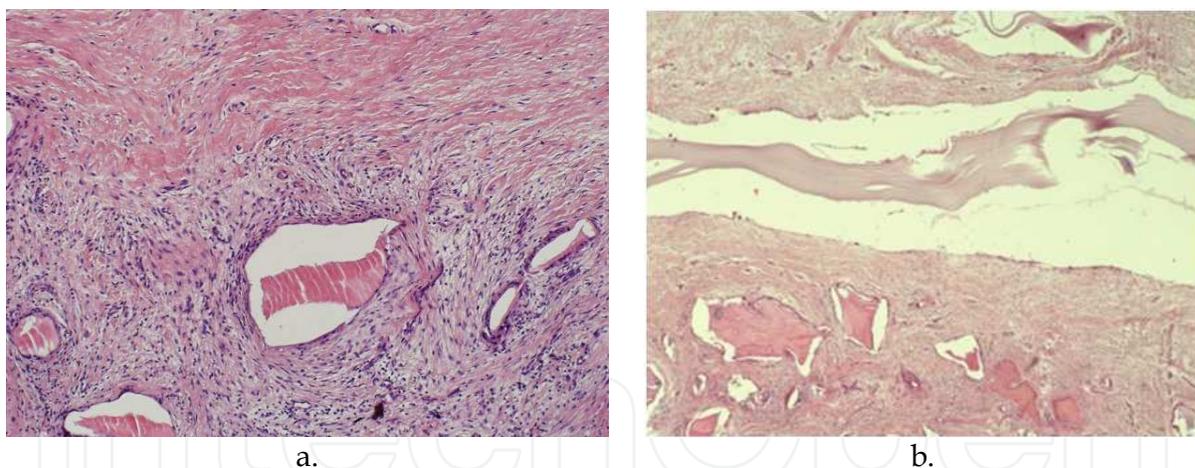


Fig. 10. Histological examination of non perforated sites 6 months after membranes placement. a. the native collagen membrane (BioGide®) completely disintegrated, and its location can be identified by the abrupt transition from mature supramembraneous connective tissue to young cellular submembraneous connective tissue b. the cross-linked membrane (Ossix™) maintained integrity and separates between the mature supramembraneous connective tissue and the newly established submembraneous connective tissue.

The findings described by Tal *et al.* (2008a,b) are in agreement with the report of von Arx *et al.* (2005) who claimed that in the rabbit calvarium cross-linked collagen membranes clearly displayed prolonged membrane integrity compared with non-crossed-linked collagen

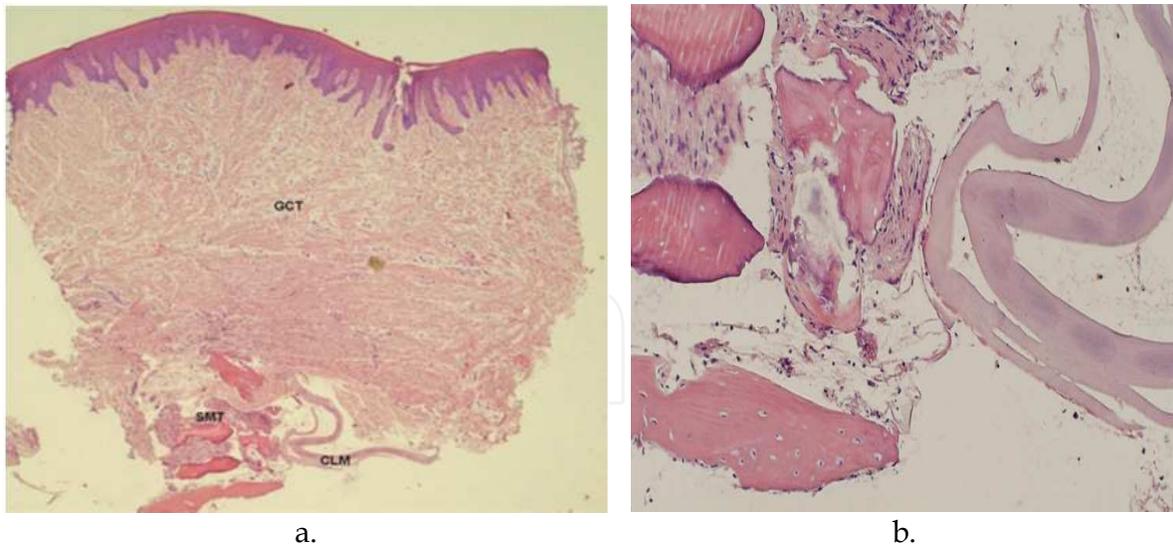


Fig. 11. a. Histological aspect of CLM remnants in a perforated site. The membrane (CLM) is interrupted under the perforation and identified at the peripheral borders of the specimen (H&E, original magnification x20). b. Higher magnification (x40) from a. showing membrane remnants (right) associated with graft particles and new bone growth.

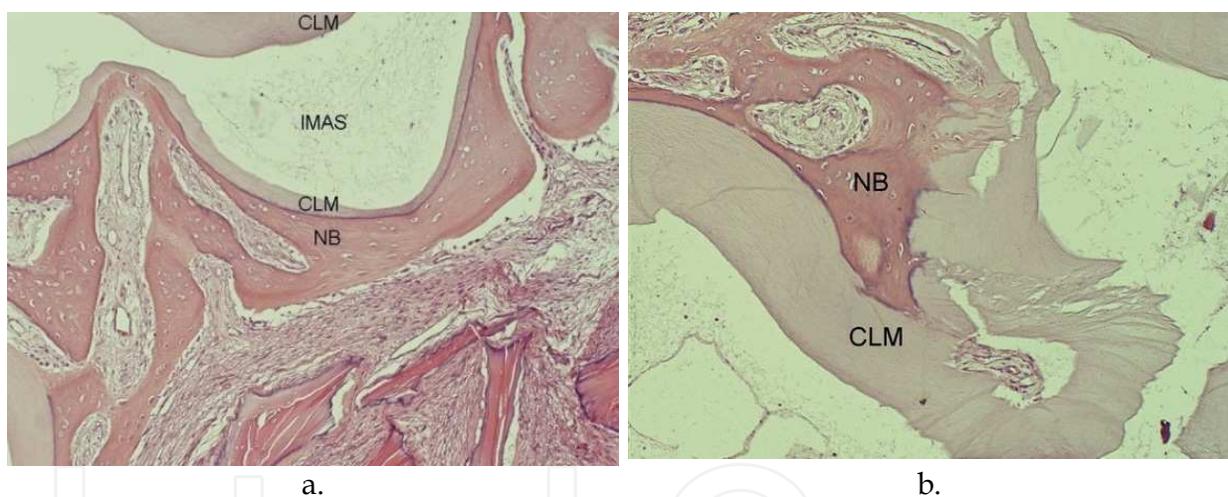


Fig. 12. Histologic view of CLM remnants in a non-perforated site showing a. new bone adhered to the membrane, (H&E, original magnification x20). b. Occasionally bone growth is observed between the membrane leaflets penetrating the membrane (CLM) and perhaps replacing it (H&E, original magnification x40).

membrane. However, since their experiment was performed in a close wound, no tissue inflammatory response was involved. The studies of Tal et al (2008a,b) disagree with the interpretation that in prematurely exposed cases crossed-linked collagen membranes have “the capacity to withstand bacterial collagenolytic degradation” when exposed by soft tissue dehiscence (Moses *et al.* 2005). It is noteworthy that long epithelial ridges were observed in several of the healed perforation lesions associated with the crossed-linked group (FIG 14). Similar epithelial ridges have been previously described by Tal and Dayan (2000) and Tal *et al.* (2001) in human specimens retrieved from mucosal early perforations over submerged implants. While no scientific proof is available, it may be logical to assume that these

epithelial ridges are remnants of epithelial down-growth that separates the exposed membrane from the perforated gingival connective tissue during early healing and before the exposed membrane resorbs.

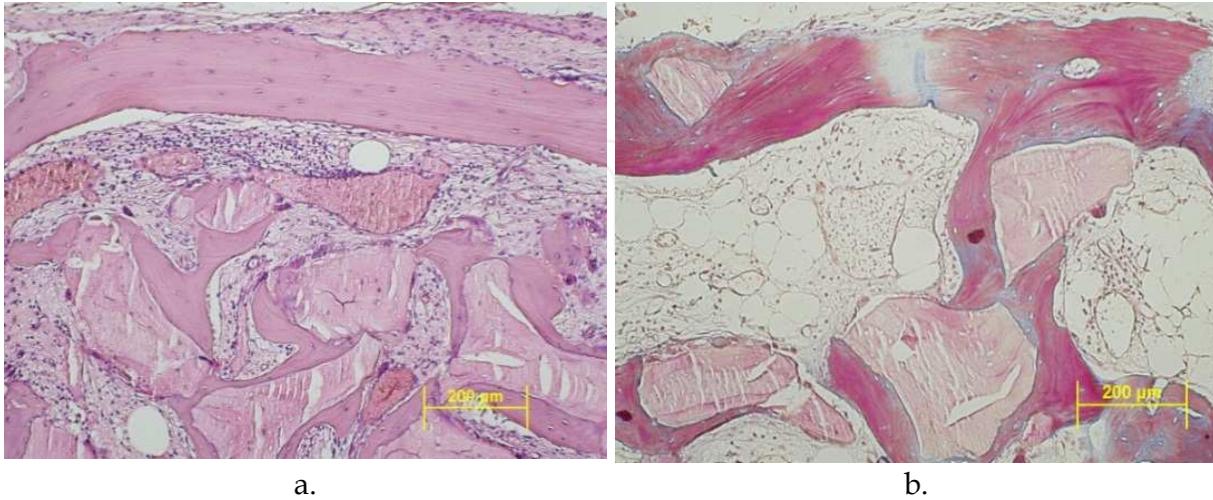


Fig. 13. Histologic view of new bone adhered to an ossified CLM in a non-perforated GBR site; new bone in intimate contact with graft particles and the resorbed membrane is observed. a. H&E, original magnification (x100). b. (x100).

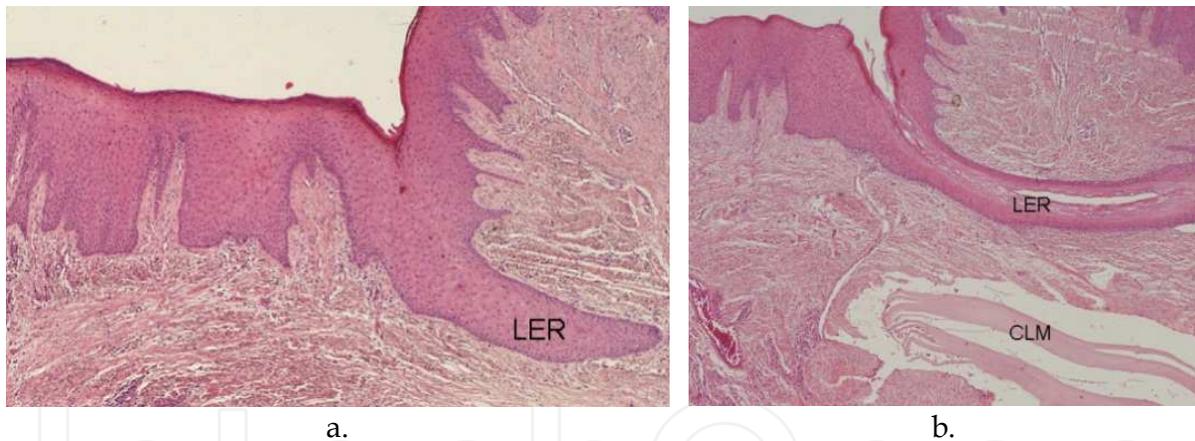


Fig. 14. Histological aspect of a CLM specimen from a perforated site. Long epithelial ridge (LER) is shown at the periphery of the healed perforation. b. Long epithelial ridge (LER) is shown along the cross-linked membrane remnants (CLM), peripheral to the perforation site. A thin layer of connective tissue separates it from the membrane (H&E, original magnification x20).

To further understand the bio-degradation of cross-linked and non-cross-linked collagen membranes, an animal study was undertaken in which membranes were experimentally exposed to the oral environment (Tal et al 2008b). In 8 cats, 48 surgical procedures were performed, 3 along each side of the palate: 32 soft tissue perforations were made, and 16 full thickness mini-flaps were raised. Cross-linked and non-cross-linked collagen membranes discs were placed either under the perforations and peripheral mucosa and left exposed (experimental), or covered by the flaps (controls) (FIG.15).



Fig. 15. Clinical view of two experimental perforation sites (top/middle) and one control site (bottom) in the cat palate. Top - Cross-linked collagen membrane disc placed under the surgically perforated mucosa; Middle - non cross-linked membrane disc placed under the surgically perforated mucosa; Bottom - membrane disc placed under surgically elevated mucosal flap and then covered (control).

The four treatment modalities were equally distributed among 8 animals. Study design provided 7 and 28 days histological specimens for each treatment modality. Histological observations revealed that cross-linked and non-cross-linked collagen membranes remained intact in the control sites during the 28 days study period. At 7 and 28 days, Cross-linked membranes appeared interrupted in 3 and 2 experimental sites, respectively, and were undetected in the remaining experimental sites. There was no statistical difference between control specimens and between CLM and NCLM of the different treatment modalities (Fig. 16). It was concluded that if covered, both cross-linked and non-cross-linked membranes were resistant to tissue degradation and maintained continuity throughout the study. However, none of the membranes was resistant to degradation when exposed to the oral environment, even after 7 days.

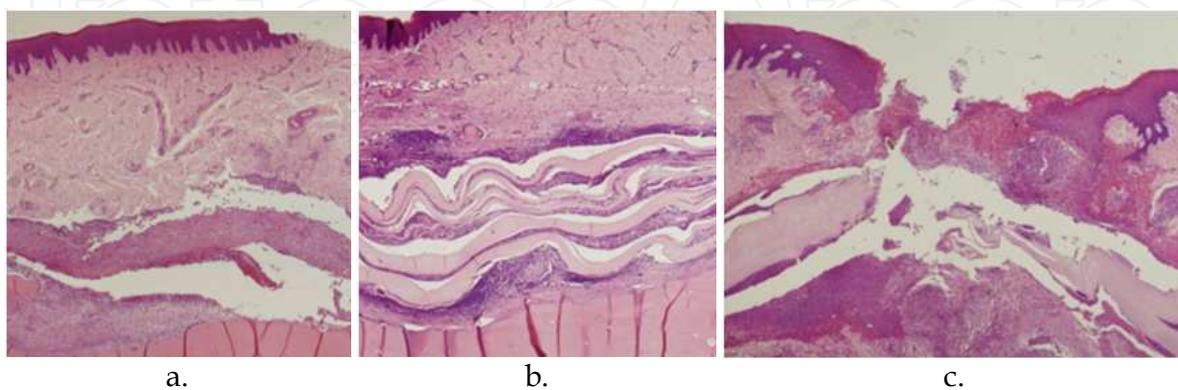


Fig. 16. Histologic view of a. 28-day control NCLM specimen. The NCLM presents intimate contact with the palatal bone on the one side and with the soft connective tissue on the other side. b. 28-day control CLM specimen. The CLM presents intimate contact with the palatal

bone on the one side and with the soft connective tissue on the other side. Connective tissue penetration between the membrane and the bone is present and membrane separation to leaflets is observed. c. 7-day experimental CLM specimen showing disintegration of the membrane under the perforation. Submembranous granulation tissue growth and peripheral epithelial migration are observed. Inflammatory infiltrate is abundant around and between the membrane layers.

6. Increasing the longevity of non cross-linked collagen membranes

6.1 Double layer native non cross-linked collagen preparation

In search for formulas that will extend the functional activity of collagen membranes *in vivo*, Kozlovsky et al (2009) have evaluated the bio-degradation of a two layer preparation of a native Type I and Type III porcine collagen membrane (Bio-Gide® (BG) (Geistlich, Wolhusen, Switzerland)) and compared it with that of the commercially available single layer membrane. It has been shown that four weeks after subcutaneous implantation of BG membrane in the rat clavaria, reduction in membrane thickness combined with nearly complete biodegradation was reported (Zhao et al 2000, Rothamel et al 2005). When applied in surgical pouches in the palate of mongrel dogs (Owens & Yukna 2001) moderate to complete degradation was reported 4-8 weeks following implantation. It has been suggested that application of a second layer of Bio-Gide® (BG) membrane (double layer technique) may reduce micro movement and improve its stabilization, thus enhancing its protective effect in the sub-membranous augmented area (von Arx & Buser 2006). Kozlovsky et al (2009) investigated the influence of the additional layer of the barrier membrane on its durability by measuring quantitatively the *in vivo* degradation of barriers composed of one vs. two layers of Bio-Gide®. The degree of membrane disintegration was histochemically measured based on the amount of residual membrane collagen labeled with biotin (Zohar *et al.* 2004). Two circular bony defects, 5-mm in diameter, were made in the calvaria of Wistar rats. Bio-Gide® membrane 5-mm diameter discs, labeled with biotin, were placed in these defects either as a mono layer (MLM) or as a double layer (DLM) (Fig 17).

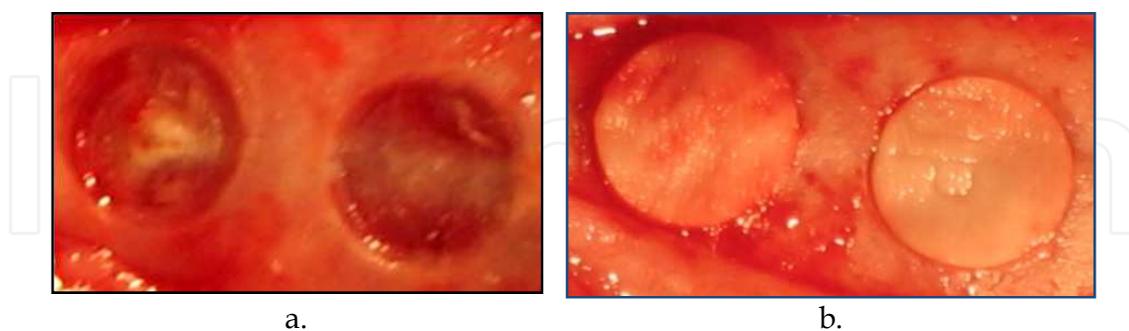


Fig. 17. Photographs of the surgical procedure. (a) on the mid-line of the parietal bone, (along the sagittal suture) two similar, 5-mm diameters, approximately 1 mm deep) bony defects are prepared. (b) The labeled membrane discs are laid over one defect as a mono layer membrane (MLM) (right) and over the other defect as double layer membrane (DLM) (left)

Rats were sacrificed after 4 and 9 weeks and histology performed. Membranes were stained with Horseradish peroxidase-conjugated Streptavidin and Aminoethyl Carbazole as substrate for detection of biotinylated collagen. Collagen degradation in the mono layer BG

ranked 60% during 4 weeks post application. The use of two layers of membranes resulted in a similar (60%) resorption rate of the initial collagen content however, since the initial total collagen content in the double layer membrane was roughly twice the amount of the monolayer one, the amount of residual collagen at 4 weeks was significantly greater in the double layer sites. The rate of collagen degradation for mono layer and double layer membrane sites at 9 weeks was similar (~80%), with twofold amount of residual collagen content at the DLM sites. In addition, the residual thickness of the DLM compared with the MLM was 81% and 74% after 4 and 9 weeks respectively. Thus, it has been shown that despite of similar collagen degradation rates of both MLM and DLM, the application of a second layer of Bio-Gide® (BG) membrane (double layer technique) results in a significantly greater residual amount of collagen, at least up to 9 weeks post surgery in rats. Since membrane thickness was reduced by ~30% between 4 and 9 weeks while the collagen surface area was reduced by 50-60% during that period, it may be suggested that the main pattern of resorption is an internal rather than an external one. Since the Bio-Gide® membrane is structured like an interconnected porous system with large interstices, it is most suitable for transmembranous formation of blood vessels (Schwarz et al 2006), facilitating its resorption (Rothamel et al 2004, Schwarz et al 2006). Indeed the transmembranous vascularization of the membrane was manifested histologically already 4 weeks following implantation and become well-defined through all the layers of the membrane 9 weeks following implantation (Fig 18).

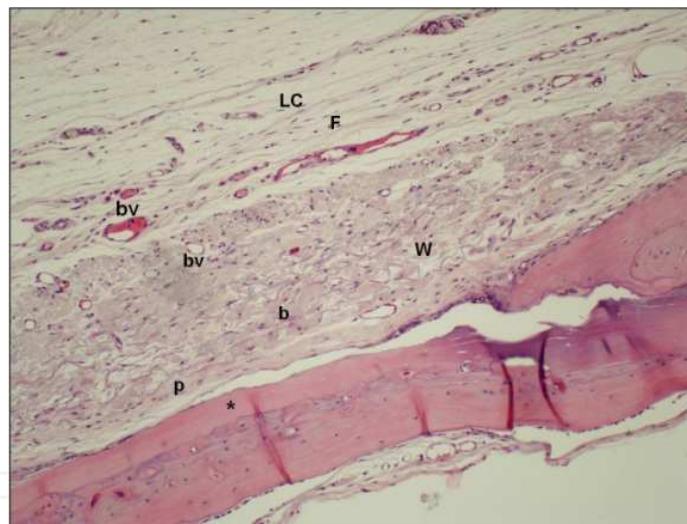


Fig. 18. Photomicrographs of hematoxylin and eosin-stained mono-layer membrane (MLM), 9-weekpost-implantation. Original magnification $\times 100$. bv- blood vessel; p- periosteum-like structure; b- eosinophilic collagen bundles; w- wavy short fibers; LC- loose connective tissue;

Schwarz et al (2006) proposed that the vascularization process may also contribute to membrane degradation since the monocytes penetrating through the blood vessel wall may differentiate into macrophages. Kozlovsky et al (2009) reported that in spite of the difference in the thickness of the 2 membrane preparations, similar degradation rate of 80% for both membranes was measured at 9 weeks. Since the trans-membranous formation of blood vessels is essential for collagen resorption (Schwarz et al 2006), it seems that the vascularization of the double layer membrane was not impaired by its increased thickness.

It has been claimed that increasing the density of cross links between collagen molecules has a negative effect on membrane biocompatibility (Schwarz et al 2006, Rothamel et al 2004), membrane to tissue integration and vascularization, and inhibits attachment and proliferation of PDL fibroblasts and osteoblasts (Rothamel *et al.* 2004, 2005). Using a second layer of resorbable, cross-linked membrane avoids these disadvantages, while extending membrane longevity. In the double layer 9 weeks membrane specimens, central intra - membrane neo-ossification was clearly identified with collagen fibers embedded in the osteoid (Fig 19), confirming a phenomena previously described by others (Taguchi et al 2005, Tal et al 2008).

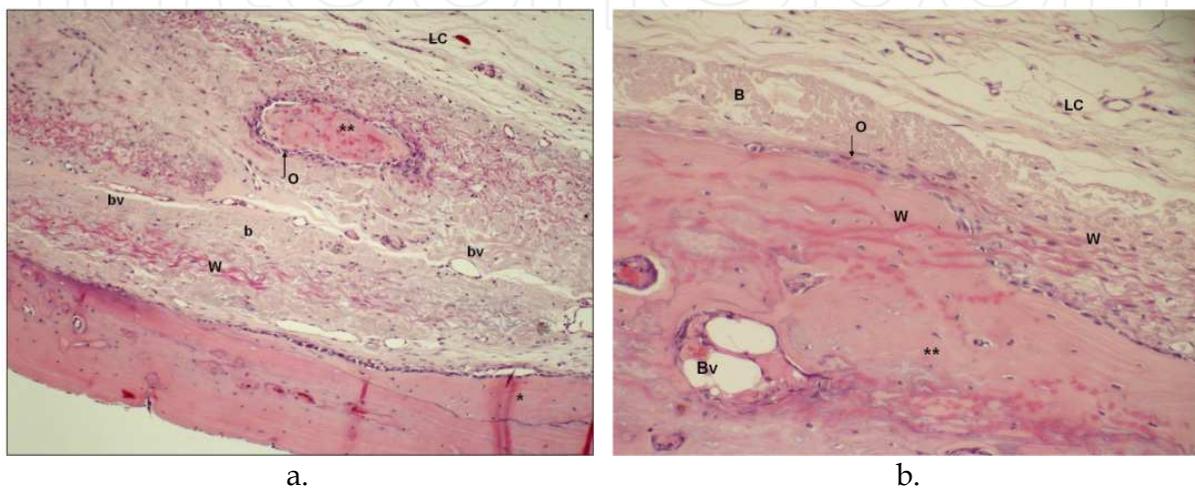


Fig. 19. Photomicrographs of a double-layer membrane (DLM) membranes 9-week post-implantation. a. P-periosteum-like structure; B - eosinophilic collagen bundles; W - wavy short fibers; LC - loose connective tissue ;BV-blood vessel; O - osteoblasts. *Newly formed bone at the floor of the defect; **central ossification. (Hematoxylin and eosin Original magnification $\times 100$). b. Newly formed bone (**) adjacent to the membrane collagen bundles (B) with osteoblasts (O) arranged on the surface. Strongly eosinophilic wave-like long collagen fibers (W) of the membrane penetrate the osteoblastic layers and incorporate into the bone matrix of the centrally neo-ossified membrane. BV, blood vessel; LC, loose connective tissue (Hematoxylin and eosin; Original magnification $\times 200$).

While this finding has never been fully understood, it may be speculated that the significant increase in membrane thickness and longevity result in increasing angiogenesis and cellular population of the collagen matrix, leading to cell proliferation, differentiation and ossification.

6.2 Increasing collagen structural stability by collagen membranes immersion and systemic tetracycline administration

In vivo breakdown of medical collagen has thoroughly been investigated. Collagenase, the enzyme responsible for collagen biodegradation, belongs to the matrix metalloproteinases (MMP's) family of enzymes which are normally present in Mammalian tissues. Collagenase which is produced by a number of body tissues and cells, degrades collagen as part of the physiological connective tissue remodeling. Neutrophils, monocytes and fibroblasts are believed to play a major role in collagen degradation mechanisms in wounds healing by releasing MMPs (Reynolds 1994, Armstrong 2002). In vivo biodegradation of collagen

membranes depends on collagenolytic activity; it has, therefore, been suggested that in addition to increasing the collagen structural stability by cross-linking, inhibition of MMP's may further slow down the collagen degradation (Golub *et al* 1998). It has been shown that Tetracycline, an antibiotic with anti-collagenolytic properties, inhibits MMPs activity (Golub *et al* 1997, Greenwald *et al* 1998). It has also been shown that the use of tetracycline-coated expanded polytetrafluoroethylene (ePTFE) barrier membranes results in additional gain of clinical periodontal attachment, most likely due to its antimicrobial activity (Zarkesh *et al.* 1999). Modulation of wound-healing has been successful applying sub dosage formulation of TTC, lacking antimicrobial activity while retaining the MMP inhibitory capacity. This phenomenon probably occurs due to TTC inhibition of extra-cellular MMP, likely due to its chelating activity on Ca^{++} and Zn^{++} ions (Golub *et al.* 1987). Furthermore, inflammatory cytokines including TNF-alpha, IL-1 beta, and IL-6 are markedly down-regulated in patients during treatment with tetracyclines. This phenomenon also reduces the amount of MMP's present in inflamed tissues, contributing to a reduction of the collagenolytic activity (Chung *et al* 1997). The effect of immersing collagen membranes in varying TTC concentration solutions on the in vitro degradation rate was evaluated (Moses *et al.* 2001) . Membranes were incubated in either phosphate buffered saline or with TTC-HCl dissolved in concentrations of 5 mg/ml, 50 mg/ml or 100 mg/ml. The TTC impregnated and non-impregnated membranes were incubated with either bacterial collagenase or cultures of human bone lineage cells. Membrane degradation was examined on days 2, 4, 7, and 14. It was concluded that collagen membranes immersed in 50 mg/ml TTC solution exhibited the longest degradation time, both in the clostridial collagenase and the human bone cell lineage assays. Immersion in a 50 mg/ml TTC solution before implantation was, therefore, considered most effective in delaying collagen membranes degradation (Moses *et al.* 2001) [FIG 20].

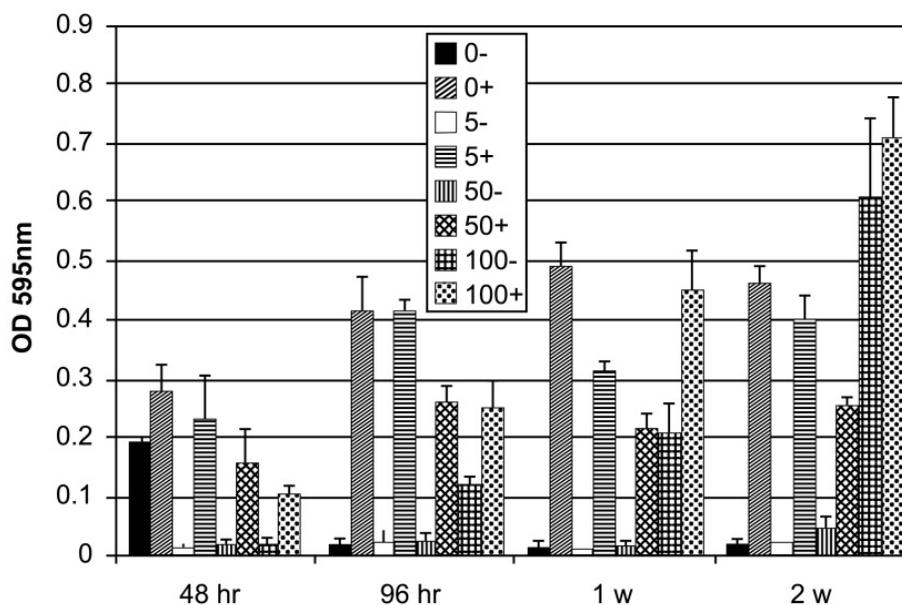


Fig. 20. Degradation of collagen membrane (measured as optical density (OD) at the 595 nm wave length with (+) and without (-) collagenase in different Tetracycline concentration.

These findings suggested the hypothesis that immersion of collagen in TTC prior to implantation could delay collagen degradation also in-vivo. The effect of soaking collagen membranes in different concentrations of tetracycline hydrochloride solutions (TTC) on membranes degradation rate in the rat calvaria (Zohar *et al.* 2004) was evaluated. Prior to implantation in the rat calvaria membranes were labeled with Biotin. The histological slides were stained with Avidin and horseradish peroxidase (HRP) to detect remnants of biotinylated collagen. Staining intensity was correlated to the amount of the remaining collagen and analyzed by image-analysis software. It was found that the staining intensity of membranes that were soaked in 50 mg/ml of TTC exhibited > 11-fold higher intensity than PBS soaked membranes (control) (Figs. 21-23). It was concluded that soaking collagen membranes in 50 mg/ml TTC solution is an effective, practical, and simple tool to reduce membrane degradation rate in vivo.

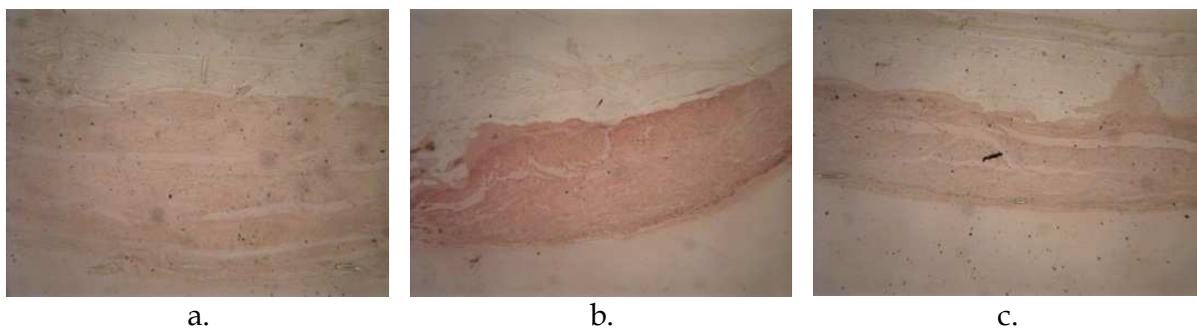


Fig. 21. Histological view (x40) of the membranes 7 days after implantation with different tetracycline (TTC) concentrations. a. Phosphate-buffered saline alone (0 mg/mL TTC); b. 50 mg/mL TTC. c. 100 mg/mL TTC. Collagen stained in red/brown with Avidin-Biotin-HRP reaction.

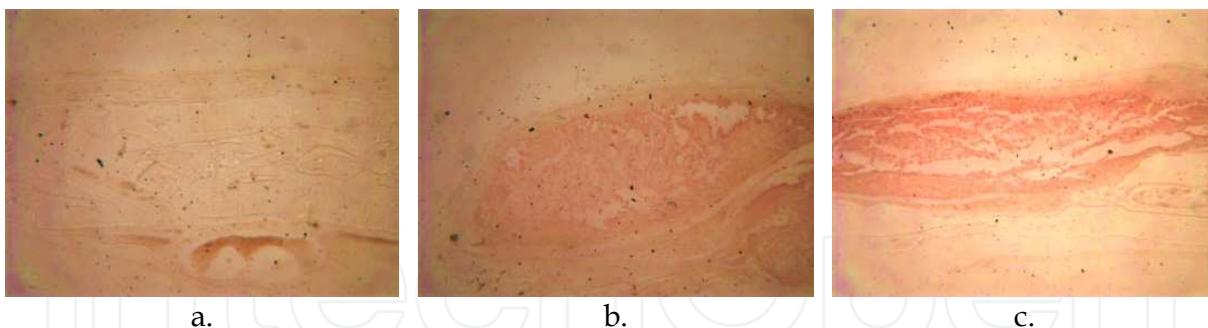


Fig. 22. Histological view (x40) of the membranes 21 days after implantation with different tetracycline (TTC) concentrations. a. Phosphate-buffered saline alone (0 mg/mL TTC); b. - 50 mg/mL TTC. c. 100 mg/mL TTC. Collagen stained in red/brown with Avidin-Biotin-HRP reaction.

The question of whether administration of subantimicrobial dose of TTC may have an effect on the degradation of collagen membranes in vivo still remained. Evaluation of the in vivo degradation of collagen membranes treated by combined TTC immersion and systemic administration in the rat have showed that immersion of collagen membranes in TTC solution prior to their implantation and systemic administration of TTC significantly decreased membranes' degradation (Figs. 24, 25). It was therefore concluded that this

technique may offer a treatment alternative to reduce bio-degradation and enhance bio-durability of certain collagen membranes.(Moses *et al.* 2010).

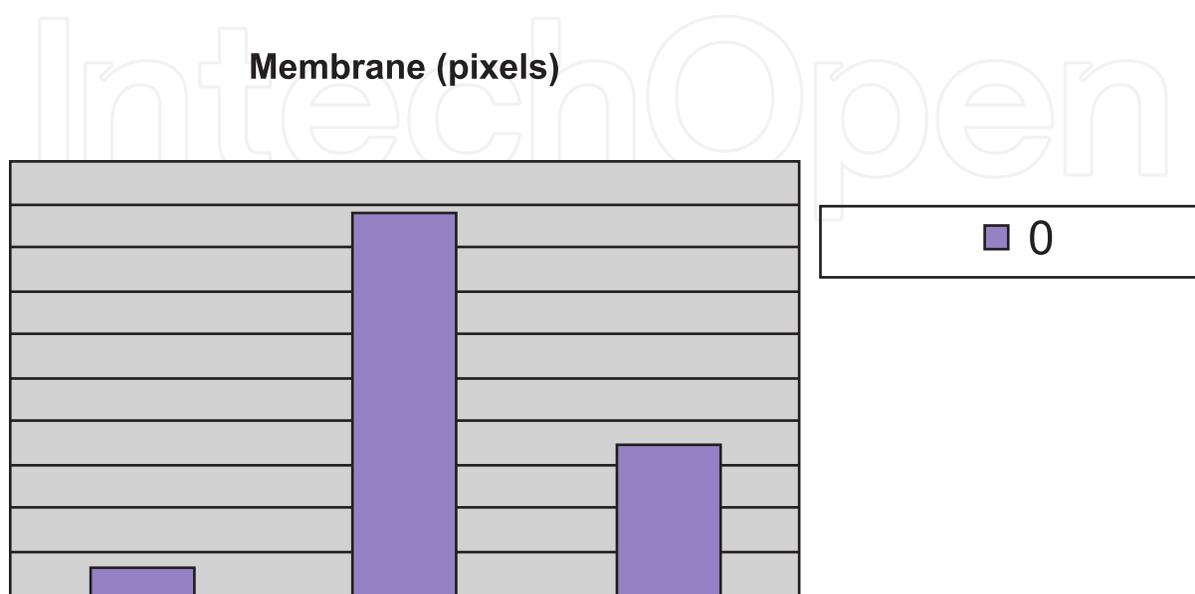


Fig. 23. Color intensity of biotinylated material as measured by number of colored pixels within collagen membrane discs from all 3 groups, 3 weeks.

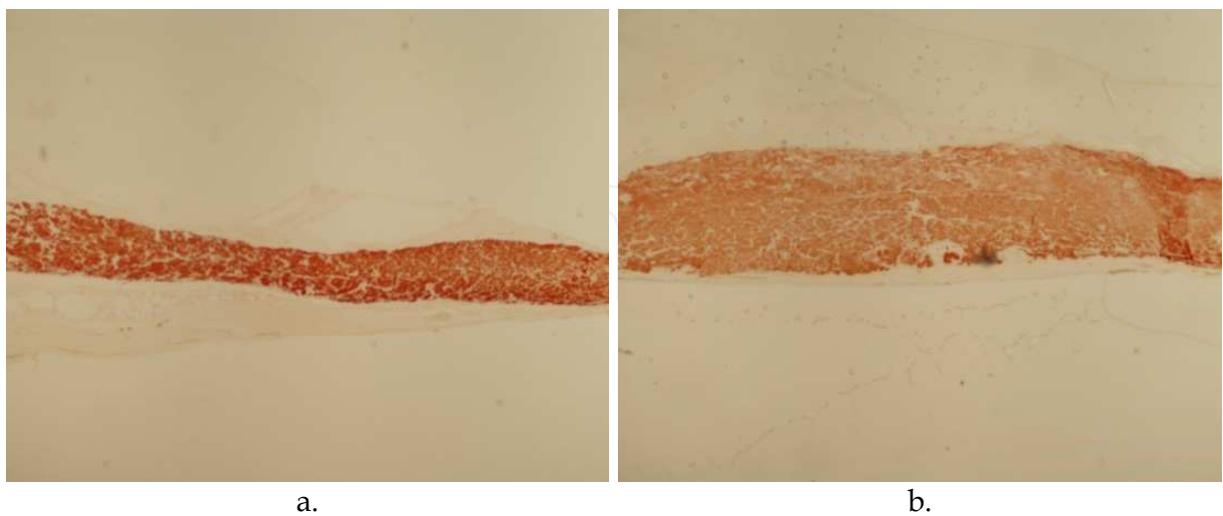


Fig. 24. Histological view (x40) of the membranes 21 days after implantation with different tetracycline (TTC) concentrations with systemic administration of TTC. a. Phosphate-buffered saline alone (0 mg/mL TTC); b. 50 mg/mL TTC. Collagen stained in red/brown with Avidin-Biotin-HRP reaction.

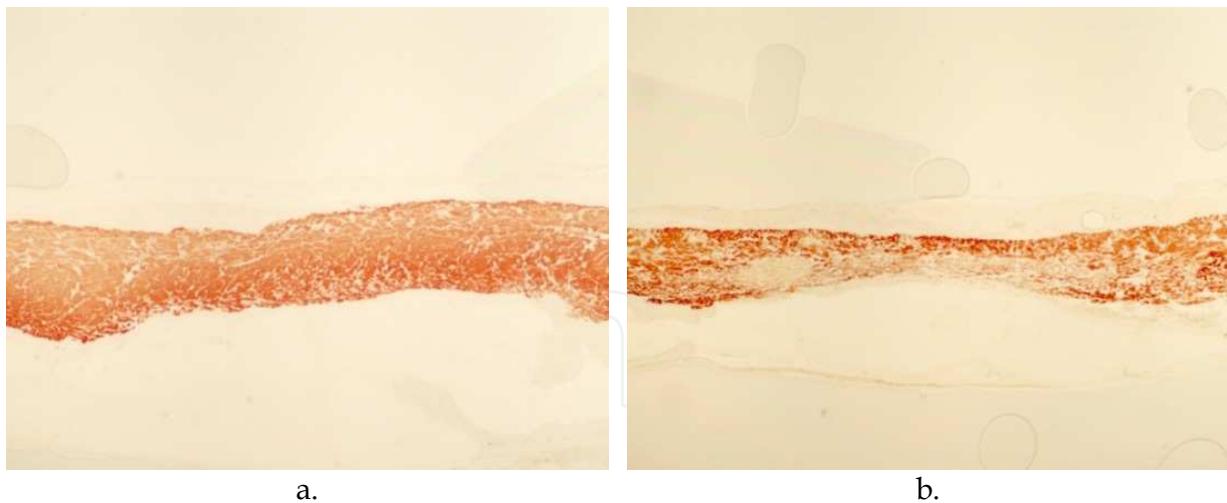


Fig. 25. Histological view ($\times 40$) of the membranes 21 days after implantation with different tetracycline (TTC) concentrations without systemic administration of TTC: a. Phosphate-buffered saline alone (0 mg/mL TTC); 2. 50 mg/mL TTC. b. Collagen stained in red/brown with Avidin-Biotin-HRP reaction.

7. Membrane supporting materials

Published reports have shown that a wide range of membrane supporting materials are suitable fulfilling the requirements set, making harvesting of autogenous bone unnecessary for most procedures (See Chapters 11-14). It has been shown that in self membrane-supporting defects, where there is no need to support the membrane in order to provide space, tissue regeneration is superior to that achieved by using membrane supporting agents; in fact, the optimal osteoconductive membrane supporting material is one that interferes the least with bone regeneration within the membrane occluded space i.e. blood clot (Fig. 26). Among many materials, deproteinized bovine bone mineral, a fully synthesized homogenous hydroxyapatite and beta tricalcium phosphate, and demineralized and mineralized freeze

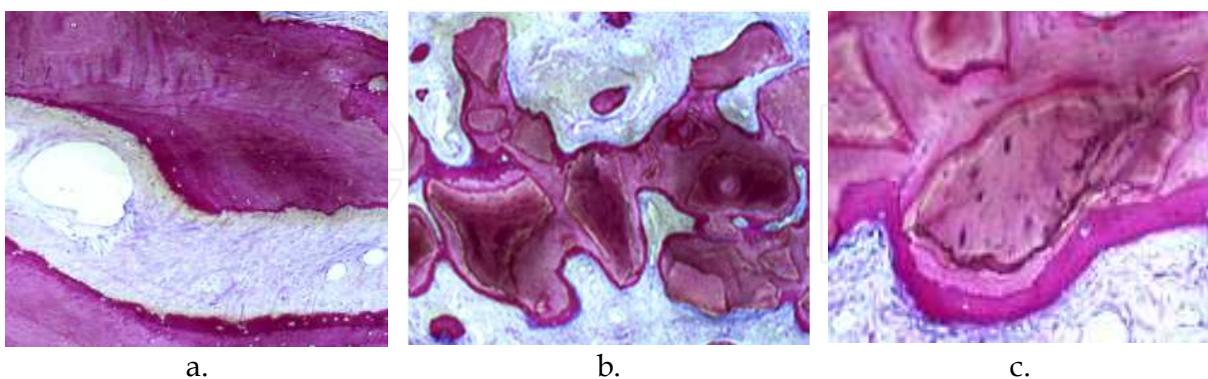


Fig. 26. Experimental guided periodontal regeneration in the dog. a. new bone growth (above) in a membrane provided space filled with natural coagulum. New cementum (below) and separating periodontal ligament were also regenerated. b. GTR of the same space filled with deproteinized Bovine Bone Mineral. Particles of the xenograft intimately surrounded by bone are observed c. higher magnification from b. showing a particle of deproteinized Bovine Bone Mineral intimately surrounded by new bone.

dried bone allografts, consistently demonstrated satisfactory clinical results. While this statement is applicable for osteoconductive materials, current research is focused on the application of growth and differentiation factors - natural proteins and polypeptides that regulate tissue regeneration. To learn more about these, the reader is referred to chapters 1-5.

8. Conclusions

Bioresorbable and non-resorbable membranes can successfully be used for bone regeneration. Bioresorbable membranes generally show better clinical performance compared with non-resorbable membranes and are the barriers of choice wherever possible. The parameters for selecting membranes also include mechanical properties, risk for spontaneous early exposure and ease of clinical handling. The search for improved barriers is underway; presently, collagen's biocompatibility, biodegradability and low immunogenicity render it advantageous for application in pharmaceutical or biotechnological disciplines in general, and in guided bone regeneration in particular. Among the membranes examined by our group *Ossix*^{TM*} (heavily cross-linked collagen) was found to be the most resistant to biodegradation but showed the least tissue integration and vascularization, while *BioGide* (native, non-cross-linked collagen) presented high tissue integration and vascularization. Clinically, *Ossix*^{TM*} was associated with a higher rate of soft tissue dehiscence followed by rapid resorption following membrane exposure to the oral environment.

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* *Ossix-P*TM Colbar, Ramat Hasharon, Israel

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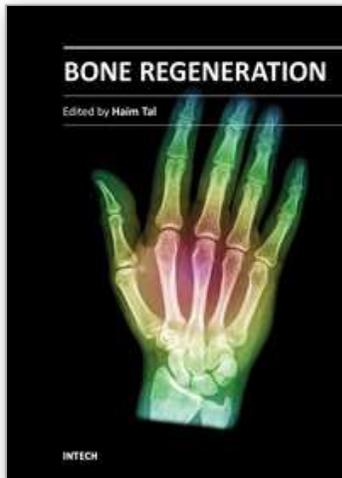
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Bone is a specialized connective tissue, most prominently characterized by its mineralized organic matrix that imparts the physical properties that allow bone tissue to resist load, to support functional organs, and to protect highly sensitive body parts. Bone loss and bone damage may occur as a result of genetic conditions, infectious diseases, tumours, and trauma. Bone healing and repair, involves integrative activity of native tissues and living cells, and lends itself to the incorporation of naturally derived or biocompatible synthetic scaffolds, aimed at replacing missing or damaged osseous tissues. There are several modalities of bone regeneration including tissue engineering, guided bone regeneration, distraction osteogenesis, and bone grafting. This book concentrates on such procedures that may well be counted among the recent outstanding breakthroughs in bone regenerative therapy.

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