Physiochemical properties and resorption progress of porcine skin-derived collagen membranes: In vitro and in vivo analysis

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The aim of the present study was to evaluate the physiochemical properties and resorption progress of two cross-linked, porcine skin-derived collagen membranes and compare their features with those of a membrane without cross-linking (Bio-Gide® [BG], Geistlich Biomaterials, Wolhusen, Switzerland). Three porcine skin-derived collagen membranes, dehydrothermally (DHT) cross-linked (experimental), DHT and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (DHT/EDC) cross-linked (experimental) and BG were investigated for their morphology, enzyme resistance, and tensile strength in vitro and biodegradation in vivo. DHT and DHT/EDC membranes exhibited irregular, interconnected macro- and micropores that formed a 3D mesh, whereas BG exhibited individual collagen fibrils interlaced to form coarse collagen strands. In enzyme resistance and tensile strength tests, DHT and DHT/EDC membranes demonstrated good resistance and mechanical properties compared with BG. In vivo, all three membranes were well integrated into the surrounding connective tissue. Thus, the DHT membrane exhibited its potential as a barrier membrane for guided bone and tissue regeneration.

Keywords: Animal study, Biodegradation, Carbodiimide, Collagen, Dehydrothermal

INTRODUCTION

The goal of periodontal regenerative surgery is reconstruction of periodontal tissues that are broken down by periodontal disease and promotion of new alveolar bone, periodontal ligament, and cementum formation1-3). Studies investigating commonly used techniques for tissue regeneration, including guided bone regeneration (GBR) and guided tissue regeneration (GTR), have emphasized the need for barrier membranes to prevent the apical growth of unwanted cells (epithelium and gingival connective tissue) within the wound area and maintain space for an adequate period during the early healing phase4-5). Barrier membranes used in GBR/GTR procedures should satisfy several physical and chemical requirements, including good biocompatibility, tissue integration and cell occlusivity, and easy manipulation in the clinic6). Currently, barrier membranes are classified into two major groups: nonresorbable and resorbable. Expanded polytetrafluoroethylene (ePTFE) is a typical example of a nonresorbable membrane7). Although ePTFE membranes remain stable throughout an extended period, their main limitation is the requirement for additional surgery for retrieval8). Furthermore, early spontaneous membrane exposure to the oral environment and subsequent bacterial colonization have been reported to be common phenomena associated with nonresorbable membranes, which frequently require early, premature retrieval9). Therefore, resorbable membranes with comparable clinical outcomes have become popular.

Collagen membranes have been widely investigated in animal studies and human clinical studies, and have demonstrated excellent biocompatibility and cell affinity10,11). These membranes differ according to their tissue of origin. Purified bovine and porcine collagen derived from tendons, dermis (skin), pericardium and other regions has been shown to be suitable as donor material12). However, the major drawback of native collagen is rapid biodegradation by the enzymatic activity of macrophages and polymorphonuclear leucocytes13,14). One key factor regarding collagen membranes used for GBR/GTR procedures is that the membrane must remain intact in the wound area for an adequate duration to prevent apical migration of the epithelium during the early healing phase. If the membrane dissolves quickly, treatment goals will not be achieved15). To decelerate resorption, various cross-linking techniques have been developed. These include ultraviolet irradiation, dehydrothermal (DHT) treatment, and treatment with cross-linking agents such as glutaraldehyde, hexamethylene diisocyanate and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC)16-17).

DHT treatment is a common physical cross-linking method for delaying biodegradation, and many studies have confirmed the superiority of the technique. In
vitro studies have demonstrated that DHT membranes showed favorable mechanical properties due to the formation of ester and amide bonds\(^1\). In a clinical study, Lee et al.\(^2\) reported that DHT membranes possessed more stable maintenance characteristics than non-cross-linked collagen membranes.

In general, chemical cross-linking is the most widely used technique in the production of collagen membranes. EDC has been identified to be a satisfactory chemical cross-linking agent to modify collagen scaffolds\(^3\). A previous study demonstrated that EDC membranes were safe as biomaterials, with adequate tissue integration capacity\(^4\). However, when cross-linking using EDC only, the major disadvantage is the large volume of solvent required. Furthermore, cross-linking with improper concentrations of EDC may alter the three-dimensional (3D) structure of the membrane itself, making it “watery” in consistency and, in turn, difficult to use in clinical practice\(^5\).

Collagen membranes with different cross-linking structure exhibit significant differences in biodegradation pattern, resorption time, and extent of inflammatory cell invasion\(^6\). Despite the widespread use of cross-linking treatments for collagen membranes, DHT cross-linking only or multi-cross linking of DHT and EDC has seldom been performed on porcine skin-derived collagen membranes. In the present study, we evaluated the physicochemical properties and resorption progress of DHT cross-linked and multi-cross-linked porcine skin-derived collagen membranes and compared their features with those of a membrane without cross-linking.

**MATERIALS AND METHODS**

The following three porcine skin-derived collagen membranes (thickness, 0.4 mm) were investigated in the present study: DHT cross-linked membrane; DHT and EDC cross-linked membrane; and Bio-Gide® (BG, Geistlich Biomaterials, Wolhusen, Switzerland). The former two membranes were the experimental membranes (monolayer). BG, a bilayered collagen membrane derived from porcine type I and type III collagens without cross-linking, was used as a control membrane. Both experimental membranes (Neobiotech, Seoul, Korea) were derived through the purification of type I collagen from porcine dermis (PD).

**Fabrication of the two experimental collagen membranes**

Fresh PD was harvested from a slaughterhouse. First, PD samples were soaked in a 1.5 M sodium chloride solution and incubated on a shaking platform at 37°C for 72 h to remove residual blood vessels and foreign materials. After washing with distilled water, the samples were frozen at -60°C for 1 h. Subsequently, the adipose layer was removed using a slicer and decellularized in a solution of 1.5 M sodium hydroxide and 99.8% acetone (ratio 6:4) at 150 rpm for 2 h at 4°C on a shaking platform, followed by soaking in a solution of 99% ethanol, and incubated for an additional 16 h.

Finally, the samples were homogenized in a lysis buffer including 0.12% (v/v) Triton X-100, 10 mM Trizma® base, and 4 mM sodium dodecyl sulfate to eliminate residual RNA and DNA. After removal from the solution, the samples were transferred onto wire mesh racks and lyophilized at -60°C for 24 h: these were the prototype experimental collagen membranes. Next, the DHT membrane was prepared by compression for 18 h in a vacuum oven, followed by cross-linking using DHT treatment in a vacuum at 110°C. The oven temperature was decreased to room temperature before releasing the vacuum, which completed the DHT membrane fabrication process.

For fabrication of the DHT/EDC membrane, the fabricated DHT samples were used in the EDC reaction. First, the DHT samples were immersed in a solution consisting of 99% ethanol and deionized water (ratio 5:5) and incubated for 1 h at 4°C. Subsequently, 2.5 mM EDC solvent was added to the solution for cross-linking for 24 h at 4°C at 150 rpm on a shaking platform. After this step, the samples were washed with deionized water three times (15 min per wash) and then washed with 1× phosphate-buffered saline (PBS, pH 7.4) for 1 h at 4°C to eliminate the remaining solvents. After a thorough wash out, samples were frozen at -60°C for 1 h and lyophilized at low chamber temperature (-86°C). This completed the DHT/EDC membrane fabrication process.

**Morphological analysis**

For the evaluation of morphological characteristics, three dry samples of each membrane were cut to a specific size using scissors and observed under scanning electron microscopy (SEM; Hitachi S-350, Tokyo, Japan). The SEM images of the surface and cross-sectional view of each membrane were compared.

**Enzyme resistance test**

For evaluation of degradation properties of the three membranes, samples of each membrane (n=10) were cut to a size of 10×10 mm², and immersed in a 0.25% porcine trypsin solution (Sigma-Aldrich, St. Louis, MO, USA), and incubated on a shaking platform (bioreactor) at an ambient temperature of 37°C. After 4, 8, 12, and 24 h, the trypsin solution was removed carefully through suction, and the samples were dried for 24 h at 37°C. Next, the weight of the dried samples was measured, and the weight of the remaining membrane was expressed as a percentage (%).

**Tensile strength test**

Collagen membranes were prepared in strips of 20×4 mm and subjected to uniaxial tensile strength tests using a texture analyzer (TA-CT, Stable Microsystems, Brookfield, Middleboro, MA, USA). The tensile strengths of dry and wet (soaked in PBS for 24 h) membranes under repeated loading and unloading at a rate of 5 mm/ min were compared. All tests were performed at room temperature.
Observation of biodegradation in vivo

1. Experimental animals
Thirty albino male Wistar rats (age, 11±0.5 weeks; weight, 350±20 g) were used in the present study. Animal selection, management, preparation, and surgical protocols were evaluated and approved by the Institutional Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea (approval number 2014-0332). In total, 90 membranes were allocated to three representative groups, which were further divided into five subgroups according to the time point of assessment: 1, 2, 4, 8, and 12 weeks after membrane implantation.

2. Surgical procedure
The animals were sedated in a chamber with 4% isoflurane (Ifran, Hana Pharm, Kyonggi-Do, Korea) in 100% O₂, followed by anesthetization using an intraperitoneal injection of 15 mg/kg zoletil (Zoletil 50, Virbac, Carros cedex, France) and 10 mg/kg rompun (Bayer®, Ansan, Gyeonggi-do, Korea). A rectangular area on the back of each rat, measuring approximately 6 and 5 cm in length and width, respectively, was depilated using an electronic shaver. After disinfection of the back using povidone iodine (Povidin, Firson, Cheonan, Chungcheongnam-do, Korea), a paramedian skin incision was made exactly along the vertebral column, followed by the separation of three unconnected subcutaneous pouches, two on the right side of the incision line and one on the left side, and vice versa. Subsequently, the membranes were randomly allocated to the resulting 90 pouches (Fig. 1). Primary wound closure was achieved using horizontal mattress 4.0 vicryl sutures (Resorba, Nurnberg, Germany). To minimize postoperative pain and prevent infection, all rats received subcutaneous antibiotics (enflaxacin [10 mg/kg], once per day for 5 days) and analgesics (meloxicam [1 mg/kg], once per day for 5 days). The rats were housed in cages under specific pathogen-free conditions, during which time they had ad libitum access to standard laboratory food pellets and water. At 1, 2, 4, 8, and 12 weeks after implantation, the animals in the respective groups were humanely euthanized in a carbon dioxide chamber. The remaining membranes with the surrounding soft tissue were removed and fixed in 10% neutral buffered formalin for 1 week.

3. Histological processing and histomorphometric analysis
All formalin-fixed samples were embedded in paraffin. Three sections (4 µm thick) were cut from each block and stained with Masson’s trichrome. Digital images were acquired using an optical microscope (Olympus BX41, Olympus Optical, Tokyo, Japan) equipped with an Olympus DP 72 camera. After conventional microscopic examination, images of the slides were evaluated using a software program (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA). The thickness of the membrane body was measured linearly in five randomly selected fields (Fig. 2). All measurements were performed by one blinded and calibrated examiner who had no knowledge of the study design. Membrane thickness was used as a sign of resorption progress, and volume stability, tissue integration, and vascularization of the membrane body were descriptively assessed.
**Statistical analysis**

All statistical analysis were performed using commercially available software (SPSS version 20.0, SPSS, Chicago, IL, USA). Histomorphometric records for the residual membranes were used to calculate the mean values and standard deviations (SD) for each group. Analysis of variance (ANOVA) and post hoc testing using Bonferroni's correction for multiple comparisons were used for comparisons among different time points. p<0.05 was considered to be statistically significant.

**RESULTS**

*Morphological findings*

SEM images are shown in Fig. 3. Distinct differences were detected between the two experimental collagen membranes and BG. Analysis of the surface images revealed that DHT and DHT/EDC membranes exhibited irregular macro- and micropores that were interconnected to form a 3D mesh, while BG exhibited individual collagen fibrils interlaced to form coarse collagen strands. Analysis of the cross-sectional views revealed that the DHT membrane had only one layer with closely aligned collagen fibers. The DHT/EDC membrane had a loose-structure system, while BG had a bilayer structure with one compact and one porous layer.

*Enzyme resistance test*

The results of the enzyme resistance test are presented in Fig. 4. During the first 12 h of immersion in the trypsin solution, DHT and DHT/EDC membranes exhibited relatively slow degradation, whereas BG exhibited rapid degradation. Within the next 12 h, the DHT membrane (physical cross-linking) exhibited fast biodegradation compared with the DHT/EDC membrane (multi-cross-linking), which exhibited a stable resorption rate and a remaining weight of >50% after 24 h. BG was not visible after 24 h of immersion. A significant difference between the two experimental membranes (i.e., DHT and DHT/EDC) and the BG membrane were observed after 4 h.

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Fig. 3 SEM findings for DHT cross-linked membrane (experimental), DHT/EDC cross-linked membrane (experimental), and BG (control, no cross-linking). (A) DHT. (B) DHT/EDC. (C) BG. (1) surface view. (2) cross-sectional view.

Fig. 4 Degradation properties of DHT cross-linked membrane (experimental), DHT/EDC cross-linked membrane (experimental), and BG (control, no cross-linking) by immersion in 0.25% porcine trypsin solution. *p<0.05 relative to BG group at the same time point; **p<0.05 relative to DHT group at the same time point.
immersion in the porcine trypsin solution (p<0.05).

**Tensile strength test**

The mean±SD values for the DHT, DHT/EDC, and BG membranes were 70.9±1.6, 77.2±1.7, 72.0±2.1 MPa, respectively. The three membranes exhibited a similar tensile strength in a dry state (Fig. 5). When the membranes were compared after soaking in PBS, the average strength values for DHT, DHT/EDC, and BG membranes were 58.6±1.8, 35.4±1.4, and 17±2.3 MPa, respectively. Clearly, the tensile strength of the two experimental membranes was significantly higher than that of the BG membrane (p<0.05).

**Clinical in vivo observations**

Postsurgical wound healing was uneventful in all groups throughout the study, and no infections or other complications related to the surgery or implanted collagen membrane, such as abscess, dehiscence, or allergic reactions, were observed.

1. Histological analysis

At 1 week after implantation, all three membranes exhibited a physiological post-traumatic inflammatory reaction in the superficial portion, with mononuclear cells as the most prominent cell types with sparsely distributed macrophages. In the DHT and DHT/EDC membranes, scattered distribution of vascular endothelial cells was observed; however, no vascular endothelial cells were captured in BG. The formation of a connective capsule around the membrane and a distinct boundary between the membrane body and connective tissue was observed in all three membranes (Figs. 6A–C).

At 2 weeks, the DHT/EDC membrane began to break down, whereas the DHT and BG membranes maintained their original structure. All three membranes were well integrated into the surrounding connective tissue, with mononuclear cells invading the outer portion of the membrane body. Macrophages were detected in some areas of the outer parts of the DHT/EDC membrane. In particular, the degree of mononuclear cell infiltration was greater in DHT/EDC than in the DHT and BG membranes. During these 2 weeks, more vascular endothelial cells were formed in the loose space of the periphery of the two experimental membranes. Vascular endothelial cell ingrowth from the surrounding connective tissue was also detected in BG. The number of mononuclear cells decreased and that of fibrocytes in the connective tissue gradually increased. Scattered plasma cells were also observed (Figs. 6D–F).

At 4 weeks, vascular endothelial cells had transformed into mature blood vessels, and cell numbers were increased in all three membranes. Fibrocyte invasion was also detected in large areas of the membrane body. BG exhibited a slight change in the thickness of its membrane body, otherwise exhibiting a stable structure. However, the DHT/EDC membrane exhibited >50% biodegradation and a loose body structure, with the absence of macrophages in the inner part of its structure. Although the DHT membrane appeared to be thinner than it was at 1 week after implantation, it did not exhibit many structural changes and appeared to be relatively stable, with blood vessels observed in its inner part (Figs. 6G–I).

At 8 weeks after implantation, the structure of all three membranes could still be recognized and the membrane body was gradually replaced by newly formed connective tissue. In DHT and DHT/EDC membranes, a high concentration of macrophages was detected where the collagen fibers remained. Blood vessel invasion was also identified in the central part of the DHT and BG membranes (Figs. 6J–L).

At 12 weeks, all three membranes were completely biodegraded, and only a thin collagen fiber/layer was visible. However, the membrane body remained distinguishable from the surrounding connective tissue. The matured blood vessels were observed far from the membrane. DHT and DHT/EDC membranes exhibited more scattered macrophages in the center of the membrane body, representing a foreign body reaction. The BG membrane was homogenously invaded by vascular endothelial and inflammatory cells inside the entire membrane body (Figs. 6M–O).

2. Histomorphometric analysis

Membrane thickness varied among time points and among rats at the same time point. The residual membrane thickness for each group at each time point is presented in Fig. 7. Histomorphometric analysis revealed a significant decrease in the thickness of the DHT and DHT/EDC membranes at 4 weeks after implantation (p<0.05). However, no significant change was observed for the remainder of the observation period (p>0.05). Meanwhile, BG exhibited a significant change in its thickness between 4 and 8 weeks after implantation (p<0.05).
Fig. 6 Histological findings for DHT cross-linked membrane (experimental), DHT/EDC cross-linked membrane (experimental), and BG (control, no cross-linking) at 1, 2, 4, 8, and 12 weeks after implantation in rat (trichrome stain, original magnification, ×200) CM: collagen membrane, CT: connective tissue, S: space, Black arrow: vascular endothelial cells, Blue arrow: macrophage.

(A) Scattered distribution of vascular endothelial cells in the surrounding portion of the DHT membrane at 1 week after implantation. (B) Sparsely distributed macrophages were observed in the DHT/EDC membrane at 1 week after implantation. (C) Membrane has not integrated well into the surrounding connective tissue in BG. The space is visible between the connective tissue and membrane body under microscope at 1 week after implantation. (D) DHT membrane was integrated well with surrounding connective tissue at 2 weeks after implantation. (E) More macrophages could be observed in the outer portion of DHT/EDC membrane at 2 weeks after implantation. (F) Vascular endothelial cells were initially noticed in the surrounding connective tissue of BG at 2 weeks after implantation. (G) Thickness of DHT membrane at 4 weeks is thinner than that at 1 week after implantation; however, it’s structure relatively stable. (H) DHT/EDC membrane exhibited extensive biodegradation, and only a thin layer was visible at 4 weeks after implantation. (I) More blood vessels could be observed around the BG membrane at 4 weeks after implantation. (J) The structure of DHT membrane was maintained after 8 weeks implantation. (K) Almost complete biodegradation of DHT/EDC membrane was observed and was replaced by newly formed connective tissue at 8 weeks after implantation. (L) Mature blood vessels could be identified in the inner part of BG at 8 weeks after implantation. (M) The structure of membrane was destroyed at 12 weeks after implantation. (N) DHT/EDC membrane exhibited more scattered macrophages in the center of the membrane body at 12 weeks after implantation. (O) Homogenous inflammatory cell invasion could be observed inside entire BG membrane at 12 weeks after implantation.
and Boyce23) proposed that appropriate porosity should be >55%. Enea et al.24) reported that an interconnected 3D architecture was important for functional performance. To some extent, however, any cross-linking technique may have uncertain effects on the structure of a membrane.

A distinct difference was detected in morphology between the two experimental collagen membranes (DHT and DHT/EDC) and BG. DHT and DHT/EDC membranes comprised irregular macro- and micropores that were interconnected to form a 3D mesh, while BG exhibited individual collagen fibrils interlaced to form coarse collagen strands. Practically, DHT and DHT/EDC membranes exhibited similar morphology after cross-linking; the difference between two experimental membranes, however, was that the DHT membrane exhibited more compact structure than the DHT/EDC membrane. The possible reason was that simultaneously performing EDC may lead to re-hydration and lyophilization, which lead to a loose structure of DHT/EDC25).

Our in vitro study involved enzymatic degradation in a bioreactor to simulate the oral environment for assessing degradation in the three membranes. Within the first 12 h, DHT and DHT/EDC membranes exhibited a relatively slow degradation, whereas BG exhibited a rapid resorption rate. Within the next 12 h, DHT/EDC membranes exhibited slower degradation than the DHT membrane, and the BG membrane was completely degraded. The results of the present study are consistent with the previous study18). Increasing the cross-linking density of collagen membranes may promote biostability. Therefore, DHT cross-linking may contribute to a decrease in the amount of free amino acids; therefore, the DHT membrane exhibited a slower degradation rate than the BG membrane. The DHT/EDC membrane exhibited the slowest degradation rate, which could be explained by a combination of increased amide and ester bond formation, leading to higher resistance to enzymatic action. However, these results can only be used as a reference because the test was performed in a sterile environment and evaluated the effects of only one enzyme on the different collagen membranes. Further studies are required to assess whether the results obtained in the present in vitro study can be applied to the human oral cavity because several studies have reported that Porphyromonas gingivalis, Treponema denticola, and Bacteroides melaninogenicus are capable of producing collagenase, which can result in premature degradation of these membranes26,27).

Generally, appropriate tensile strength is crucial for collagen membrane use in clinical practice, especially when it is applied to GBR/GTR sites. Positive correlations between cross-linking density and tensile strength have been reported18,21). In the present study, the tensile strength of two experimental membranes was evaluated. Although the DHT/EDC membrane has a higher cross-linking density than the DHT membrane, the tensile strength of the DHT/EDC membrane was lower in wet conditions. One possible reason for this phenomenon is that EDC treatment partially denatures the 3D architecture of membrane itself, causing the misalignment of side chains and leading to membrane brittleness18,28). Another possible reason could be that the re-hydration and lyophilization procedure leads to loose connection between collagen fibers25).

One pivotal issue concerning the use of collagen membranes is the duration they remain intact without degradation in GBR/GTR procedures. A previous study reported that it was crucial for the membrane to remain intact during the early wound healing period when the epithelium actively migrates along the root surface. The presence of a barrier after this initial period may delay...
connective tissue maturation during the late wound healing period\textsuperscript{30}. Other studies have also reported that, if membrane resorption occurred before 4 weeks after implantation, bone and periodontal ligament cells could not fill the space beneath the barrier membrane\textsuperscript{29}. In the present study, both the DHT and BG membranes exhibited >50\% of their thickness 4 weeks after implantation and, although the thickness of the DHT/EDC membrane was <50\%, a relatively stable biodegradation rate was observed. The cross-linking technique may influence the biodegradation of collagen membranes. Higher collagen density and compact structure may be a reason for slower biodegradation\textsuperscript{12}. Hurley\textit{et al.}\textsuperscript{30} reported that looser membrane structures enabled better nutrient transfer and organized faster than denser membranes. From our findings, a significantly different morphology was noted for the two experimental (DHT and DHT/EDC) membranes. The DHT membrane displayed compact structure and higher collagen density than that of the DHT/EDC membrane. However, loose structure and lower collagen density maybe a result of simultaneous EDC cross-linking.

Tissue regeneration depends on the reformation of soft tissues in the wound-healing phase\textsuperscript{31}. Angiogenesis is a prerequisite for wound healing, and potentially influences the level of extracellular matrix synthesis and, subsequently, connective tissue formation\textsuperscript{32}. Therefore, the formation of an adequate numbers of blood vessels can serve as a measure for tissue integration. In the present study, the membranes were implanted in subcutaneous pouches created on the backs of the animals. The rat model used in the present study is generally used for biocompatibility assays for biomaterials because it provides a tissue area that is highly vascularized, easy access to the connective tissue layer, and out of reach of the animal’s paws\textsuperscript{33,34}. However, data obtained from rat models should be cautiously applied to humans. Gottlow\textit{et al.}\textsuperscript{5} investigated bioresorbable barrier membranes in monkeys and found that the membrane was still intact after 3 months. However, when it was investigated in rabbit model, considerable degradation was observed at 3 months. Further studies are necessary to evaluate the biocompatibility and degradation of DHT and DHT/EDC membranes with or without bone grafts in different animal models, or other models mimicking the human oral cavity to provide more reliable and predictable data.

**CONCLUSION**

Within the limitations of the present study, we conclude that both DHT and DHT/EDC membranes, which are novel porcine skin-derived collagen membranes, exhibit good physiochemical properties, biocompatibility, and tissue integration. Moreover, the DHT membrane exhibited a more compact structure than the DHT/EDC membrane. The DHT membrane exhibited a relatively slower biodegradation pattern and a lower inflammatory response compared with DHT/EDC membrane; thus, supporting its potential as a barrier membrane for GBR/GTR. However, further studies are needed to assess these membranes in conjunction with bone grafts for evaluating their bone regeneration effects.

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**REFERENCES**

1) Caffesse RG, Nasjleti CE, Morrison EC, Sanchez R. Guided tissue regeneration: comparison of bioabsorbable and non-bioabsorbable membranes. Histologic and histometric study in dogs. J Periodontol 1994; 65: 583-591.
2) Garrett S. Periodontal regeneration around natural teeth. Ann Periodontol 1996; 1: 621-666.
3) Chung KM, Salkin LM, Stein MD, Freedman AL. Clinical evaluation of a biodegradable collagen membrane in guided tissue regeneration. J Periodontol 1990; 61: 732-736.
4) Hammarle CH, Lang NP. Single stage surgery combining transmucosal implant placement with guided bone regeneration and bioresorbable materials. Clin Oral Implants Res 2001; 12: 9-18.
5) Wang HL, Boyapati L. “PASIT” principles for predictable bone regeneration. Implant Dent 2006; 15: 8-17.
6) Hardwick R, Hayes BK, Flynn C. Devices for dentoalveolar regeneration: an up-to-date literature review. J Periodontol 1995; 66: 495-505.
7) Gottlow J, Nyman S, Lindhe J, Karring T, Wennstrom J. New attachment formation in the human periodontium by guided tissue regeneration. Case reports. J Clin Periodontol 1986; 13: 604-616.
8) Tal H, Kozlovsky A, Artzi Z, Nemcovsky CE, Moses O. Long-term bio-degradation of cross-linked and non-cross-linked collagen barriers in human guided bone regeneration. Clin
9) Tempro PJ, Nalbandian J. Colonization of retrieved polytetrafluoroethylene membranes: morphological and microbiological observations. J Periodontol 1993; 64: 162-168.

10) Bunyaratavej P, Wang HL. Collagen membranes: a review. J Periodontol 2001; 72: 215-229.

11) Tal H. [Healing of osseous defects by guided bone regeneration using ribose cross linked collagen membranes]. Refuat Hapeh Vehashinayim (1993) 2004; 21: 32-41, 93.

12) Rothamel D, Schwarz F, Fienita T, Smeets R, Dreiseidler T, Ritter L, Happe A, Zoller J. Biocompatibility and biodegradation of a native porcine pericardium membrane: results of in vitro and in vivo examinations. Int J Oral Maxillofac Implants 2012; 27: 146-154.

13) Schwarz F, Rothamel D, Herten M, Sager M, Becker J. Angiogenesis pattern of native and cross-linked collagen membranes: an immunohistochemical study in the rat. Clin Oral Implants Res 2006; 17: 403-409.

14) Rothamel D, Schwarz F, Sager M, Herten M, Sculean A, Becker J. Biodegradation of differently cross-linked collagen membranes: an experimental study in the rat. Clin Oral Implants Res 2005; 16: 369-378.

15) Minabe M, Kodama T, Kogou T, Tamura T, Hori T, Watanabe Y, Miyata T. Different cross-linked types of collagen implanted in rat palatal gingiva. J Periodontal 1989; 60: 35-43.

16) Quteish D, Dolby AE. The use of irradiated-crosslinked human collagen membrane in guided tissue regeneration. J Clin Periodontol 1992; 19: 476-484.

17) Hafemann B, Ghofrani K, Gattner HG, Steive H, Pallua N. Cross-linking by 1-ethyl-3-[(3-dimethylaminopropyl)-carbodiimide (EDC) of a collagen/elastin membrane meant to be used as a dermal substitute: effects on physical, biochemical and biological features in vitro. J Mater Sci Mater Med 2001; 12: 437-446.

18) Hu Y, Liu L, Dan W, Dan N, Gu Z, Yu X. Synergistic effect of carbodiimide and dehydrothermal crosslinking on acellular dermal matrix. Int J Biol Macromol 2013; 55: 221-230.

19) Lee JH, Lee JH, Baek WS, Lim HC, Cha JK, Choi SH, Jung UW. Assessment of dehydrothermally cross-linked collagen membrane for guided bone regeneration around peri-implant dehiscence defects: a randomized single-blinded clinical trial. J Periodontal Implant Sci 2015; 45: 229-237.

20) Park JY, Jung IH, Kim YK, Lim HC, Lee JH, Jung UW, Choi SH. Guided bone regeneration using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-cross-linked type-I collagen membrane with biphasic calcium phosphate at rabbit calvarial defects. Biomater Res 2015; 19: 15.

21) Li J, Ren N, Qiu J, Jiang H, Zhao H, Wang G, Boughton RL, Wang Y, Liu H. Carbodiimide crosslinked collagen from porcine dermal matrix for high-strength tissue engineering scaffold. Int J Biol Macromol 2013; 61: 69-74.

22) Keogh MB, O’Brien FJ, Daly JS. Substrate stiffness and contractile behaviour modulate the functional maturation of osteoblasts on a collagen-GAG scaffold. Acta Biomater 2010; 6: 4305-4313.

23) Powell HM, Boyce ST. EDC cross-linking improves skin substitute strength and stability. Biomaterials 2006; 27: 5821-5827.

24) Enea D, Henson F, Kew S, Wardale J, Getgood A, Brooks R, Rushton N. Extruded collagen fibres for tissue engineering applications: effect of crosslinking method on mechanical and biological properties. J Mater Sci Mater Med 2011; 22: 1569-1578.

25) Bottino MC, Jose MV, Thomas V, Dean DR, Janowski GM. Freeze-dried acellular dermal matrix graft: effects of rehydration on physical, chemical, and mechanical properties. Dent Mater 2009; 25: 1109-1115.

26) Mayrand D, Grenier D. Detection of collagenase activity in oral bacteria. Can J Microbiol 1985; 31: 134-138.

27) Sela MN, Kohavi D, Krausz E, Steinberg D, Rosen G. Enzymatic degradation of collagen-guided tissue regeneration membranes by periodontal bacteria. Clin Oral Implants Res 2003; 14: 263-268.

28) Haugh MG, Jaasma MJ, O’Brien FJ. The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds. J Biomed Mater Res A 2009; 89: 363-369.

29) Caton JG, DeFuria EL, Polson AM, Nyman S. Periodontal regeneration via selective cell repopulation. J Periodontal 1987; 58: 546-552.

30) Harlunk LA, Stinchfield FE, Bassett AL, Lyon WH. The role of soft tissues in osteogenesis. An experimental study of canine spine fusions. J Bone Joint Surg Am 1959; 41-a: 1243-1254.

31) Thoma D, Cochran D. Unlocking the mystery of periodontal regeneration—different tissues, different phenotypes. Journal de Parodontologie et Implantologie Orale 2010; 17: 23-60.

32) Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing: 2000; Elsevier; 2000. p.40-46.

33) Zafalon EJ, Versiani MA, de Souza CJ, Moura CC, Dechichi P. In vivo comparison of the biocompatibility of two root canal sealers implanted into the subcutaneous connective tissue of rats. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007; 103: e88-94.

34) Freitas RM, Spin-Neto R, Spolidório LC, Campagna-Filho SP, Marcantonio RAC, Marcantonio Jr E. Different molecular weight chitosan-based membranes for tissue regeneration. Materials 2011; 4: 380-389.