Biodegradation of Three Different Collagen Membranes in the Rat Calvarium: A Comparative Study

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Background: Collagen barrier membranes are commonly applied in periodontal and bone-regenerative procedures. Membranes differ in their resorption pattern following implantation, thus influencing clinical outcome. The purpose of this study was to quantitatively evaluate the biodegradation of three different commercially available collagen membranes.

Methods: Collagen membranes were cut into 5-mm-diameter disks and labeled with aminohexanoyl-biotin-N-hydroxy-succinimide ester. One membrane disk of each type (non-cross-linked [NCL], glutaraldehyde cross-linked [GCL], and ribose cross-linked [RCL]) was implanted on the calvaria of 20 Wistar rats. Block sections were retrieved after 2 days (baseline, two animals), 14 days (10 animals), or 28 days (eight animals). Decalcified histologic sections were stained with streptavidin horseradish peroxidase. Residual membrane thickness and area were measured. Statistical analysis consisted of analysis of variance (ANOVA) with repeated measures.

Results: Statistically significant differences in the amount of residual membrane material were recorded within each membrane (among different time points) and among different membranes at the same time points ($P<0.001$). At 28 days, the least amount of residual collagen area, expressed as the percentage of baseline, was observed in the NCL group (13.9%–10.25%), followed by the GCL (24.7% ± 35.11%) and RCL (91.3% ± 10.35%) groups. Residual membrane thickness, expressed as the percentage of baseline thickness, presented a similar pattern (31% ± 16.55%, 37% ± 41.90%, and 94.1% ± 12.22%, respectively). ANOVA with repeated measures showed a significant interaction between membranes and time ($P<0.001$).

Conclusions: The tested membranes differed in their degradation patterns and collagen contents. Membranes should be chosen for each clinical case according to the desired biodegradation characteristics.

KEY WORDS
Animal studies; bone and bones; collagen; in vivo; membrane.

guided bone regeneration, a common procedure in implant surgery,$^1$ uses barrier membranes, excluding epithelial and connective tissues, to enable bone progenitor cell proliferation and differentiation into the isolated area.$^2$-$^5$ Bioabsorbable and non-resorbable barrier membranes are effective.$^1$-$^6$ However, non-absorbable membranes, mostly made of polytetrafluoroethylene, require a second surgical procedure for their retrieval.$^7$-$^10$ Therefore, bioabsorbable membranes with comparable clinical outcomes$^6$-$^8$ have become popular in bone-regeneration procedures. Successful regeneration is possible provided cell exclusion and space maintenance are continued throughout the time needed. This can vary between 3 and 12 months, depending on the dimensions of the bony defect.$^{11}$-$^{14}$

Bioabsorbable membranes$^{11}$-$^{12}$ may be prepared from dura mater,$^{15}$ polyactic acid,$^{16}$-$^{17}$ polyglycolic acid, polyurethane,$^{17}$ and collagen.$^4$-$^8$ The ability of collagen to promote progenitor cell adhesion, chemotaxis, homeostasis, and physiologic degradation, together with its physiologic degradation, easy manipulation, and low immunogenicity make it an ideal material for barrier membrane preparation.$^{13}$-$^{19}$-$^{20}$ Types I and III collagen of bovine or porcine origin$^{13}$-$^{21}$-$^{22}$ are the main components of most commercially available collagen membranes.
Cells release matrix metalloproteinases (MMPs) to the wound area during healing, which contribute to collagen membrane degradation.\textsuperscript{23,24} Downregulation of collagen-degrading enzymes, such as MMPs, or increasing their structural stability by cross-linking, slows down the process. Because tetracycline inhibits MMP activity,\textsuperscript{25-27} immersion of collagen membranes in tetracycline solution prior to their implantation reduces their degradation in vitro\textsuperscript{28} and in vivo.\textsuperscript{29} Collagen cross-linking has been achieved with ultraviolet and gamma radiation, hexamethylenediisocyanate glutaraldehyde, diphenylphosphorilazide, and ribose;\textsuperscript{20,30-33} however, the degree of cross-linking and, therefore, membrane resistance to resorption following implantation may differ with each method.

Collagen membranes may differ in their microarchitecture (e.g., spaces between the collagen molecules, fibers, bundles, and collagen layers within the membrane) and cross-linking. Membrane microarchitecture and collagen cross-linking determine membrane characteristics, such as tensile strength, handling, tissue integration, and biodegradation characteristics.\textsuperscript{22,32,34-36} Collagen membranes with a higher degree of cross-linking remain intact for longer periods. This enables improved healing of larger defects\textsuperscript{18,22} and may preserve their integrity if exposed spontaneously.\textsuperscript{5,37,38} However, cross-linked membranes have reduced tissue integration and vascularity.\textsuperscript{22,36} and in a clinical trial,\textsuperscript{6} they showed a higher incidence of spontaneous exposure following their application in the oral cavity.

Histologic evaluation of the degradation of different collagen membranes has been performed previously;\textsuperscript{22,39} however, to the best of our knowledge, no quantitative evaluation of the degradation pattern of membranes with varying degrees of cross-linking has been performed. The purpose of this study was to quantitatively evaluate the biodegradation of three different commercially available collagen membranes.

**MATERIALS AND METHODS**

Collagen membranes, including bilayered, non-cross-linked (NCL),\textsuperscript{1} glutaraldehyde cross-linked (GCL),\textsuperscript{9} and ribose cross-linked (RCL),\textsuperscript{9} from three manufacturers were selected for the study. These membranes are widely used in clinical practice. Membrane disks were cut using a disposable biopsy punch\textsuperscript{**} into 5-mm-diameter membrane disks. The average weight for the membrane disks was 2.0 ± 0.1 mg (NCL), 3.7 ± 0.2 mg (GCL), and 1.4 ± 0.3 mg (RCL).

Membrane biotin labeling was described previously.\textsuperscript{29,40} Briefly, membrane disks were labeled with aminohexanoyl-biotin-N-hydroxy-succinimide ester\textsuperscript{††} (AH-BNHS) following a slight modification of the supplier’s protocol. Membranes were incubated with 1 mg/ml AH-BNHS biotin for 1 hour at room temperature and washed overnight with three changes of phosphate buffered saline (Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free; pH 7.4) to remove any unbound biotin. Ten biotinylated collagen membrane disks from each group were processed for histologic observation; however, only five from each group were also treated with formic acid.

The study consisted of 20 8-month-old female Wistar rats that were fed water and food ad libitum. The animals were maintained in a room with a 12-hour light/dark cycle and an ambient temperature of 22°C. The Medical Faculty Animal Research Council of Tel Aviv University approved the study. Animals were weighed and anesthetized with an intramuscular injection of ketamine chlorhydrate\textsuperscript{‡‡} at 90 mg/kg body weight and 2% xylazine\textsuperscript{§§} at 10 mg/kg body weight. The surgical protocol for membrane implantation was described previously.\textsuperscript{29} Briefly, the dorsal part of the skin covering the scalp was shaved and prepared aseptically for surgery. A U-shaped incision was made in the scalp over the parietal bone to enable elevation of a full-thickness flap. The soft tissues and periosteum were raised in two layers. A high-speed, water-cooled, diamond wheel-shaped bur was used to create three similar, 5-mm-diameter, shallow (~1 mm deep) bony defects on the midline of the parietal bone.

Bone always covered the dura mater. One disk of each membrane type was placed in one defect (three disks per animal). The soft tissues were repositioned, first the periosteum, to cover the implanted membranes, and the dermal tissues were sutured with bioabsorbable sutures.\textsuperscript{i}

An overdose of ketamine chlorhydrate and xylazine and asphyxiation with carbon dioxide (CO\textsubscript{2}) were used to sacrifice the animals. The first two animals were sacrificed after 2 days and served as the baseline for all measurements. Ten animals were sacrificed after 14 days, and the remaining eight were sacrificed after 28 days. Dermal tissues were dissected, leaving the periostea undisturbed and covering the membrane disks lying in the calvaria defects. Block sections containing the calvaria and surrounding tissues were retrieved, fixed in 10% neutral buffered formalin, decalcified for 2 weeks in 5% formic acid, washed, dehydrated in ethanol and xylene, and embedded in paraffin; 4- to 6-µm anteroposterior sections were cut. Sections including the central area of the three bony defects were chosen for comparative
analysis of collagen membrane degradation. Detection of biontinylated collagen was carried out with streptavidin horseradish peroxidase, according to the supplier’s protocol. Slides were treated with a solution of one drop of enzyme in 1 ml 10 mM phosphate buffered saline, pH 7.4, and incubated for 3 minutes at room temperature. Biontinylated collagen was identified with a 3-amino-9-ethylcarbazole substrate (red) kit and mounted with an aqueous solution of glycerol vinyl alcohol.

Stained sections were photographed with a digital camera mounted on a light microscope at a magnification ×100. Multiple digital images were taken and stored for each sample and analyzed for staining intensity. The central part of the membrane in each block was identified by measuring the underlying bone defect, and a region of interest (ROI) was created. Because membranes differed in their thickness, a specific ROI was defined at baseline for each membrane; its long axis corresponded to the greatest membrane thickness encountered among the sections of a certain membrane, and the width was arbitrarily established at 0.5 mm. This ROI was overlaid onto each of the sectioned disks in five different areas. Where an artifact was present, it was avoided and not included in the ROI or compensation was made by eliminating the artifact at the time of the measurement. The number of pixels occupied by biontinylated collagen and the actual membrane thickness were determined for each of the five measurements. For each membrane type (NCL, GCL, or RCL), measurements were taken in each section, and their calculated mean served as the unit for statistical analysis. Subsequently, at 14 or 28 days, the collagen area of each disk was calculated as the percentage of the respective baseline measurements according to the formula: amount of collagen at baseline and at 14 and 28 days divided by the amount of collagen at baseline and multiplied by 100. Several slides from each specimen were stained with hematoxylin to identify the membrane, surrounding tissues, and underlying bone defect.

Statistical Analysis
Formic acid–treated and non-treated, non-implanted membranes were compared to the Student t test. The mean for each slide was considered an independent sample. The percentage of collagen within the membrane was calculated according to the following formula: amount of collagen at baseline – measured amount of collagen in a certain slide divided by the amount of collagen at baseline and multiplied by 100. No statistical analysis was performed comparing the results at 14 and 28 days to baseline; therefore, the small number of animals in the baseline group had no influence on the statistical findings. Furthermore, no statistically significant differences in the collagen contents were found between the non-implanted collagen membranes and the 2-day post-implantation collagen membrane as evaluated with the Student t test. Data were expressed for each group as mean ± SD and analyzed with analysis of variance (ANOVA) with repeated measures, where time was the between-subjects factor and membranes were the within-subjects factor. Paired comparisons among membranes were performed.

RESULTS
No statistically significant differences were noted between the formic acid–treated membranes and the non-treated, non-implanted membranes. Wound healing was uneventful in all animals. At the time of block section retrieval, membranes appeared integrated with surrounding tissues with no adverse effects.

Table 1 shows the amount of biontinylated collagen in each membrane at baseline and at 14 and 28 days. Figure 1 shows the percentage of collagen in each group at each time point relative to the baseline percentage of collagen. At 14 days, the least area of residual collagen was observed in the GCL group followed by the NCL and RCL groups. At 28 days, the least area of residual collagen was observed in the NCL group followed by the GCL and RCL groups. ANOVA with repeated measures showed a statistically significant difference among membranes (P < 0.001) and within membranes at the different time points (P < 0.001) and a significant interaction between membranes and time (P < 0.001) (Figs. 2 and 3).

Membrane thickness at each time point is presented in Table 2. At 14 days, minimal differences were found among the membranes; this was not the case at 28 days. ANOVA with repeated measures revealed a statistically significant difference among membranes (P = 0.002) and for the interaction between membranes and time (P < 0.001).

DISCUSSION
The biodegradation of three different, commercially available collagen membranes implanted and maintained in a closed, non-contaminated, extraoral environment was evaluated. The results might not be directly applied to guided bone regeneration procedures in the oral cavity, especially the way in which these membranes would function if spontaneously exposed to oral bacteria. No analysis of cell colonization of the membranes or examination with a scanning
Labeled membranes were subject to decalcification during histologic preparation. Although it was not proven in this study, according to the manufacturer’s specifications, the biotin link to collagen is irreversible and is not affected by biomineralization or histologic preparation. Apparently, decalcification has minimal influence on the quality of collagen labeling with biotin. Although it has been suggested that histologic processing including decalcification could affect the nature of cross-linking in mineralizing collagen, biotin binding to the collagen membranes used in this study did not seem to be affected by histologic processing with formic acid. The in vitro assay did not show any statistically significant difference between membranes with and without formic acid processing. The biodegradation pattern of the membranes was clearly different: RCL showed minimal structural alterations, whereas NCL and GCL lost most of their collagen after 28 days. These findings are in agreement with a previous report that found that RCL presented the least degradation among a series of membranes after subcutaneous implantation in the rat. Although GCL is a chemically induced cross-linked membrane, it had lost most of its collagen at 28 days. Furthermore, GCL has low biocompatibility because of cross-linking through glutaraldehyde.

The membranes varied in thickness, and the amount of collagen within each was statistically significantly different. RCL contained the largest relative amount of collagen per surface but weighed the least. All membranes lost part of their collagen and the thickness was reduced over time. However, most of the original thickness was preserved in RCL throughout the study but not in GCL or NCL, for which differences over time were statistically significant ($P = 0.018$). However, changes in membrane thickness cannot be directly interpreted as membrane degradation. Membranes may simultaneously lose collagen and increase in thickness. In these cases, there were larger amounts of voids within the membranes (Figs. 2 and 3). In our study, a large decrease in membrane thickness was noted between 14 and 28 days. This finding is in disagreement with a previous study that showed almost no change in membrane thickness between the 2- and 4-week evaluations; different results might

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<tr>
<th>Table 1.</th>
<th>Amount of Collagen (in pixels; mean ± SD) Measured in Each Group at Baseline and 14 and 28 Days</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>NCL</td>
<td>1,123,927.9 ± 45,798.30</td>
</tr>
<tr>
<td>GCL</td>
<td>1,480,900.2 ± 407,118.34</td>
</tr>
<tr>
<td>RCL</td>
<td>679,844.2 ± 55,092.84</td>
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Figure 1. Percentage of collagen remaining in each membrane group at each time point relative to baseline in the same membrane. The differences between RCL and NCL ($P = 0.035$) and RCL and GCL ($P < 0.001$) were statistically significant.

Figure 2. Histologic appearance of the three membranes 14 days after implantation: RCL, GCL, and NCL (from left to right). Collagen stained in red/brown with avidin/biotin reaction. Bar = 1 mm.
be due to the different membrane visualization techniques applied. Biotin/avidin staining clearly demarks the limits of the original membrane, differentiating it from the host connective tissues. The success of bone-augmentation procedures, using barrier membranes, depends on cell exclusion and space maintenance for a sufficient period to allow for new bone formation. Early exposure of barrier membranes to the oral environment jeopardizes results because of infection, mostly of non-resorbable membranes, or early bioabsorbable membrane disintegration. In large bony defects, premature membrane resorption leads to less than optimal results. Collagen cross-linking may prolong the membrane barrier function.

The three collagen membranes used in the present study differed in their structure and physical characteristics. A larger membrane porosity (less collagen content per area) could allow for cell ingrowths within the membrane, thus enabling better tissue integration and cell growth within the membrane, but it also could result in reduced barrier function.

Membrane degradation starts shortly after implantation. The present study showed that all membranes lost collagen by 28 days. Although RCL lost the least amount and NCL and GCL lost the most, differences within and among membranes were statistically significant. This difference in the degradation pattern may have clinical implications. In large, non-self-contained bone defects, where prolonged membrane barrier functions are desirable, certain cross-linked membranes may offer advantages. Membranes should be chosen according to the clinical demands of each case. Barriers with high degradation rates might have a shorter than indicated effect.

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