Non–Cross-linked Versus Cross-linked Collagen Membrane in Maxillary Sinus Perforation Repair: A Comparative Histologic Study in a Rat Model

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Purpose: To compare and evaluate maxillary sinus perforation repair, bone regeneration, and membrane degradation with cross-linked and non–cross-linked collagen membranes in rat sinuses at 2, 4, and 10 weeks, respectively. Materials and Methods: Fifty-one Sprague-Dawley rat models were included in the study. Bilateral maxillary sinus perforations were made with a straight bur. In the control site, cross-linked collagen membrane (Ossix Plus) was placed, and in the test site, non–cross-linked collagen membrane was used (Pro-Tiss). Euthanasia was carried out under carbon dioxide asphyxia where 17 rats were sacrificed at weeks 2, 4, and 10. Histologic evaluation of the specimens was subsequently done. Results: At 2 (P = .001), 4 (P = .031), and 10 (P = .024) weeks, there was a significant regeneration of maxillary sinus lining in sites treated with non–cross-linked collagen membrane over the cross-linked collagen membrane. No significant differences were observed in measures of bone regeneration (P = .92; 10 weeks) and membrane degradation (P = .06; 4 weeks) at the end of the study period between the two groups. Conclusion: The non–cross-linked collagen membrane appears to be more beneficial in maxillary sinus repair. However, it does not seem to confer additional benefits in bone regeneration or membrane degradation over cross-linked collagen membranes. Int J Oral Maxillofac Implants 2020;35:91–99. doi: 10.11607/jomi.7600

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is that it may be beneficial over cross-linked collagen membrane by virtue of its enhanced tissue integration, rapid vascularization, and nearly complete biodegradation with no observable foreign body reactions.\(^4\)\(^-\)\(^6\)

Animal models are considered to be excellent biologic tools to study the pathogenesis of wound repair.\(^6\)\(^,\)\(^7\) Several studies on wound healing have been done in the past using the rat model.\(^6\) Since it is easy to handle and has a maxillary sinus anatomy similar to humans, the rat model can be efficiently implemented for sinus repair studies.\(^6\)\(^,\)\(^7\) In the rat, the maxillary sinus is the only paranasal sinus\(^5\) that extends predominantly posteriorly from its ostium with minimum superior or lateral spread. The surgical access to this area is easier, and the sinus lining resembles a simple columnar ciliated epithelium.\(^5\)\(^,\)\(^6\) Also, wound healing is accelerated in a rat model, which makes it possible to study the process in shorter time periods compared with human subjects.\(^5\)\(^,\)\(^6\)

As resorbable membranes are commonly used for repairing sinus membrane perforations,\(^1\)\(^-\)\(^3\) the research question in this study revolved around comparing two distinct types of resorbable membranes: cross-linked and non–cross-linked collagen membranes.\(^4\)\(^-\)\(^6\) The purpose of this study was to compare and evaluate maxillary sinus perforation repair, bone regeneration, and membrane degradation with cross-linked and non–cross-linked collagen membranes in rat sinuses. The outcomes were evaluated histologically at three different time intervals.

**MATERIALS AND METHODS**

**Participants and Eligibility Criteria**

Animals were selected after approval from the Institutional Animal Ethics Committee (SVSMC/IAEC/2017(2)–107), and the study was done in an accredited animal lab. Fifty-one healthy Sprague-Dawley rats within the age group of 6 to 9 weeks and weighing 200 to 250 g were included in the study.

**Sample Size Calculation**

A sample of 17 animals per time interval (2 sites in each animal; 34 sites per time interval) were required for an anticipated effect size of 1, a probability level of .05, and a desired statistical power of 0.8.

**Interventions**

**Presurgical Protocol.** The animals were kept in isoflurane chamber for 5 to 10 minutes to achieve conscious sedation. They were then placed in dorsal recumbency. The operative field was prepared in a standard manner, and the surgical procedures were carried out using aseptic methods.

**Surgical Procedure**

A 2-mm punch biopsy instrument was used to create a perforation on the right and left premaxillary areas to reach the bony covering of the maxillary sinus. Then, a straight bur of 2-mm diameter was placed over the bone, and the sinus was intentionally perforated under medium speed. Relieving incisions were placed on the ridge around the perforations to elevate the flap and to place the cross-linked collagen membrane (Ossix Plus, Datum Dental) over the left perforation and a non–cross-linked collagen membrane (Pro-Tiss, AzureBio) on the right perforation. The cross-linked collagen membrane served as a positive control, while the non–cross-linked collagen membrane was considered to be the experimental material. Tension-free primary closure was obtained after membrane placement. This procedure was performed bilaterally in 51 rats.

**Postsurgical Management**

Soon after the surgery, the animals were caged individually at a temperature of 20°C to 24°C and at 30% to 70% relative humidity and were kept on a 12-hour light/12-hour dark cycle. The animals were kept on ground pellet food and water and were monitored for signs of disease regularly. Subsequently, 17 rats were sacrificed at 2, 4, and 10 weeks, respectively, under carbon dioxide asphyxia.

**Tissue Processing and Histologic Analysis**

The specimens were harvested from each animal at 2, 4, and 10 weeks postoperatively and were fixed in 10% formalin for at least 24 to 48 hours. The biopsy specimens were excised as follows. The postoperative scar was considered to be the epicenter of the region of interest, and a 4.1-mm soft tissue punch (Nobel Biocare) was used through the uppermost soft tissue layers until the bone was felt. A 4.1-mm bone trephine (Straumann Dental India) was then used to penetrate the sinus floor until a “drop” was felt. The soft tissue punch was reinserted to separate any soft tissue in the sinus, and the core was gently separated and excised from the surrounding tissues. Coronally to apically,\(^6\)\(^,\)\(^7\) the core had three distinct layers: oral mucosa, the osseous floor of the sinus, and the sinus lining. A water-cooled diamond-grinding blade was then used to allow slicing without separating the soft tissues from the nasal bone in a longitudinal plane. The oral mucosa, the osseous floor of the sinus, and the sinus lining were then evaluated for membrane degradation, bone regeneration, and maxillary sinus perforation repair, respectively.

Specimens were demineralized with 5% nitric acid with formalin until the tissue was decalcified. Then, they were dehydrated in a series of increasing ethanol concentrations (from 70% to 100%) followed by a
clearing cycle in xylene for 1 hour. Then, the specimens were impregnated in wax bath overnight. The following day, they were embedded in paraffin, and serial sections (5 μm) were made with microtome and mounted onto the slides. Staining of the sections was done with hematoxylin and eosin (H&E) stain and mounted using DPX. The histologic specimens were observed using ProgRes CapturePro/Janoptik Optical microscope (Advanced Imaging Concepts) at 10× magnification.

**Outcomes**

Histologically, sinus repair, membrane degradation, and bone regeneration at three time intervals were assessed based on the following criteria:

- The maxillary sinus repair was assessed based on the epithelium healing index by Landry, Turnbull, and Howley, where the scores are as follows: 1, no epithelialization; 2, no epithelium with exposed connective tissue; 3, epithelium with no exposed connective tissue.
- Newly formed bone was assessed based on the histologic scoring method for bone regeneration by Han et al, where the scores are as follows: 0, no evidence of newly formed bone; 1, ≤ 10% of the original bone defect; 2, ≤ 20% of the original bone defect; 3, ≤ 30% of the original bone defect; 4, ≤ 40% of the original bone defect; 5, ≤ 50% of the original bone defect; 6, ≤ 60% of the original bone defect; 7, ≤ 70% of the original bone defect; 8, ≤ 80% of the original bone defect; 9, ≤ 90% of the original bone defect; and 10, ≤ 100% of the original bone defect.
- Quantification of membrane degradation was done by application of a classifier that had been generated by a pathologist using the machine learning Weka plugin in FIJI (Fig 1). Evaluation of membrane degradation by Weka analysis was performed as follows. In each slide, images were split into tiles of 10× magnification, and the classifier looked for a rounded connective tissue capsule with a clear split from the surrounding tissues suggestive of membrane degradation products. The areas of interest were automatically segmented into images. The area occupied by the membrane degradation product was calculated and expressed as (degradation product/total area) × 100 as per a previously reported protocol.
Statistical Analysis
Data were analyzed using Prism8 (GraphPad Software). Data were summarized by median ± IQR or mean ± SD depending on the parameters. Intragroup comparison for measures of maxillary sinus repair and bone regeneration at 2, 4, and 10 weeks was done by the Kruskal-Wallis test. Intragroup comparison for measures of membrane degradation at 2 and 4 weeks was done by the paired t test. Intergroup comparison between control and test groups for measures of maxillary sinus repair and bone regeneration was done by the Mann Whitney test. Intergroup comparison between control and test groups for measures of membrane degradation was done by the unpaired t test for score data.

RESULTS
Intragroup Comparison of Maxillary Sinus Perforation Repair, Bone Regeneration, and Membrane Degradation at Different Time Intervals (Test Group)
At 2 weeks, the given H&E-stained sections showed pseudo-stratified ciliated columnar epithelium at few sites and underlying loose connective tissue stroma with multiple blood capillaries engorged with red blood cells and an intense chronic inflammatory cell infiltrate chiefly of lymphocytes. The H&E sections also showed connective tissue stroma with areas of newly formed bone with osteocytic lacunae and marrow spaces. Remnants of the membrane were seen as collagen bundles arranged in concentric layers with a central area showing inflammatory cells and extravasated RBCs and peripheral areas showing mild inflammatory cells with blood vessels. In deeper sections, there was evidence of muscle tissue, some arranged parallel to the collagen bundles. At 4 weeks, the given specimens showed pseudo-stratified ciliated columnar epithelium with a slight discontinuity in some areas indicating healing of maxillary sinus epithelium. The H&E sections showed connective tissue stroma and areas of woven bone formation with marrow spaces and mild inflammatory cell infiltrate. Membrane remnants were seen at this time as well as constricted collagen bundles in underlying connective tissue with numerous inflammatory cells, muscle tissue, and blood vessels. At 10 weeks, the given specimens showed...
pseudo-stratified ciliated columnar epithelium with underlying connective tissue stroma with numerous blood vessels and inflammatory cell infiltrate. H&E sections of hard tissue showed connective tissue stroma with areas of mature bone with osteocytic lacunae and marrow spaces. No membrane remnants were seen histologically at this time (Figs 2 and 3).

In a comparison of Landry, Turnbull, and Howley’s scores for maxillary sinus repair, a significant amount of progressive healing was noted from 2 weeks to 10 weeks ($P = .036$). Analysis of Han et al’s score for bone regeneration showed a significant increase in the amount of bone regeneration from 2 weeks to 10 weeks ($P = .042$). Membrane degradation analysis showed a highly significant reduction in degradation product volume from 2 weeks to 4 weeks ($P = .001$).

**Intragroup Comparison of Maxillary Sinus Perforation Repair, Bone Regeneration, and Membrane Degradation at Different Time Intervals (Control Group)**

Sections from the control group revealed a histologic appearance comparable to that of the test group with some notable exceptions.

At 2 weeks, no pseudo-stratified ciliated columnar epithelium was seen in most of the specimens. The given H&E-stained soft tissue specimens showed connective tissue stroma with inflammatory infiltrate and spicules of lamellar bone showing few lacunae with osteocytes within them. Remnants of the membrane were seen as smaller collagen bundles arranged in concentric layers with a well-defined central area. At 4 weeks, the given H&E-stained soft tissue specimens showed abundant connective tissue stroma with a well-defined osteoid-like matrix. Membrane remnants were seen at this time as well as smaller but numerous constricted collagen bundles. At 10 weeks, the given H&E-stained specimens showed pseudo-stratified ciliated columnar epithelium, and the rest of the section showed connective tissue stroma with numerous blood vessels and inflammatory cell infiltrate. No membrane remnants were seen histologically at this time (Figs 2 and 3).

In a comparison of Landry, Turnbull, and Howley’s scores for maxillary sinus repair, a significant amount of progressive healing was noted from 2 weeks to 10 weeks ($P = .047$). Analysis of Han et al’s score for bone regeneration showed a highly significant increase in...
the amount of bone regeneration from 2 weeks to 10 weeks ($P = .001$). Membrane degradation analysis showed a highly significant reduction in degradation product volume from 2 weeks to 4 weeks ($P = .001$).

**Intergroup Comparison of Maxillary Sinus Perforation Repair, Bone Regeneration, and Membrane Degradation at Different Time Intervals (Test vs Control Groups)**

Landry, Turnbull, and Howley’s scores for maxillary sinus repair at 2 weeks showed a highly significant difference in the amount of sinus healing ($P = .001$) between the test and control groups. Contrary to this, at 4 weeks ($P = .031$) and 10 weeks ($P = .024$), a significant difference in the amount of sinus healing was seen between the test and control groups. Analysis of Han et al’s score for bone regeneration at 2 weeks revealed a highly significant difference in bone regeneration between both groups ($P = .001$). At 4 weeks ($P = .128$) and 10 weeks ($P = .92$), no significant difference was observed. Membrane degradation analysis at 2 weeks showed a highly significant amount of degradation product volume in the control group ($47.22 \pm 11.64$) compared with the test group ($36.82 \pm 12.82$) ($P = .001$). However, at 4 weeks, there was no significant difference in the amount of degradation product volume between the test ($22.76 \pm 9.29$) and control groups ($23.87 \pm 9.56$) ($P = .06$) (Table 1).

**DISCUSSION**

Rodents are known to have small maxillary sinuses; the volumes of the anterior maxillary sinus and posterior maxillary sinus in the rat are 8.6 mm$^3$ and 7.7 mm$^3$, respectively. In sites treated with both cross-linked and non–cross-linked collagen membranes, a significant amount of progressive healing of the sinus membrane was noted from 2 weeks to 10 weeks. This is in accordance with various human and animal studies that have observed progressive healing of the sinus lining at various time intervals. In sites treated with the cross-linked collagen membranes, at 2 weeks, no pseudo-stratified ciliated columnar epithelium was seen in most of the specimens, and there was a highly significant difference in the amount of sinus healing ($P = .001$) between sites treated with cross-linked and non–cross-linked collagen membranes. The non–cross-linked collagen membranes are subject to a rapid degradation process, which may lead to re-epithelialization of the sinus lining. Khalmuratova et al conducted a study on the healing of nasal mucosa in Sprague-Dawley rats after a simple mechanical injury and at 2 weeks found epithelial thickening and ciliated cells, which was in agreement with the histologic findings seen in 2-week specimens from sites treated with the non–cross-linked collagen membranes. At 4 weeks, the respiratory mucosa in their study was restored to a near-normal anatomy ($P \leq .05$), which was also observed in the present study in both control ($P = .045$) and test groups ($P = .036$).

Hosemann et al reported that re-epithelialization of the injured basement membrane is caused by cell migration rather than cell proliferation at the beginning. Cross-linking enhances the inhibitory effect on epithelial migration and this may be the reason behind significantly higher Landry, Turnbull, and Howley’s scores at 2 weeks (1 vs 0; $P = .001$) in the test group (treated with non–cross-linked collagen...
membranes) compared with the control group. At this step, although the wound appears to be closed, the epithelial integrity has still not been completely restored. Even at 4 weeks, a similar trend was observed in both groups; however, complete epithelium formation was not achieved, but connective tissue integrity was maintained with a well-developed connective tissue stroma (1 vs 1; \( P = .031 \)). This is in agreement with the study of Athanasiadis et al\(^2^4\) in a sheep model, where they reported that complete formation of epithelium was still not observable by 28 days. In their study, epithelium formation was incomplete even after 84 days, which is similar to the observations at 10 weeks in the present study, where the sites treated with non–cross-linked and cross-linked collagen membranes showed unexposed connective tissue but incompletely formed epithelium (2 vs 1; \( P = .024 \)).

Analysis of scores for bone regeneration at 2 weeks revealed a highly significant difference in bone regeneration between the test and control groups (5 vs 1; \( P = .001 \)). At 4 weeks (\( P = .128 \)) and 10 weeks (\( P = .92 \)), though, no significant differences were observed. This is in agreement with the study of Brunel et al\(^3^0\) who observed that membrane-protected defects show significantly more bone regeneration; however, the degree of collagen cross-linking does not affect the bone gain achieved through guided bone regeneration. Both Pro-Tiss and Ossix Plus are collagen-derived materials differing only in terms of cross-linking. Sadeghi et al\(^3^2\) in their study on bone regeneration in rabbits, observed that materials with similar composition, when compared with each other, rarely show marked differences in bone formation at 4 and 10 weeks. In the sites treated with non–cross-linked and cross-linked collagen membranes, there was a limited amount of bone formation at 2 weeks and 4 weeks. At 10 weeks, a significant increase in bone formation in both groups was observed. This is in agreement with the study of Kim et al\(^3^1\) in rat premaxillary bone defects, where they observed the formation of very thin and loose connective tissues with a limited amount of new bone formation in the defect at 2 and 4 weeks. At 6 weeks, there was better bone formation, but the loose nature of the connective tissues was still observed.

The collagen membrane must remain intact for a minimum of 4 weeks to achieve optimum periodontal regeneration.\(^4^,5,3^3–3^7\) The non–cross-linked collagen membranes are subject to a rapid and uncontrollable degeneration process, and studies seem to suggest that the membranes made of non–cross-linked collagen possibly lack sufficient resistance to degradation.\(^5,3^4–3^7\) In the present study, the test material was Pro-Tiss, which is a non–cross-linked collagen membrane of porcine origin, and Ossix Plus was the control, which is a sugar–cross-linked collagen membrane. In a study by Ghanaati,\(^3^5\) the non–cross-linked, porcine-derived collagen membranes showed neither a rapid vascularization nor an early breakdown of membranes. There was only a mild vascularization observed, especially at the membrane interfaces, which supports the hypothesis that membranes integrate into the surrounding connective tissue, adapting to the vascularization of the latter.\(^3^5,3^6\) Membrane remnants, suggestive of incomplete degradation of Pro-Tiss, which is the non–cross-linked collagen membrane of Types I and III collagen used in the present study, were seen in the histologic sections of the 4-week test and control groups. Ghanaati et al\(^3^6\) observed connective tissue cells penetrating collagen membranes, which then become more dispersed throughout.\(^3^6,3^7\) This is similar to the observations in test specimens at 4 weeks, where membrane remnants were seen as smaller but numerous constricted collagen bundles.

In the present study, after placing the membrane, both materials underwent an early integration into the surrounding tissues. Membrane degradation analysis at 2 weeks showed a highly significant amount of degradation product volume in the control group (47.22 ± 11.64) compared with the test group (36.82 ± 12.82). However, at the 4-week interval, there was no significant difference in the amount of degradation product volume between the test (22.76 ± 9.29) and control groups (23.87 ± 9.56). Bozkurt et al\(^3^4\) compared the membrane degradation rates of the two non–cross-linked collagen membranes and found membrane remnants at 20 weeks. This is contrary to the present study, where 10-week specimens from the test and control groups did not show any remnants of the membrane. The reason for the complete degradation of the membranes before 10 weeks can be because of the rapid metabolic rate in rats compared with humans.\(^6,7\) Few studies\(^5,3^8\) observed a foreign body reaction with cross-linked membranes in the rat model; however, no such reaction was observed in the histologic sections from the test and control groups.

The potential limitations of this study are as follows:

- The time of total membrane degradation could not be determined due to the larger interval between 4 weeks and 10 weeks.
- Use of 10x magnification in the present study could not properly differentiate woven bone from mature bone.
- As none of the rodent cavities are completely enclosed by bone, they are technically not true sinus cavities, as opposed to the human maxillary sinus, which is completely enclosed by a single bone (maxilla).\(^6,7\)
• The studies employing animal models can only be applied to human tissues with certain limitations, with different animal species showing characteristic healing processes. Both of the membranes did not lead to complications such as postoperative exposure, hemorrhage, thermal injury, or secondary granulation tissue formation.

CONCLUSIONS

When compared with cross-linked collagen membranes, non–cross-linked collagen membranes were found to be more beneficial in terms of maxillary sinus perforation repair. The use of non–cross-linked collagen membranes may also lead to better outcomes in a maxillary sinus with a perforated sinus membrane. However, the use of non–cross-linked collagen membranes does not seem to confer added benefits over cross-linked collagen membranes in terms of bone regeneration and membrane degradation at different time intervals.

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REFERENCES