Opposing Effects of Diabetes and Tetracycline on the Degradation of Collagen Membranes in Rats

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Background: Increased collagenolytic activity, characteristic of uncontrolled diabetes, may compromise collagen membrane (CM) survival. Tetracycline (TCN) possesses anticollagenolytic properties and delays CM degradation in healthy animals. This study evaluates the degradation of TCN–immersed and -non-immersed CMs in rats with diabetes compared to those with normoglycemia.

Methods: Diabetes was induced in 15 12-week-old male Wistar rats by injection of 65 mg/kg streptozotocin. The control group consisted of 15 rats with normoglycemia. Sixty bilayered CM disks were labeled before implantation with aminohexanoyl-biotin-*N*-hydroxy-succinimide ester, of which 30 were immersed in 50 mg/mL TCN solution (experimental) or phosphate-buffered saline (PBS) (control). In each animal, two disks (control and experimental) were implanted in two midsagittal calvarial defects in the parietal bone. Similar non-implanted disks served as baseline. After 3 weeks, animals were euthanized, and the calvaria and overlying soft tissues were processed for demineralized histologic analysis. Horseradish peroxidase-conjugated streptavidin was used to detect the biotinylated collagen. The area of residual collagen within the membrane disks was measured and analyzed with a digital image analysis system. Several slides from each specimen were also stained with hematoxylin and eosin. Statistical analysis consisted of paired and unpaired *t* tests.

Results: The amount of residual collagen in PBS-immersed disks was lower in rats with diabetes compared to rats with normoglycemia (69% of baseline versus 93%, respectively, $P < 0.001$). TCN immersion increased the amount of residual collagen contents in both diabetic (83% of baseline) and healthy (97.5% of baseline) animals ($P < 0.0001$).

Conclusion: Diabetes increases CM degradation, whereas immersion in 50 mg/mL TCN solution before implantation presents an opposite effect. *J Periodontol 2013;84:529-534.*

KEY WORDS

Collagen; diabetes mellitus; membranes; rats; streptozotocin; tetracycline.

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Barrier membranes are routinely applied in guided tissue regeneration and guided bone regeneration (GBR) procedures. Barrier membranes prevent site repopulation by rapidly growing cells (i.e., epithelial, fibroblasts), thus enabling mesenchymal progenitor cell proliferation and differentiation into osteoblasts, periodontal ligament fibroblasts, and cementoblasts.[1,2] Progenitor cells, given the space and sufficient time, can restore lost attachment around teeth or regenerate mineralized tissue in bony defects.[3-6] Collagen membrane (CM) degradation plays a crucial role in the success of the regenerative procedures.[6-10] Premature exposure of barrier membranes to the oral environment and their consequent resorption has a detrimental effect on bone regeneration.[11-17] Cells within the surgical site release matrix metalloproteinases (MMPs) to the wound area during healing, which contributes to CM degradation.[18,19] Downregulation of collagen-degrading enzymes, such as MMPs, by specific tissue inhibitors of matrix metalloproteinases (TIMPs)[20] or increasing the structural stability of the membranes by crosslinking slows down the process.[21]

Tetracycline (TCN) inhibits MMP activity.[22-24] We had reported previously that immersion of collagen membranes in TCN solution before their implantation reduces their degradation in vitro[25] and in vivo.[26] Decreased levels of TIMP-1 and

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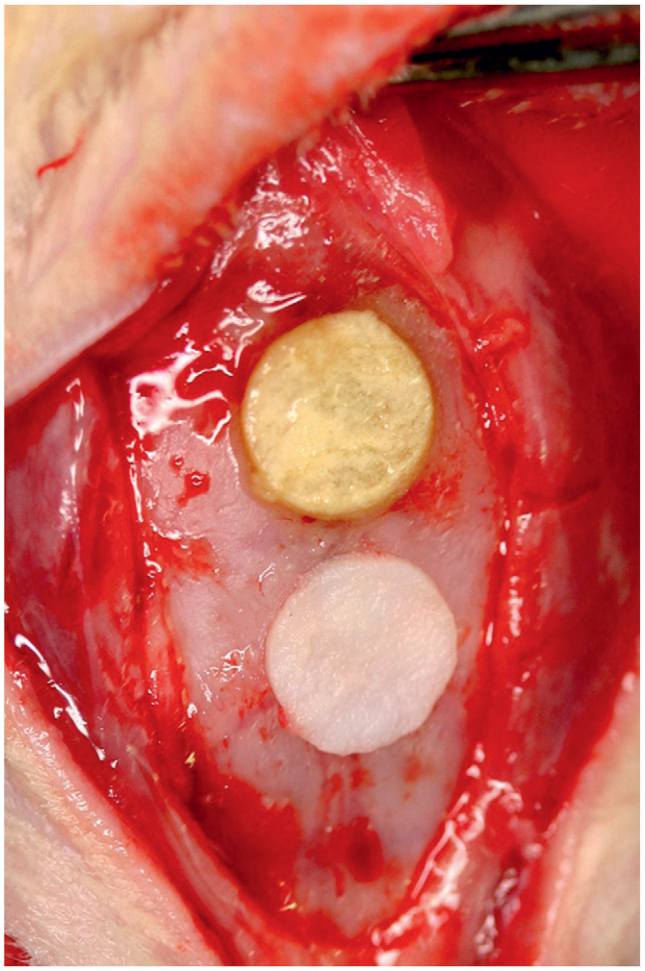

Figure 1.
Surgical site of experiment. TCN-immersed (yellow) and PBS-immersed (white) collagen disks both located in a surgically created intrabony defect to contain the disks.

elevated activity of MMPs were found in rats with streptozotocin (STZ)-induced diabetes.[27] Although enhanced CM degradation could be expected in individuals with diabetes, there are still no data confirming this hypothesis. CM degradation depends on collagenolytic activity, and TCN, an antibiotic agent with anticollagenolytic properties, could slow down the degradation of CMs also in rats with diabetes.

The purpose of the present study is to evaluate CM degradation in rats with diabetes versus healthy rats and, at the same time, evaluate the effect of soaking the membranes in TCN solution before their implantation.

MATERIALS AND METHODS

The study comprised 30 12-week-old male Wistar rats. The Institutional Animal Care and Use Committee of Tel Aviv University, Tel Aviv, Israel, approved the study.

Diabetes was induced in 15 animals with a single intraperitoneal administration of the diabetogenic agent STZ (65 mg/kg body weight)§ diluted in citrate buffer (0.01 M, pH 4.3). Blood glucose level was evaluated at regular intervals using a glucometer‖ according to the instructions of the manufacturer. The rest of the animals were given similar volumes of citrate buffer and served as normoglycemic controls.

Bilayered CMs¶ were cut with a disposable biopsy punch# to 5-mm-diameter disks. The average weight of the disks was 2.0 ± 0.1 mg. Membrane labeling with biotin has been described previously.[25,28] Briefly, all disks were labeled with aminohexanoyl-biotin-*N*-hydroxy-succinimide ester (AH-BNHS),** following a slight modification of the protocol of the supplier. Membranes were incubated with 3 mg/mL AH-BNHS for 1 hour at room temperature and then washed overnight with three changes of phosphate-buffered saline (PBS) (Ca^{2+}–Mg^{2+} free, pH 7.4) to remove any unbound biotin. Half of the biotin-labeled membrane disks were immersed in TCN (50 mg/mL)†† for 1 hour as described previously,[24] followed by washing in PBS. This TCN concentration was found to be the most effective to delay CM degradation.[24] The remaining disks were immersed only in PBS. All of the above membrane processing was performed in an aseptic environment; therefore, there was no need for membrane resterilization. Mechanical membrane properties after TCN were not evaluated.

All animal surgeries were performed by the same experienced operator (CN). The surgical protocol for membrane implantation has been described previously.[24] Briefly, the dorsal part of the skin covering the scalp was shaved and aseptically prepared for surgery. A U-shaped incision was made in the scalp between the eyebrows caudally connecting two sagittal incisions extending posteriorly over the parietal bone to enable elevation of a full-thickness flap. A high-speed, water-cooled diamond wheel-shaped bur was used to create two similar, 5-mm-diameter, shallow (≈1 mm deep) bony defects on the midline of the parietal bone. Bone was always left to cover the dura mater, which was not involved. Two different CM disks (one immersed in TCN and the other in PBS) were placed in each animal (Fig. 1). Soft tissues were repositioned, first the periosteum, covering the implanted membranes, and then the dermal tissues were sutured with resorbable sutures .‡‡

§ Sigma Chemical, St. Louis, MO.
‖ Accu-Chek, Roche Diagnostics, F. Hoffmann-La Roche, Basel, Switzerland.
¶ Bio-Gide, Geistlich Pharma, Wolhusen, Switzerland.
# Miltex Instrument Company, Lake Success, NY.
** Zymed Laboratories, San Francisco, CA.
†† Tevacycline, Teva Pharmaceutical Industries, Petah Tikva, Israel.
‡‡ VICRYL RAPIDE, Ethicon, Madrid, Spain.

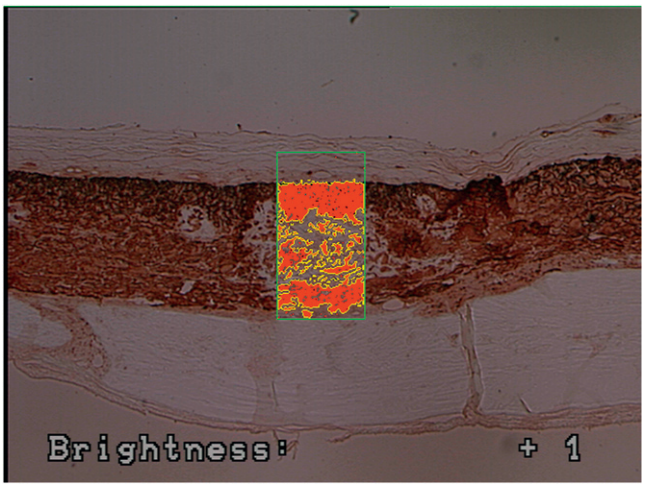

Figure 2.
Histologic appearance of the central part of the membrane. An ROI shows as a green rectangle. The red–brown pixels within the ROI represent the collagen content registered. Magnification ×40; biotin–streptavidin–HRP.

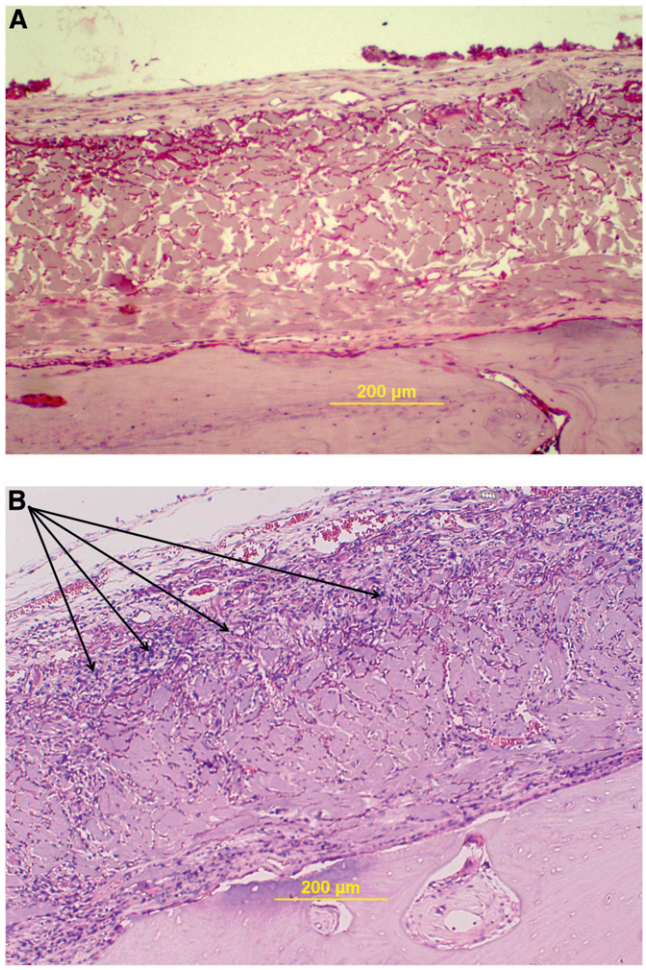

Figure 3.
A) *No inflammatory infiltrate can be noticed around and within the membrane in the animals with normoglycemia.* ***B)*** *Marked inflammatory infiltrate, primarily consisting of mononuclear cells (some examples highlighted by black arrows), is apparent within the membrane and surrounding tissues in the diabetic group. Magnification ×100 (A) and ×200 (B); hematoxylin and eosin.*

Animals were sacrificed after 21 days with an overdose of ketamine chlorohydrate[§§] at 90 mg/kg body weight and xylazine[‖‖] at 10 mg/kg body weight, followed by asphyxiation with carbon dioxide. Just before the animals were sacrificed, blood was collected from the animals' tail vein for final glucose and hemoglobin A1c (HbA1C) measurements. The latter was performed with affinity columns.[¶¶] Dermal tissues were dissected, leaving the periosteum undisturbed, covering the membrane disks lying in the calvaria defects. The calvaria and surrounding tissues were retrieved, fixed in 10% neutral buffered formalin, decalcified for 5 weeks in 10% EDTA solution, washed, dehydrated in ethanol and xylene, and embedded in paraffin. Sagittal 5-µm sections were made, and those that included the central area of each of the two bony defects were selected for comparative analysis of CM degradation. Horseradish peroxidase (HRP)-conjugated streptavidin[##] was used according to the protocol of the manufacturer to detect biotinylated collagen. Slides were incubated with a solution of one enzyme drop in 1 mL 10 mM PBS (pH 7.4) for 5 minutes at room temperature, followed by detection with a 3-amino-9-ethylcarbazole (red) substrate kit, *** and mounted with an aqueous solution of glycerol vinyl alcohol.[†††]

Histologic evaluations were performed by the same experienced investigator (OM). Stained sections were photographed with a digital camera mounted on a light microscope,[‡‡‡] at a magnification of ×100. Multiple digital images were taken and stored for each sample and analyzed for staining intensity and area by an image analysis system.[§§§] In each block, the central part of the membrane was identified by measuring the underlying bone defect. A region of interest (ROI) was determined as a rectangle, 1.2 × 0.5 mm, with an area of 0.6 mm^2, which was superimposed on five different areas in each disk. The number of pixels that were positively stained within the ROI was registered (Fig. 2). Two non-implanted biotin-labeled disks (one immersed in TCN and the other in PBS) were processed in the same manner and served as baseline. The residual collagen area measured in each section

§§ Rhone Merieux, Lyon, France.
‖‖ Vitamed, Bat-Yam, Israel.
¶¶ GLYCO-Tek column kit, Helena Laboratories, Beaumont, TX.
Zymed Laboratories.
*** Zymed Laboratories.
††† Invitrogen, Camarillo, CA.
‡‡‡ Laborlux K, Leitz, Wetzlar, Germany.
§§§ NOVA, R & M Biometrics, Nashville, TN.

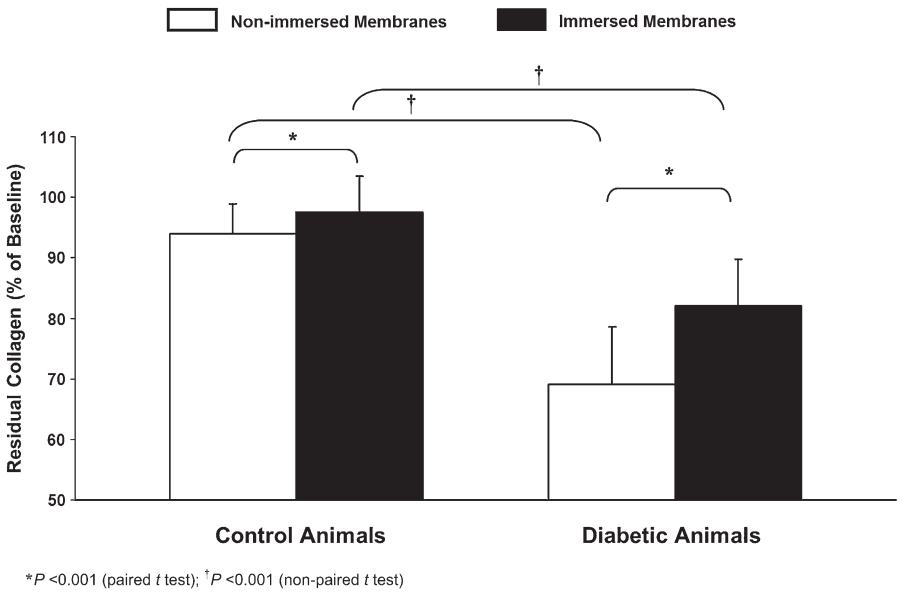

Figure 4.
Percentage of membrane collagen remaining in the diabetic and the normoglycemic groups related to baseline contents of collagen with or without TCN. *P <0.001 *(paired t tests);* †P <0.001 *(non-paired t tests).*

was calculated as percentage of the respective baseline measurements according to the following formula: collagen area at 21 days ÷ baseline collagen area × 100. In each slide, several measurements were taken for each membrane, and their calculated mean served as a unit for statistical analysis. Several slides from each specimen were also stained with hematoxylin and eosin to identify the surrounding tissues.

Data obtained from disks implanted within the same animals (PBS versus TCN) were statistically analyzed by paired *t* tests, whereas comparison between animals (hyperglycemic versus normoglycemic) was performed by non-paired *t* test.

RESULTS

All 15 STZ-injected rats demonstrated a glucose level >250 mg/dL after 4 days and, accordingly, were considered diabetic. Animals that were injected with citrate buffer and did not develop diabetes conformed to a matched normoglycemic control group. Healing after the surgical procedures for the collagen disk implantation was uneventful in all animals.

By the end of day 21, the mean level of HbA1C in the rats with diabetes was 11.536 ± 1.328, whereas in the rats with normoglycemia, it was 5.254 ± 0.961. Differences between groups were statistically significant (P <0.001).

At tissue harvest, clinically, membranes appeared well integrated with the surrounding tissues.

Although not measured microscopically, voids within the membranes in the rats with diabetes appeared larger compared to the rats with normoglycemia, whereas in the TCN-immersed CM disks, voids appeared smaller in both rats with diabetes and control animals compared to the non-immersed membranes. In the animals with normoglycemia, no inflammatory infiltrate was observed around the membranes of both TCN-immersed and control animals. However, a marked inflammatory infiltrate (Fig. 3), primarily consisting of mononuclear cells, was apparent within the tissues surrounding the membranes in the diabetic group.

To verify that the decalcification process with EDTA does not modify the biotin labeling of collagen, a preliminary in vitro assay including 10 biotin-labeled collagen disks was performed, among which five were exposed to EDTA. Avidin–HRP staining was then performed to visualize the biotin-labeled collagen. No statistically significant differences were noted between EDTA-treated and non-treated membranes (not shown).

Results are summarized in Figure 4. The amount of residual collagen in PBS-immersed disks in rats with diabetes (86,095 ± 11,881 pixels/field [≈69% of baseline]) was much lower than that in the healthy rats (117,655 ± 6,070 pixels/field [≈93% of baseline]; P <0.001).

TCN immersion significantly increased the residual collagen contents of the CM disks in both animals with diabetes (to 113,489 ± 10,818 pixels/field [≈83% of baseline]; P <0.0001), as well as in healthy animals (to 135,575 ± 8,364 [≈97.5% of baseline]; P<0.0001) (Figs. 5 and 6)

DISCUSSION

The main findings of this study show for the first time that collagen barrier membrane degradation is markedly enhanced in uncontrolled STZ-induced diabetes compared to a normoglycemic situation. Immersion of the membranes in 50 mg/mL of TCN before implantation significantly reduces their degradation in both healthy animals and those with diabetes.

Various reports have shown that, in diabetes, the level of MMPs is higher and the level of TIMPs is lower.[27,29] The inflammatory infiltration of the tissues surrounding the CMs was more marked in the animals with diabetes compared to those with normoglycemia. Previous reports have found that macrophage infiltration into the kidney is increased in rats with STZ-induced diabetes,[30] and diabetes may lead to enhanced monocyte chemoattractant protein-1 levels

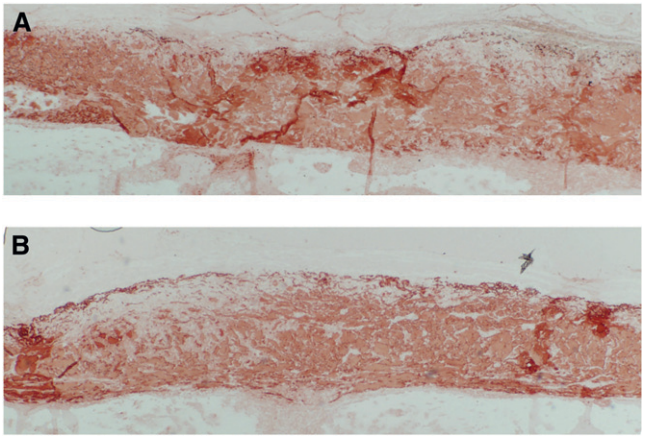

Figure 5.
Histologic view (×40) of collagen membranes stained in red–brown with avidin–biotin–HRP reaction, 21 days after implantation in rats with STZ-induced diabetes. ***A)*** *Membrane without immersion in TCN.* ***B)*** *Membrane immersed in 50 mg/mL TCN.*

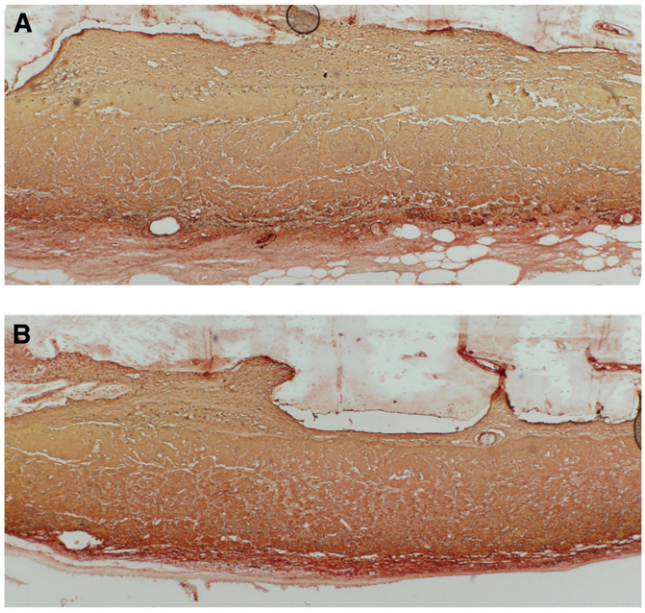

Figure 6.
Histologic view (×40) of collagen membranes stained in red–brown with avidin–biotin–HRP reaction, 21 days after implantation in a rat with normoglycemia. ***A)*** *Membrane without immersion in TCN.* ***B)*** *Membrane immersed in 50 mg/mL TCN.*

that may be responsible for the recruitment of these inflammatory cells.[31] The existence of high levels of inflammatory cellular and molecular infiltrate, such as MMPs or low levels of TIMPs in diabetes mellitus,[27] may contribute to the enhanced degradation of CMs compared to normoglycemic conditions.

TCN and its chemically modified non-antimicrobial derivatives inhibit the catalytic activities of human collagenases and gelatinases, especially the neutrophil MMP.[32-34] Previous findings from our research group have shown that immersion of a bilayered porcine CM in a 50 mg/mL TCN solution significantly delays its degradation after implantation in the rat calvaria.[25] The present study confirms these findings in both rats with STZ-induced diabetes and rats with normoglycemia. This could be advantageous in certain bone regenerative procedures in which a prolonged barrier function is indicated. In patients with diabetes, faster membrane resorption could be detrimental after bone augmentation procedures.

CONCLUSIONS

The main conclusions of the present study are that diabetes increases CM degradation, whereas immersion in 50 mg/mL TCN solution before implantation presents an opposite effect. However, it should be noted that, in the present study, membranes are implanted in surgically created shallow defects in the rat calvaria, which is a closed, non-contaminated, extraoral environment. Therefore, results might not be directly extrapolated to GBR procedures in the oral cavity, especially regarding the possibility of membrane exposure to oral bacteria.

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The authors report no conflicts of interest related to this study.

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