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 (TTC) possesses anti-collagenolytic properties and delays CM degradation in healthy animals. This study evaluated the degradation of TTC-immersed and non-immersed CMs in diabetic, compared with normoglycemic rats.

Materials & methods: Diabetes was induced in fifteen 12-week old male Wistar rats by injection of 65 mg/kg Streptozotocin. Control group consisted of 15 normoglycemic rats. 60 bi-layered collagen membrane discs were labeled prior to implantation with Aminohexanoyl-Biotin-N-Hydroxysuccinimide Ester, 30 of those were immersed in 50 mg/mL TTC solution (experimental) or PBS (control). In each animal, 2 discs (control and experimental) were implanted in 2 mid-sagittal calvarial defects in the parietal bone. Similar non-implanted discs served as baseline. After 3 weeks, animals were euthanized and the calvaria and overlying soft tissues processed for demineralized histological analysis. Horseradish Peroxidase-conjugated streptavidin was used to detect the biotinylated collagen. The area of residual collagen within the membrane discs was measured and analyzed with a digital image analysis system. Several slides from each specimen wee also stained with H&E. Statistical analysis consisted of paired and unpaired t-tests.

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

Conclusions: Diabetes increases collagen membranes degradation while immersion in 50mg/mL TTC solution previous to implantation presents an opposite effect.

KEY WORDS

collagen, in-vivo, membrane, tetracycline, Streptozotocin, Diabetes.

Barrier membranes are routinely applied in guided tissue regeneration (GTR), 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 degradation plays a crucial role in the regenerative procedures success [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 collagen membrane degradation.[18-19] Down-regulation 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 cross-linking, slows down the process [21].

Tetracycline (TTC) inhibits MMP activity [22-24.] We had previously reported that immersion of collagen membranes in tetracycline solution prior to their implantation reduces their degradation in vitro [25] and in vivo[26]. Decreased levels of TIMP-1 and elevated activity of MMPs were found in rats with STZ-induced diabetes[27]. Although, enhanced collagen membrane degradation could be expected in diabetic subjects, there are still no data confirming this hypothesis. Collagen membranes degradation depends on collagenolytic activity, TTC, an antibiotic agent with anti-collagenolytic properties could slow down the degradation of collagen membranes also in diabetic rats.

The purpose of the present study was to evaluate collagen membrane degradation in diabetic versus healthy rats and, at the same time, evaluate the effect of soaking the membranes in TTC solution prior to their implantation.

MATERIALS & METHODS

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

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

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

All animal surgeries were performed by the same experienced operator (CN). The surgical protocol for membrane implantation has been previously described [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 2 similar, 5-mm diameter, shallow (approximately 1 mm deep) bony defects, on the mid-line of the parietal bone. Bone was always left to cover the dura mater, which was not involved. Two different

collagen membrane discs (1 immersed in TTC 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 [‡‡].

Animals were sacrificed after 21 days, with an overdose of Ketamine chlorhydrate [§§] at 90 mg/kg body weight and Xylazine [‖‖] at 10 mg/kg body weight, followed by asphyxiation with carbon dioxide (CO_2). Just prior to euthanasia, blood was collected from the animals' tail vein for final glucose and HbA1C measurements. The latter was performed with affinity columns [¶¶]. Dermal tissues were dissected leaving the periosteum undisturbed, covering the membrane discs 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 collagen membrane degradation. Horseradish Peroxidase-conjugated streptavidin [##] was used according to the manufacturer's protocol to detect biotinylated collagen. Slides were incubated with a solution of one enzyme drop in 1 mL of 10 mM PBS, pH 7.4 for 5 min at room temperature, followed by detection with an AEC (red) substrate kit, containing Aminoethyl Carbazole Substrate [##] 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 x100. 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 mm x 0.5 mm, with an area of 0.6 mm^2, which was superimposed on 5 different areas in each disc. The number of pixels that were positively stained within the ROI was registered. (Fig.2) Two non-implanted biotin-labeled discs, (one immersed in TTC and the other in PBS), were processed in the same manner and served as baseline. The residual collagen area measured in each section was calculated as percentage of the respective baseline measurements according to the formula: collagen area at 21 days divided by baseline collagen area and multiplied by 100. In each slide, 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 discs implanted within the same animals (PBS vs. TTC) were statistically analyzed by paired t tests, while comparison between animals (hyperglycemic vs. normoglycemic) was performed by non-paired t test.

RESULTS

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

By the end of the 21st day, the mean level of HbA1C in the diabetic rats was 11.536±1.328, while in the normoglycemic rats 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 diabetic rats appeared larger compared to the normoglycemic rats, while in the TTC immersed collagen membrane discs voids appeared smaller both in diabetic and control animals compared to the non-immersed membranes. In the normoglycemic animals, no inflammatory infiltrate was observed, around the membranes both TTC immersed and control. However, a marked inflammatory infiltrate (Fig 3A, 3B), largely consisting of mononuclear cells was apparent within the tissues surrounding the membranes in the diabetic group.

In order 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 5 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 the non-treated membranes.

Membrane Degradation

Results are summarized in Figure 4. The amount of residual collagen in PBS-immersed discs in diabetic rats [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), ($P<0.001$).

TTC-immersion significantly increased the residual collagen contents of the collagen membrane discs both in diabetic animals [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, $P<0.0001$)]. (Fig 5A, 5B, 6A, 6B)

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 as compared to a normoglycemic situation. Immersion of the membranes in 50 mg/mL of TTC prior to implantation significantly reduces their degradation both in healthy and diabetic animals.

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 collagen membranes, , was more marked in the diabetic animals compared to the normo-glycemic ones. Previous reports have found that macrophage infiltration into the kidney is increased in STZ induced diabetic rats,[30] diabetes may lead to enhanced monocyte chemo-attractant protein-1 (MCP-1) levels 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 collagen membranes compared to normoglycemic conditions.

TTC and their 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 collagen membrane in a 50 mg/mL TTC solution significantly delays its degradation after implantation in the rat calvaria[25]. The present study confirms those findings both in STZ induced diabetic and in normoglycemic rats. This could be advantageous in certain bone regenerative procedures were a prolonged barrier function is indicated. In Diabetic patients faster membrane resorption could be detrimental following bone augmentation procedures.

The main conclusions of the present study were that diabetes increases collagen membranes degradation while immersion in 50mg/mL TTC solution previous to implantation presents an opposite effect. However, it should be noted that in the present study, membranes were implanted in surgically created shallow defects in the rat calvaria which is a closed, non-contaminated, extra-oral 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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Submitted March 19, 2012; accepted for publication May 07, 2012.

Figure 1.

Surgical site of experiment: TTC immersed collagen disc (yellow), PBS immersed (white), both located in a surgically created intrabony defects to contain the discs.

Figure 2.

Histologic appearance of the central part of the membrane. A region of interest (ROI) shows as a green rectangle. The red-brown pixels within the ROI represent the collagen content registered. (Magnification x40. Staining: Biotin-streptavidine-HRP)

Figure 3.

Fig 3A. No inflammatory infiltrate can be noticed around and within the membrane in the normoglycemic animals. Fig 3B. Marked inflammatory infiltrate, largely consisting of mononuclear cells (few examples highlighted by black arrows), is apparent within the membrane and surrounding tissues in the diabetic group. (Staining: H&E. Magnification 3A: x100, 3B: x200)

Figure 4.

Percentage of membrane collagen remaining in the diabetic and the normoglycemic group related to baseline contents of collagen with or without tetracycline (TTC).

Figure 5.

Histological view (x40) of collagen membranes stained in red/brown with Avidin-Biotin-HRP reaction, 21 days after implantation with in STZ induced diabetic rat. A.: membrane without immersion in TTC, B.: membrane immersed in 50 mg/mL TTC.

Figure 6.

Histological view (x40) of collagen membranes stained in red/brown with Avidin-Biotin-HRP reaction, 21 days after implantation in a normoglycemic rat: - A. membrane without immersion in TTC. B – membrane immersed in 50mg/mL of TTC.

[§] Sigma Chemical Co., St Louis, MO, USA

[‖] Accu-Chek, Roche Diagnostics, F. Hoffmann-La Roche, Basel, Switzerland

[¶] BioGide®, Geistlich Pharma, Wolhusen, Switzerland

[#] Miltex Instrument Company, Lake Success, NY, USA

[**] Zymed Laboratories, Inc., San Francisco, CA, USA

[††] Tevacycline, Teva Pharmaceutical Industries, Ltd., Petah Tikva, Israel

[‡‡] Vycril Rapid, Ethicon, Madrid, Spain

[§§] Rhone Merieux, Lyon, France

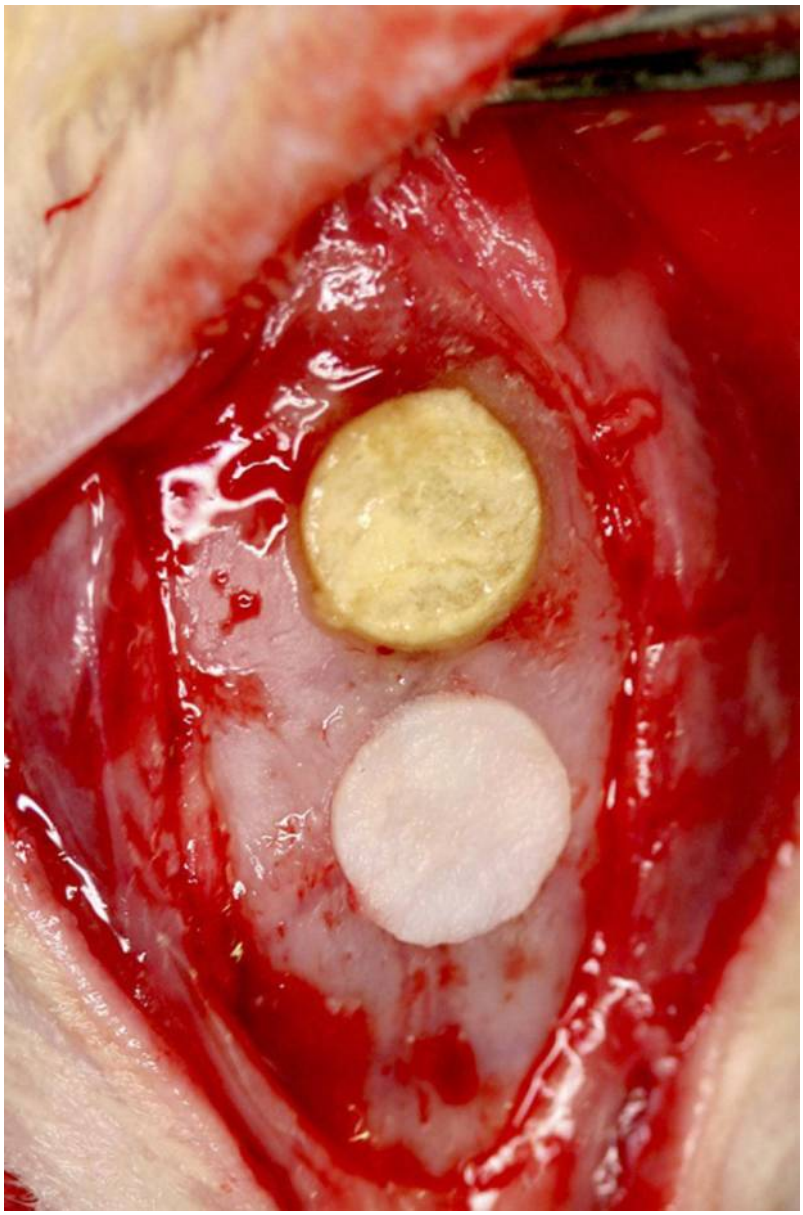
[‖‖] Vitamed, Bat- Yam, Israel

[¶¶] Glyco-tek column kit, Helena Laboratories, Beaumont, Texas, USA

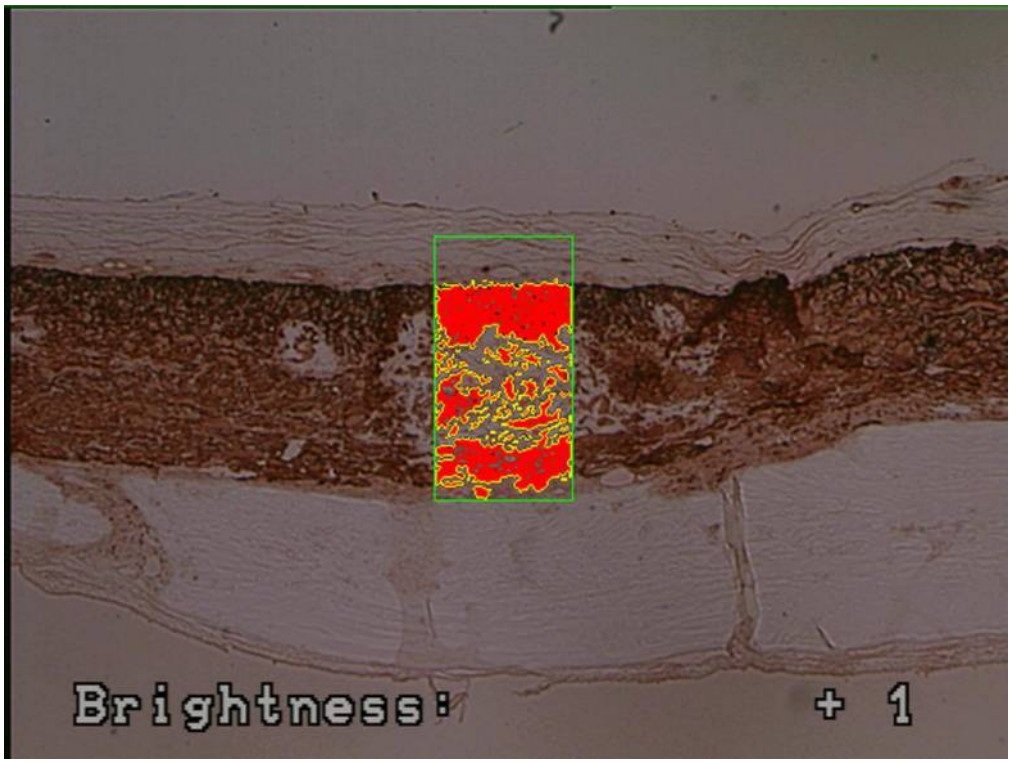
[##] Zymed Laboratories Inc., San Francisco, CA, USA

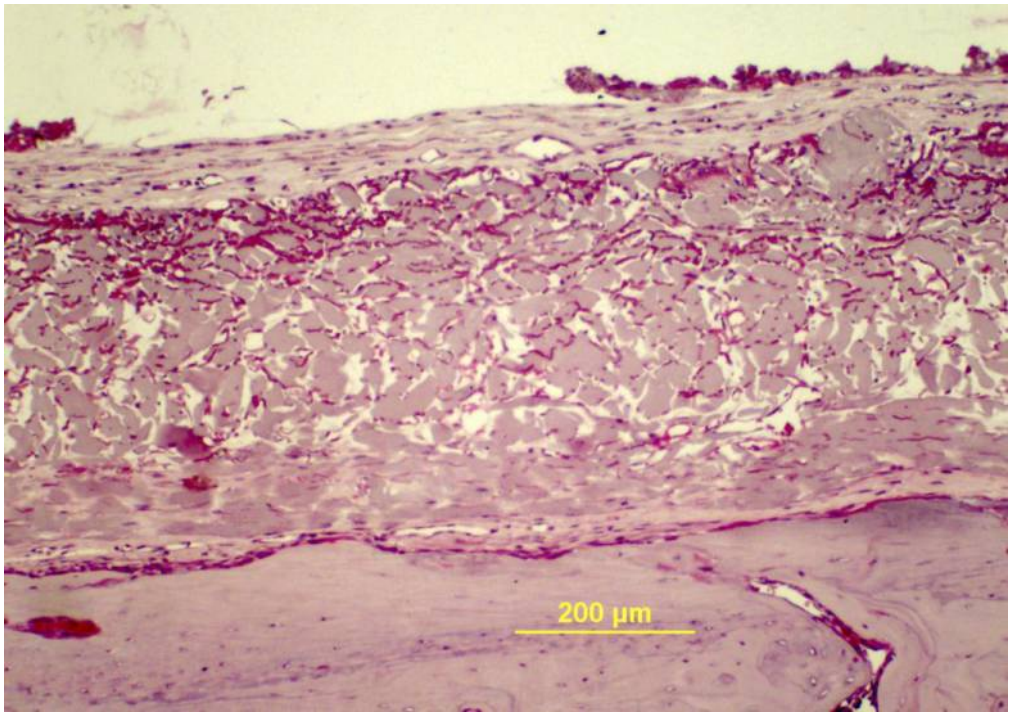
*** Laborlux K, Leitz, Wetzlar, Germany

††† NOVA, R&M Biomatrics, Nashville, TN, USA



 DOI: 10.1902/jop.2012.120188

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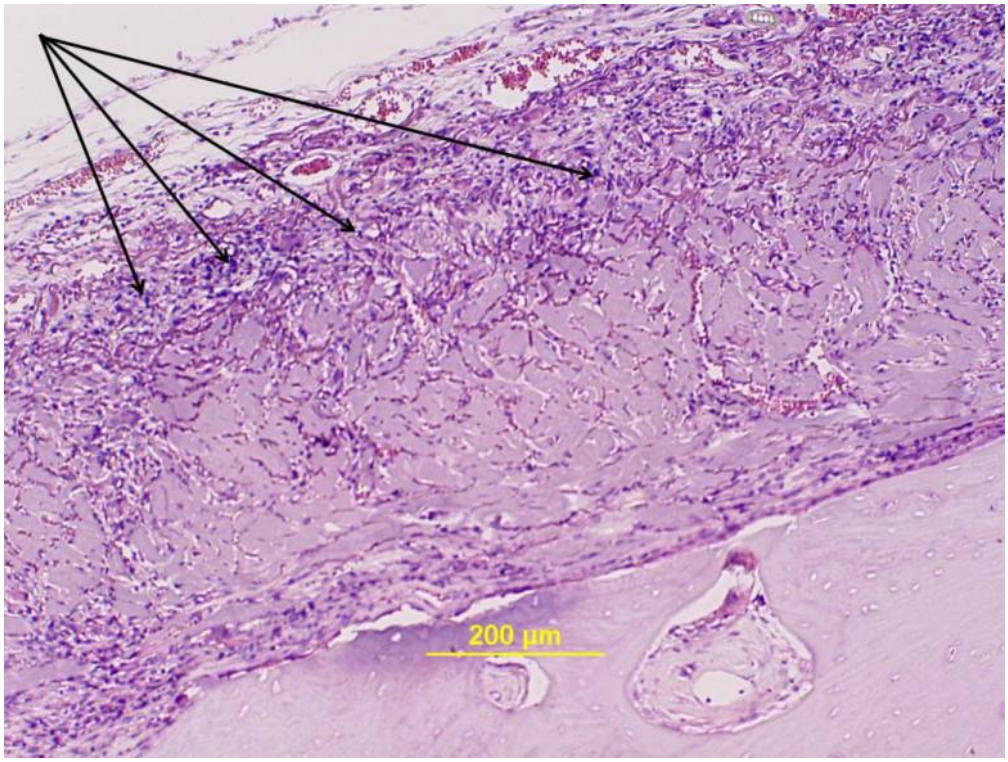
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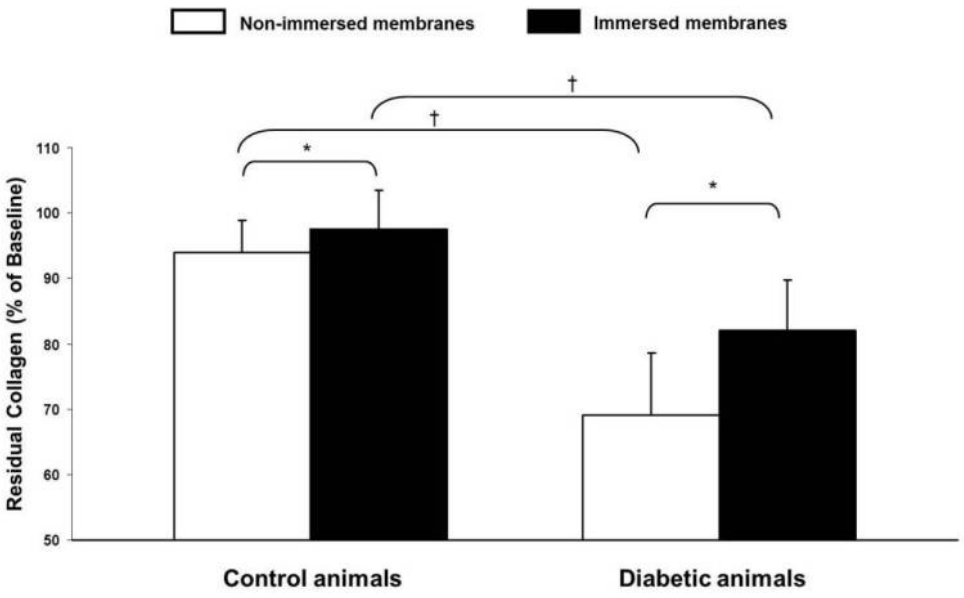

For legends: *, P<0.001 (paired t-test). †, P< 0.001 (non-paired t-test)