

# Tetracycline impregnation affects degradation of porcine collagen matrix in healthy and diabetic rats

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## Abstract

**Objectives** The present study evaluated the degradation of collagen matrix (CM) immersed in tetracycline (TTC) or phosphate-buffered saline (PBS) in diabetic and normoglycemic rats.

**Materials and methods** Diabetes was induced in 15 rats by systemic streptozotocin (STZ) (experimental); 15 healthy rats served as controls. One day before implantation 60 CM disks, 5 mm in diameter, were labeled with biotin: 30 were immersed in tetracycline (TTC) and 30 in PBS. One disk of each type was implanted subdermally in each rat. Animals were euthanized after 3 weeks, and tissue specimens containing the disks were prepared for histologic analysis. Horseradish peroxidase (HRP)-conjugated streptavidin was used to detect the remaining biotinylated collagen. Residual collagen area within the CM disks was analyzed and compared to baseline.

**Results** Diabetes significantly increased the CM degradation. Immersion of the CM disks in a 50-mg/mL TTC solution before implantation decreased its degradation both in diabetic and normoglycemic rats.

**Conclusions** Diabetes significantly increases collagen matrix degradation; immersion of collagen matrix in TTC before

implantation decreases its degradation in both diabetic and normoglycemic conditions.

**Clinical relevance** Immersion of medical collagen devices in TTC may be an effective means to decrease their resorption rate and increase their effectiveness, especially in situations with increased degradation such as diabetes.

**Keywords** Animal model · Biodegradation · Collagen · Diabetes · Membranes

## Introduction

Implantation of medical collagen products is a routine procedure in oral surgery [1]. Most commonly, it is used in the form of collagen barrier membranes in guided tissue regeneration (GTR) and guided bone regeneration (GBR) procedures. Collagen is a natural choice for many xenogeneic tissue matrices due to its high biocompatibility, low antigenicity, as well as the possibility of safely controlling its biodegradability by cross-linking reagents [2–8].

Collagen membranes have also been used with varying degrees of success as an alternative to autologous tissue grafts, aiming to improve the gingival biotype by increasing the thickness and width of the keratinized mucosa around teeth and implants [9, 10]. Recently, a novel collagen matrix (CM) (Mucograft<sup>®</sup>, Geistlich Pharma AG Wolhusen, Switzerland) has been designed for similar purposes, with application in soft tissue augmentation procedures. The CM consists of pure porcine type I and III collagen extracted and purified without additional cross-linking [11, 12]. It is subsequently engineered into a bi-layered matrix of approximately 3.5–4.0 mm thickness. The outer smooth layer is thin and made up of tightly packed collagen fibers to promote tissue adherence and facilitate wound healing. The inner roughened surface is a thick

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porous structure, which is placed against the host tissue, providing a space, initially for blood clot formation and then for tissue in-growth [11–15].

Although CM was primarily designed for oral soft tissue defect grafting, providing an adequate alternative to autologous soft tissue, it has also been shown to eliminate adverse effects such as disruption of the adjacent soft tissue architecture, loss of vestibular depth, and needs for a second surgical site, thus reducing morbidity [11–16]. An extended survival time of the CM seems to be important to improve soft tissue in-growth into the implanted collagen scaffold.

In vivo collagen implant degradation and remodeling depend on several tissue characteristics [17]. During healing, cells within the surgical site release matrix metalloproteinases (MMPs) which contribute to implant degradation [18, 19]. The catabolic effect of MMPs on implanted collagen may also be delayed by collagen stabilization such as increasing intermolecular cross-linking [2–5, 20–22]. Immersion of collagen membranes in tetracycline (TTC) has been suggested as an additional means to delay the degradation of non-cross-linked collagen membranes both in vitro and in vivo, probably due to its anti-collagenolytic activity [23–26].

Diabetes is a prevalent and debilitating disease affecting a multitude of organs. The common denominator for most of the diabetic complications is an exaggerated and prolonged inflammatory response, including excessive production of pro-inflammatory cytokines, such as tumor necrosis factor  $\alpha$  and interleukin-1 [27, 28]. The diabetic condition is also accompanied by increased numbers of immune cells, such as neutrophils and macrophages [27] as well as excessive amounts and activity of MMPs [29]. Together with a reduction in tissue inhibitors of metalloproteinases (TIMP) activity [30], these changes result in exaggerated collagenolytic activity in diabetic tissues that may potentially jeopardize the integrity and longevity of implanted collagen devices. Indeed, we have recently shown that uncontrolled diabetes increases the degradation rate of collagen membranes implanted in rats and that immersion in TTC delayed their degradation both in normoglycemic and diabetic animals [31]. The present study was undertaken to evaluate the effects of diabetes and immersion in TTC on the degradation of collagen matrix in vivo.

## Materials and methods

This animal research comprised of 30 12-week-old male Wistar rats. Methods were in strict compliance with the protocol approved by the Institutional Animal Care and Use Committee of the Tel Aviv University. Diabetes was induced in 15 animals (experimental group) with a single intraperitoneal administration of streptozotocin (STZ, Sigma Chemical, St. Louis, MO), (65 mg/kg body weight) diluted in citrate buffer (0.01 M, pH 4.3). Blood glucose levels were evaluated

once a week using a glucometer (Accu-Chek, Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The remaining 15 rats were given similar volumes of citrate buffer and served as normoglycemic controls.

CM sheets were used to aseptically prepare 5-mm-diameter disks using a disposable biopsy punch (Miltex Instrument Company, Lake Success, NY). The disks were labeled with biotin as previously described [23, 31]. Briefly, 1 day before implantation, the disks were incubated with aminohexanoyl-biotin-*N*-hydroxy-succinimide ester (Zymed Laboratories, San Francisco, CA) at 3 mg/mL for 1 h at room temperature and then washed overnight with three changes of sterile phosphate-buffered saline (PBS) ( $\text{Ca}_{2+}/\text{Mg}_{2+}$  free, pH 7.4). Half of the biotin-labeled CM disks were immersed in TTC (Tevacycline, Teva Pharmaceutical Industries, Petah Tikva, Israel) (50 mg/mL) for 1 h, followed by washing in PBS. The other half was immersed solely in PBS. The TTC concentration of 50 mg/mL was chosen based on previous in vitro and in vivo experiments that found it to be the most effective [23, 24].

A rectangular area measuring approximately 15 × 30 mm along the animal's back was shaved and aseptically prepared for surgery. Two dermal pouches were surgically created over the paravertebral area, 5 and 15 mm caudally to an imaginary line between the ears. Semi-lunar incisions were made perpendicular to the midsagittal line, and a full-thickness dermal flap was elevated by separating the skin from the underlying fascia within the epifascial plane. Pouches were made slightly larger than the disks to enable passive CM flat placement. One disk was placed in each pouch, with the smooth surface facing the skin. Each disk was stabilized by a 6–0 nylon suture (Assut, Pully-Lausanne, Switzerland) to the underlying tissues. The dermal flaps were repositioned and sutured with resorbable 4–0 Vicryl sutures (Vicryl Rapide, Ethicon, Madrid, Spain).

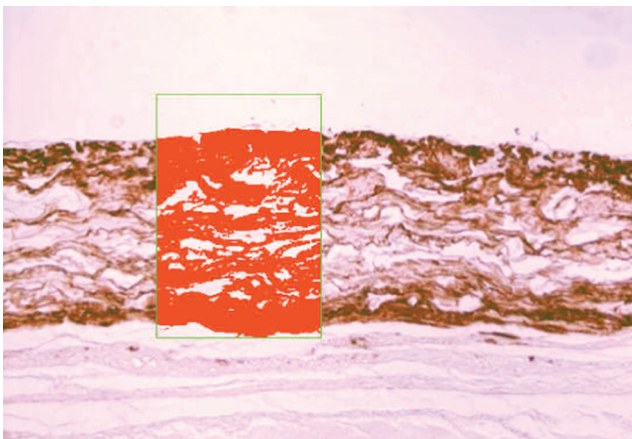
Animals were sacrificed 21 days after surgery with an overdose of ketamine chlorohydrate (Rhone Merieux, Lyon, France, 90 mg/kg body weight) and xylazine (Vitamed, Bat-Yam, Israel, 10 mg/kg body weight), followed by asphyxiation with carbon dioxide. A single evaluation time was chosen at 3 weeks since due to animals' uncontrolled diabetic condition, a longer observation was not possible. Prior to euthanasia, blood was collected from the tail vein for final glucose measurements. The surgical scars were identified, and the skin over the sites was dissected out by separating the dermal tissue together with the underlying fascia and the disks and removed en bloc.

The tissue blocks were trimmed, separated in two parts (experiment/control), and fixed in 10 % neutral buffered formalin. After fixation, the sutures were carefully removed and the specimens were washed and dehydrated in ethanol and

xylene and embedded in paraffin. Sagittal 5- $\mu$ m-thick sections were made, and those including the central area of each disk were selected for analysis. Horseradish peroxidase (HRP)-conjugated streptavidin (Zymed Laboratories) was used according to the manufacturer's protocol to detect biotinylated collagen. The slides were incubated with a solution of one drop of enzyme and washed with PBS for 5 min at room temperature, followed by detection with a substrate kit containing 3,3'-diaminobenzidine (DAB, ScyTek, Logan, UT, USA) as substrate and mounted with an aqueous solution of glycerol vinyl alcohol (Zymed). Two similar disks (immersed in PBS or TTC) that had not been implanted were processed for histology and served as baseline.

Blinded histometric evaluation was performed by one single investigator (AS). Stained sections were photographed with a digital camera mounted on a light microscope (Laborlux K, Leitz, Wetzlar, Germany) at a final magnification of  $\times 40$ . Digital images were taken from the most central section of each sample by selecting the longest CM profiles and analyzed with an image analysis system (OSTEO, R & M Biometrics, Nashville, TN). A region of interest (ROI) was determined as a rectangle (0.7 mm $\times$ 0.45 mm), which was superimposed on five adjacent areas in each disk. The number of pixels that were positively stained within the ROI was recorded (Fig. 1).

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 $\times$ 100/baseline collagen area. For statistical purposes, measurements from each CM disc were averaged and evaluated with two-way ANOVA with repeated measures. Several sections from each specimen were also stained with hematoxylin and eosin (H&E) or with picro-sirius red (PSR) to be viewed under polarized light for identification and histological observations of the surrounding tissues.



**Fig. 1** Method for measuring the residual collagen contents. A rectangular ROI (green) is superimposed over a CM stained with biotin-avidin-HRP. The red (detected) pixels are calculated as percentage of the ROI area

## Results

One animal from the diabetes group died 3 days after diabetes induction. All the other animals injected with STZ became hyperglycemic (blood glucose  $>250$  mg/dL) by day 4. Post-surgical healing was uneventful in the remaining 29 animals. During dissection, the dark nylon sutures were visible and the disks were identifiable. All 58 CM specimens were prepared for histology. Four specimens (2 from the diabetic TTC-treated group and 2 from the diabetic non-TTC-treated group) were damaged during the preparation process: these and their counterparts were dropped from further study, thus, only 25 pairs of CM discs were available for analysis.

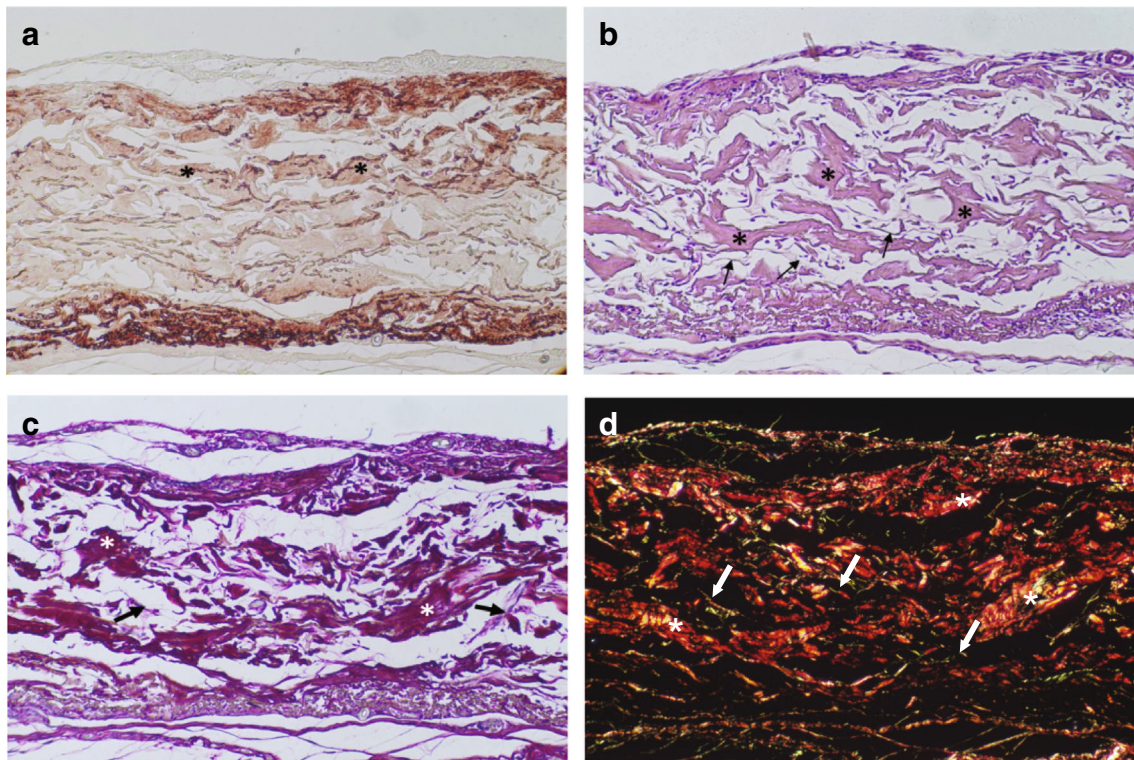
Histological observation of sections stained with avidin, H&E, and PSR revealed CM in the form of relatively thick collagen bundles, twisting and entangling along the disk, regardless of the experimental conditions (asterisk in Fig. 2a–d). Thin fibrils were also present (arrows in Fig. 2b–d) among the thick fibers. Their presence was confirmed by the PSR-stained specimens observed under polarized light (Fig. 2d), in which the thick fibers appeared dark red (representing mature [implanted] collagen), while the thin fibrils appeared light green/yellow (representing young collagen). These findings are consistent with an in-growth of new connective tissue into the implanted CM. The collagen bundles of the CM were more condensed peripherally, with no visible difference between the smooth “denser” and rough “spongy” zones in most specimens (Fig. 2b). The borders of the CM disk showed little integration with the surrounding connective tissue. Small clusters of inflammatory cells were located within several CM disks (Fig. 3a, b).

Observation of sections stained with biotin-avidin, which showed only the implanted matrix in the various groups, revealed significant gaps within the residual collagen. The extent of gaps in CM that had been implanted in diabetic rats appeared greater than those in normoglycemic rats (Fig. 4). This was borne out by histometric measurements, which revealed that the mean collagen content in PBS-immersed disks declined from  $57.5\pm 4.4\%$  in normoglycemic animals to  $44.7\pm 6.4\%$  in hyperglycemic rats ( $P<0.001$ ). Pre-implantation incubation of CM in TTC increased the area of residual collagen in both groups: to  $73.4\pm 7.3\%$  in normoglycemic rats and to  $55.5\pm 6.3\%$  in diabetic rats ( $P<0.001$ ) (Table 1). Differences were between groups (normoglycemic and hyperglycemic) and between TTC- and PBS-immersed disks; within each group were statistically significant.

## Discussion

Collagen matrix such as the one used in our study has previously been evaluated after implantation in animals and humans [11–16]. To our knowledge however, this is the first



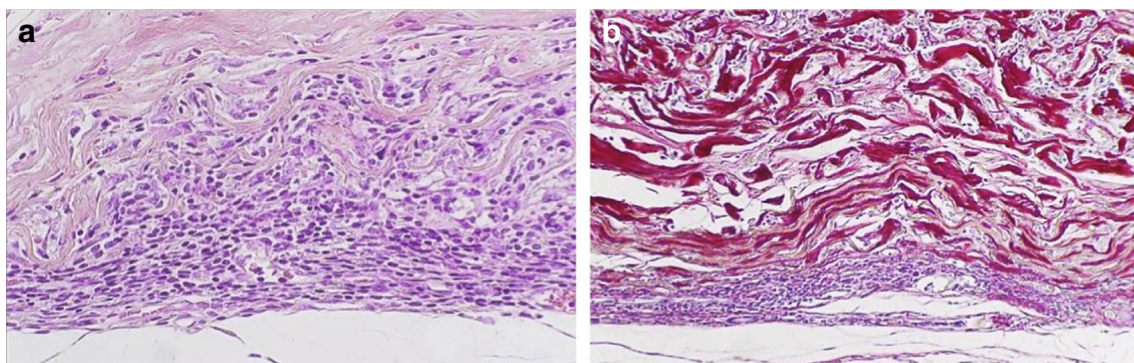


**Fig. 2** Photomicrographs of CMs stained with avidin-biotin-HRP (a), H&E (b), and PSR (c, d), showing the thick fibers of the implanted collagen (asterisks) and thin fibrils from the invading connective tissue (arrows). a–c Bright-field illumination. d Polarized light. Final magnification= $\times 100$

study analyzing the degradation of subcutaneously implanted untreated and TTC-treated CM in both normoglycemic and diabetic animals. The animal model of STZ-induced type 1 diabetes in Wistar rats and the biometric evaluation of the residual collagen in the implanted CM disks were chosen based on previous experiments, in which native collagen barriers were stained before implantation with biotin and evaluated following *in vivo* implantation [23, 24, 26, 31].

The main findings of this study were that CM degradation rate was significantly higher in uncontrolled, STZ-induced diabetes, compared to normoglycemic conditions, and that immersion of the CM in TTC at 50 mg/mL prior to implantation significantly reduced the CM degradation rate

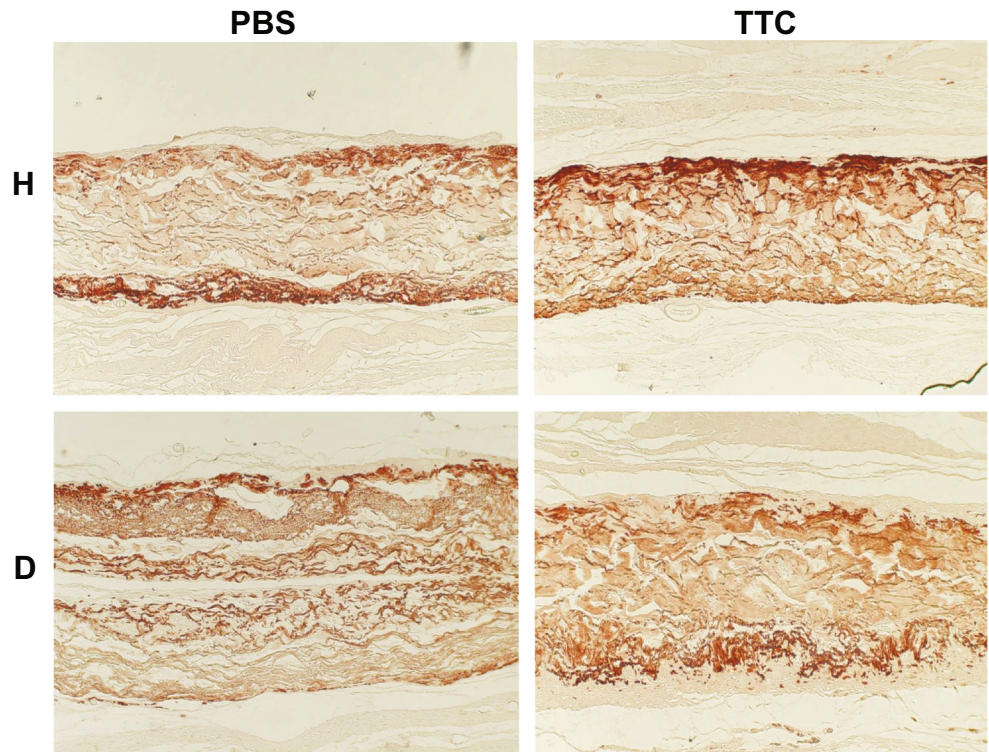
both in diabetic and normoglycemic animals. These results are comparable to similarly treated collagen regenerative membranes implanted over defects in the rat parietal bone [31]. Although both materials are engineered using a similar source of collagen, the difference in the degradation rate between the two could be expected. The regenerative membrane has a denser arrangement of collagen fibers that are designed to act as a tissue barrier and to reduce tissue and cell penetration. As such, it is expected to be more resistant to degradation and tissue integration. On the other hand, the collagen matrix contains a major component of “spongy” loose fibers, providing a resorbable scaffold which favors tissue in-growth and integration, resulting in a more rapid



**Fig. 3** Photomicrographs of CMs stained with H&E (a) and PSR (b) showing inflammatory infiltrate in and around the CM. Bright-field illumination. Final magnification= $\times 200$



**Fig. 4** Representative CMs from the various experimental groups stained with biotin-avidin-HRP (final magnification  $\times 40$ ). *PBS* PBS-immersed CM, *TTC* TTC-immersed CM, *H* healthy animal, *D* diabetic animal



degradation. It is noteworthy however, that implantation sites in the different models were not similar, making direct comparisons not appropriate.

The present histological analysis showed that CM thickness was reduced from about 4.0 mm to less than 1.0 mm. Apparently, this reduction in thickness may have occurred after wetting the disks and during handling and covering it by the dermal flap, at the expense of the spaces within the collagen network.

Our previous [31] and present data showed that diabetes increases the rate of degradation of native collagen barriers and CM. Various reports have shown that in diabetes, the levels and activity of MMPs are higher and that those of

TIMPs are lower [10, 12]. Although the present study did not quantitate the inflammatory infiltration peripheral to the CM, it appeared to be more marked in the diabetic animals compared with the normoglycemic ones. This observation is consistent with our previous findings [31]. Noteworthy, other studies have shown that macrophage infiltration into the kidney is increased in rats with STZ-induced diabetes [32] and that diabetes may lead to enhanced monocyte chemoattractant protein-1 levels that may be responsible for the recruitment of these inflammatory cells [33]. It would, therefore, follow that an increased inflammatory reaction in diabetic rats could lead to faster degradation of collagen matrix.

TTC and its chemically modified non-antimicrobial derivatives inhibit the catalytic activities of human collagenases and gelatinases, especially the neutrophil MMP [15–17]. We have previously shown that immersion of a bi-layered porcine collagen membrane in a 50-mg/mL TTC solution significantly delayed its degradation after implantation on the rat calvaria [26, 31]. The present study shows similar results when using CM both in diabetic and normoglycemic rats. This effect may be advantageous in procedures where CM is used, especially in submucosal implantation aimed to increase tissue volume, since prolonged scaffold function may enhance connective tissue in-growth. Thus, immersion of medical collagen devices in TTC may be an effective means to decrease their resorption rate and increase their effectiveness, especially in situations with increased degradation such as diabetes.

**Table 1** Percentage of matrix collagen remaining in the diabetic and the normoglycemic groups as percent of baseline

	Mean (%)	SD	N
<b>PBS-immersed</b>			
Normoglycemic	57.5	4.4	13 <sup>a,c</sup>
Diabetic	44.7	6.4	12 <sup>b,c</sup>
<b>TTC-immersed</b>			
Normoglycemic	73.4	7.3	13 <sup>a,d</sup>
Diabetic	55.5	6.3	12 <sup>b,d</sup>

Values labeled with identical letters are significantly different from each other

<sup>a,b</sup>  $P < 0.001$

<sup>c,d</sup>  $P < 0.001$

## Conclusions

Diabetes significantly increases collagen matrix degradation; immersion of collagen matrix in tetracycline before implantation decreases its degradation in both diabetic and normoglycemic conditions.

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**Compliance with ethical standards** This study was approved by Institutional Animal Care and Use Committee of the Tel Aviv University.

**Conflict of interest** The authors declare that they have no competing interests.

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