Effect of Systemic Tetracycline on the Degradation of Tetracycline-Impregnated Bilayered Collagen Membranes: An Animal Study

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ABSTRACT

Background: Premature collagen membrane degradation may compromise the outcome of osseous regenerative procedures. Tetracyclines (TTCs) inhibit the catalytic activities of human metalloproteinases. Preprocedural immersion of collagen membranes in TTC and systemic administration of TTC may be possible alternatives to reduce the biodegradation of native collagen membranes.

Aim: To evaluate the in vivo degradation of collagen membranes treated by combined TTC immersion and systemic administration.

Materials and Methods: Seventy-eight bilayered porcine collagen membrane disks were divided into three groups and were immersed in 0, 50, or 100 mg/mL TTC solution. Three disks, one of each of the three groups, were implanted on the calvaria of each of 26 Wistar rats. Thirteen (study group) were administered with systemic TTC (10 mg/kg), while the remaining 13 received saline injections (control group). Calvarial tissues were retrieved after 3 weeks, and histological sections were analyzed by image analysis software.

Results: Percentage of remaining collagen area within nonimpregnated membranes was 52.26 ± 20.67% in the study group, and 32.74 ± 13.81% in the control group. Immersion of membranes in 100 mg/mL TTC increased the amount of residual collagen to 63.46 ± 18.19% and 42.82 ± 12.99% (study and control groups, respectively). Immersion in 50 mg/mL TTC yielded maximal residual collagen values: 80.75 ± 14.86% and 59.15 ± 8.01% (study and control groups, respectively). Differences between the TTC concentrations, and between the control and the study groups were statistically significant.

Conclusions: Immersion of collagen membranes in TTC solution prior to their implantation and systemic administration of TTC significantly decreased the membranes' degradation.

KEY WORDS: bone augmentation, collagen, in vivo, membrane, tetracycline

Guided bone regeneration (GBR) is a common procedure in implant surgery.[1] GBR is based on epithelium and connective tissue exclusion by barrier membranes, resulting in selective repopulation of the defect area by bone progenitor cells.[2–5] Progenitor cells, given the space and time, are capable of restoring lost attachment around teeth or mineralized tissue in bony defects.[4–6] Resorbable and nonresorbable membranes can fulfill this purpose. Bioresorbable membranes do not require a second procedure for their removal. Commercially available bioresorbable membranes[7,8] are prepared from different materials, such as dura mater,[9] polylactic acid,[10] polyurethane,[10] or collagen.[4,5,11,12] Collagen type I, the major matrix protein in the human connective tissues, is used as the main component of

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most collagen membranes. Appropriate regenerative results are possible provided cell exclusion and space maintaining prevail for long enough period of time varying from 6 to 8 weeks for periodontal regeneration, and from 3 to 12 months for bone regeneration in edentulous areas.[13–15] Collagen appears as a natural candidate[16,17] because of its high biocompatibility, low antigenicity, ability to form composites with ceramics,[18] and the ability to control its biodegradability by cross-linking.[19]

Collagen membranes are widely used in GBR procedures.[20,21] The period in which the structural integrity of the membranes remains functional seems to be important for the success of these procedures.[22,23]

Membrane biodegradation depends on the enzymatic activity of various types of matrix metalloproteinases (MMPs) and other tissue characteristics.[24] The activity of these enzymes can be diminished through different means, such as tetracycline (TTC)[25–28] or collagen cross-linking.[19,29–31]

During wound healing, neutrophils and monocytes release MMPs into the wound area, thus contributing to collagen degradation.[32,33] Different cell types modulate MMP activity[34] by secreting MMP inhibitors (tissue inhibitors of metalloproteinases). TTC, a well-known antibacterial agent extensively used in periodontal therapy, also possesses MMP inhibitory abilities. Chemically modified TTC (CMT), which lacks antimicrobial activity while retaining the MMP inhibitory capacity, has been clinically used in periodontal treatment.[35,36]

TTC inhibits the action of extracellular MMPs. The molecular location of inhibition is the site of Ca^{++} and Zn^{++} binding.[37] TTC and CMT also block MMP production[37] by inhibiting the activation of pro-MMPs into MMPs through the delay of its oxidation process.

Immersion of collagen membranes in TTC solution prior to their implantation decreases their degradation in vitro[25] and in vivo.[28] Systemic TTC can also retard the degradation of various collagen membranes, depending on the membrane composition.[27]

The purpose of this study was to evaluate in vivo the effect of combined (immersion + injection) TTC treatment on the degradation of a bilayered native porcine collagen membrane.

MATERIALS AND METHODS

Collagen membranes (BioGide®, Geistlich, Wolhusen, Switzerland) were cut with a disposable biopsy punch (Miltex Instrument Company, Inc., Lake Success, NY, USA) to 5 mm-diameter membrane disks. The average weight of the membrane disks was 2.16 mg ± 0.152.

The membrane disks were labeled with biotin as previously described.[26–28,38] Briefly, the disks were soaked in 1 mg/mL aminohexanoyl-biotin-*N*-hydroxy-succinimide ester (Zymed Laboratories, San Francisco, CA, USA) for 1 hour at room temperature and then washed for 16 hours with three changes of phosphate-buffered saline (PBS) (Ca^{2+}, Mg^{2+}-free; pH 7.4) to remove any unbound biotin. Ten biotin-labeled, nonimplanted disks were processed for histological observation and served as baseline; half of them (five disks) were also treated with 5% formic acid prior to embedding to assess the effect of demineralization on membrane labeling. Biotin-labeled disks were incubated with TTC–HCl (Tevacycline®, Teva Pharmaceutical Industries, Ltd., Petah Tikva, Israel) dissolved in PBS at 50, 100, or 0 mg/mL (PBS alone)[25] for 1 hour. The disks were then washed overnight with PBS. Membranes soaked in TTC showed a surface pH of ~1.3 regardless of the TTC concentration.

The Animal Research Council of Tel Aviv University approved the study, which consisted of 26 4-month-old female Wistar rats that were divided into two groups (13 animals each): an experimental group, in which systemic TTC (Engemycin® 10%, Abic Veterinary Products, Netanya Israel) was administered at 10 mg/kg body weight, beginning on the day of surgery and then every 3 days until the study ended, and a control group, that received saline injections. The antimicrobial dose recommended by the manufacturer is 20 to 40 mg/kg body weight. Therefore, in this study, TTC was administered at 25 to 50% of the recommended antibacterial dose. Animals had free access to water and food, and were maintained in a room with a 12-hour light/dark cycle in an ambient temperature of 22°C. For surgery, the animals were weighed and anesthetized with intramuscular injection of ketamine chlorhydrate (Rhône Mèrieux, Lyon, France), 90 mg/kg body weight, and 2% xylazine (Vitamed, Bat-Yam, Israel) at 10 mg/kg body weight. The surgical protocol for membrane implantation has been previously described.[26–28] 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. Soft tissues were raised in two

layers: first the dermis and subdermal tissues while the periosteum remained attached to the bone, and second, the periosteum, which was elevated separately. A high-speed, water-cooled, diamond wheel-shaped bur was used to create three 5 mm-diameter, shallow (approximately 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. One membrane disk from each TTC concentration (50 and 100 mg/mL, and PBS alone) was placed in each animal. Soft tissues were repositioned over the disks, and the skin was sutured with resorbable sutures (Vycril Rapid®, Ethicon, Madrid, Spain).

The animals were sacrificed after 3 weeks with an overdose of ketamine and xylazine, and asphyxiation with carbon dioxide (CO_2). Dermal tissues were dissected, leaving the periosteum and disks undisturbed. Tissue blocks 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 embedded in paraffin. The blocks were sectioned antero-posteriorly at 4 to 6 μm thickness. Sections that included the central area of each of the three bony defects were selected for analysis of collagen membrane degradation. For biotin visualization, horseradish peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA, USA) was used according to the supplier's protocol. Sections were incubated in a solution of one enzyme drop in 1 mL of 10 mM PBS, pH 7.4, for 5 minutes at room temperature, and biotinylated collagen was identified with an aminoethyl carbazole substrate kit (Zymed Laboratories), followed by mounting with an aqueous solution of glycerol vinyl alcohol (Zymed Laboratories).

Stained sections were photographed with a digital camera mounted on a light microscope (Laborlux K®, Leitz, Wetzlar, Germany), at a ×100 magnification. Multiple digital images were analyzed for staining intensity by the NOVA® Image Analysis System (R&M Biomatrics, Nashville, TN, USA). In each specimen, the central part of the disk was identified by measuring the underlying bone defect. A region of interest (ROI), defined as a 1.2×0.5 mm rectangle (having an area of 0.6 mm^2), was superimposed to five different zones in each disk. The number of pixels occupied by stained collagen within each ROI was registered, and their calculated mean served as a unit for statistical analysis. Residual collagen area was calculated as percent of the collagen area of baseline membranes. In addition, membrane thickness was measured in the same five points, equally distributed along each specimen and, again, was calculated as percent of baseline disk thickness.

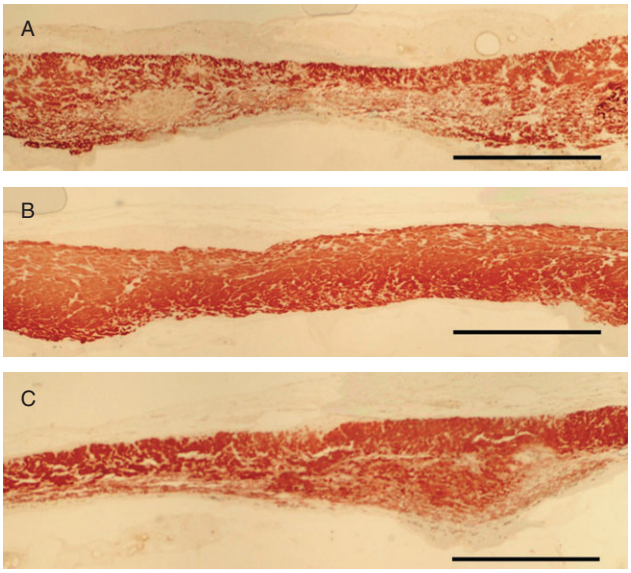

Figure 1 Histological view (×40) of the membranes 21 days after implantation with different tetracycline (TTC) concentrations without systemic administration of TTC. *A,* Phosphate-buffered saline alone (0 mg/mL TTC); *B,* 50 mg/mL; and *C,* 100 mg/mL TTC. Collagen stained in red/brown with avidin/biotin reaction. Bar = 1 mm.

Analysis of variance with repeated measures was used to analyze the effects of the within-subjects factor (TTC concentration) in which membranes were immersed prior to implantation (0, 50, and 100 mg/mL) and the between-subjects factor (systemically administered TTC).

RESULTS

Treatment of baseline membrane disks with formic acid did not change the apparent biotin staining of the disks, indicating that the demineralization process did not interfere with the membrane staining characteristics.

Figures 1 and 2 show the histological aspect of the membranes in noninjected and TTC-injected animals. The effect of TTC immersion of the membranes may be appreciated within groups. The effect of systemic TTC administration may be appreciated in between-groups' comparison.

Table 1 and Figure 3 present the quantization of the amount of remaining collagen area within the disks. Regardless of the concentration of TTC used for immersion, systemic administration of TTC increased the

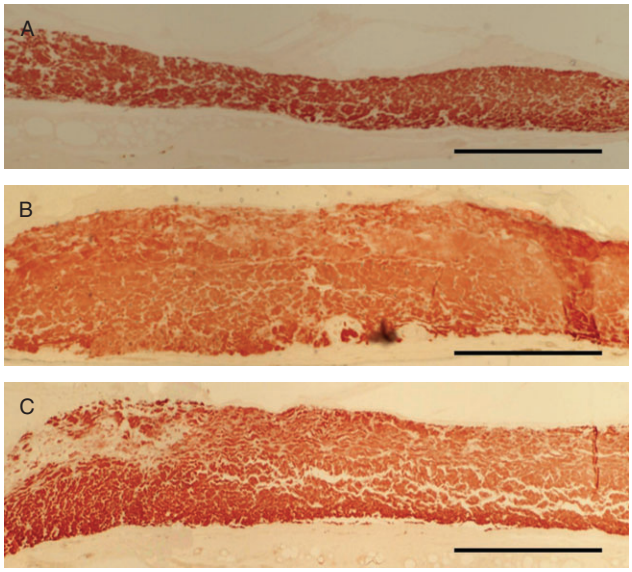

Figure 2 Histological view (×40) of the membranes 21 days after implantation with different tetracycline (TTC) concentrations with systemic administration of TTC. *A,* Phosphate-buffered saline alone (0 mg/mL TTC); *B,* 50 mg/mL; and *C,* 100 mg/mL TTC. Collagen stained in red/brown with avidin/biotin reaction. Bar = 1 mm.

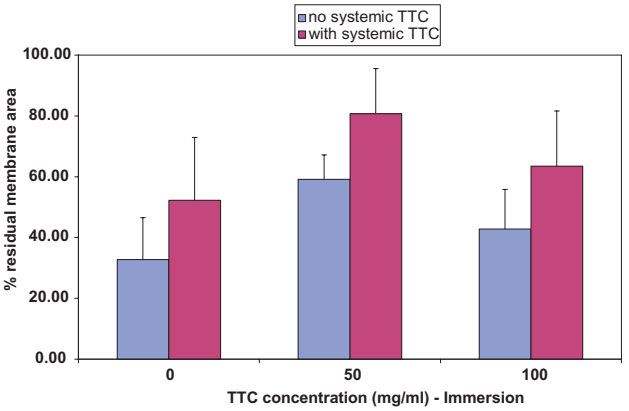

Figure 3 Percentage of collagen remaining in the membranes (related to baseline) immersed in the different tetracycline (TTC) concentrations with and without systemic TTC.

amount of residual collagen. The percentage of remaining collagen area within nonimpregnated membranes was 32.74 ± 13.81% (mean ± SD) in the control group, and 52.26 ± 20.67% in the study (TTC) group. Immersion of membranes in 100 mg/mL TTC increased the residual collagen to 42.82 ± 12.99% in the control group, and 63.46 ± 18.19% in the TTC group. Immersion in 50 mg/mL TTC yielded maximal residual collagen values: 59.15 ± 8.01% in the control group, and 80.75 ± 14.86% in the study (TTC) group. The effect of TTC immersion on residual collagen area was statistically significant ($p < .001$). Also, the effect of systemic TTC administration on residual collagen in the various TTC concentrations was statistically significant ($p = .002$). There was no interaction between the two effectors (TTC immersion and injection).

Figure 4 presents the quantization of residual membrane thickness, calculated as percent of baseline thickness. TTC immersion and injection had a similar effect on membrane thickness with similar statistical significance as those reported for the remaining collagen area within the membranes.

DISCUSSION

Success of bone augmentation procedures using barrier membranes depends on cell exclusion and space maintenance for a sufficient period of time to allow for new bone formation.[7,8,39–41] Collagen barrier membrane degradation starts shortly after implantation.[23,42,43] It has been claimed that resorbable membranes used for GBR procedures must support new bone formation and maturation for at least 6 months.[7,44] In large bony defects, premature membrane resorption may lead to less than optimal results.[44,45] Collagen membranes with a higher degree of cross-linking may serve as barriers for longer periods, thus improving the healing of larger defects.[11,19,20,30]

TTC has been widely used in periodontal treatment because of its antimicrobial and anti-collagenolytic

TABLE 1 Amount of Collagen (in Pixels), Measured in the Membranes Immersed in the Different Tetracycline (TTC) Concentrations with and without Systemic TTC

	Mean (± SD)	
TTC solution (mg/mL)	No TTC	With TTC
0	1,924,358.3 (± 811,867.16)	3,072,015 (± 1,215,267.94)
50	3,477,448.9 (± 470,615.18)	4,746,765 (± 873,798.24)
100	2,516,933.3 (± 763,922.77)	3,730,380 (± 1,069,572.31)

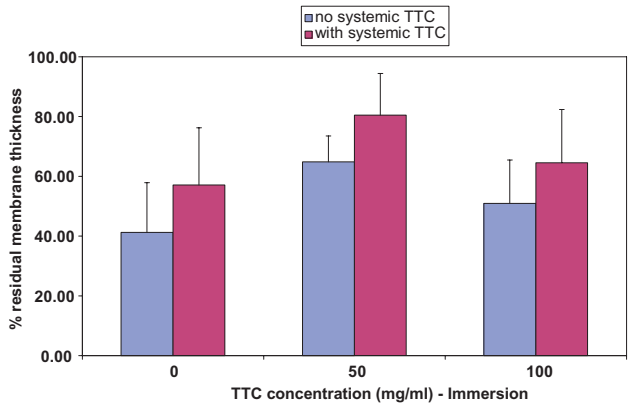

Figure 4 Remaining percentage of membrane thickness (related to baseline) for the different tetracycline (TTC) concentrations with and without systemic TTC.

effects. In sub-antimicrobial doses, TTC retains its anti-enzymatic properties, and affects the structure and function of osteoclasts.[46] In this study, TTC was administered at 25 to 50% of the recommended antibacterial dose. The rationale for this dose was to eliminate any possible antibacterial effect of the administered drug. In a previous study, we had already showed that this dose was effective in delaying different collagen membrane degradation in vivo.[27] Future studies could evaluate the effects of varying sub-antimicrobial doses. TTC and their chemically modified non-antimicrobial derivatives can inhibit the catalytic activities of human collagenases and gelatinases, especially the neutrophil MMP.[47,48] 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,[28] and that systemic administration of TTC delays degradation of three different collagen membranes.[27] The findings of the present study confirm that immersion of bilayered collagen membranes in a 50 mg/mL TTC solution prior to their implantation significantly decreases their degradation. In a previous study,[28] the pH of the TTC-impregnated collagen membranes was measured prior to implantation, and both 50 and 100 mg/mL showed a similar pH. Therefore, this dose-dependent modulating TTC effect cannot be explained on a pH basis. The lack of linear inhibitory response maybe the result of other adverse effects of high TTC concentration on the tissue, or that high TTC concentration may affect the collagen membrane integrity and enhance early membrane disintegration. In the present study, systemic administration of TTC significantly further decreased the biodegradation of TTC- and PBS-immersed membranes. Systemic administration of TTC in sub-antibacterial doses could be a possible alternative way to reduce biodegradation of certain collagen membranes[26,27] and enhance bio-durability of non-cross-linked collagen membranes; however, possible interactions with other antibiotics should be considered. The present research evaluated the effects of locally and systemically applied TTC, but not the mechanisms of action.

The present study evaluated the biodegradation of bilayered collagen membranes that were implanted and maintained in a closed, non-contaminated, extraoral environment. The results might not be directly extrapolated to GBR procedures in the oral cavity, especially as to how this membrane would disintegrate if spontaneously exposed to oral bacteria. Further studies should evaluate the effect of a prolonged barrier function on bone regeneration and the degradation of collagen membranes immersed in TTC, together with systemic TTC administration in sub-antibacterial doses in the presence of soft tissue dehiscences imitating premature exposure of barrier membranes to the oral environment.

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