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Original Article

Effect of Mitomycin C on bFGF, TGF–β1, KGF–1 Expressions after Myringotomy: An Animal Study

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OBJECTIVE: The purpose of this study was to evaluate the effect of topical mitomycin C (MMC) on growth factor expression in tympanic membrane (TM) wound healing.

MATERIALS and METHODS: Forty (20 male and 20 female) adult Wistar albino rats that varied from 250 to 300 g in weight were divided into five groups. In the first group, no intervention was performed, and the intact TMs were excised after the rats were sacrificed. In the other groups, both ears of rats underwent an electrocautery myringotomy procedure; MMC was applied to the right ears and saline to the left ones. In all groups, on the 3rd, 7th, 14th, and 30th days, macroscopic examinations of TM patency and the expressions of transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor (bFGF), and keratinocyte growth factor-1 (KGF-1) in TM epithelia, fibroblasts, and macrophages were performed by immunohistochemical staining and compared among the groups.

RESULTS: Complete healing was significantly less in the MMC group with respect to the saline group on the 7th and 14th days ($p<0.05$). On immunohistochemical study, no significant differences in the expressions of bFGF or KGF-1 were observed among the groups with one exception; on the 3rd day, the expression of TGF-β1 in macrophages was more elevated in the MMC group than in the saline group ($p=0.001$).

CONCLUSION: Application of MMC to acute perforations of the TM delays closure and has significant effects on some growth factors for certain durations.

KEYWORDS: Tympanic membrane wound healing, mitomycin C, TGF-β1, bFGF, KGF-1

INTRODUCTION

Tympanic membrane (TM) wound healing is a sophisticated process that comprises many overlapping phases that are modulated by the expression of several growth factors. These factors are secreted by the injured cells in the early stages and by fibroblasts, endothelial cells, and keratinocytes in the later stages [1].

The most important types of these factors are thought to be basic fibroblast growth factor (bFGF), keratinocyte growth factor-1 (KGF-1), and transforming growth factor-β_1 (TGF-β_1) [2-4]. bFGF is expressed by fibroblasts and endothelial cells, and it induces the proliferation of endothelial cells, keratinocytes, and fibroblasts as well as stimulates chemotaxis. It is one of the most important factors in vascularization. KGF-1 is synthesized in fibroblasts and stromal cells and acts only on keratinocytes because its target receptors are found only on keratinocyte membranes. KGF-1 stimulates the proliferation of keratinocytes and is chemotactic and mitogenic for epithelial cells. TGF-β_1 is expressed by macrophages, keratinocytes, fibroblasts, and platelets. It acts mainly in scar formation, stimulates the proliferation and migration of inflammatory cells, induces extracellular matrix formation, and increases mesenchymal tissue formation and vascularity [2, 5].

Mitomycin C (MMC) is an antineoplastic agent of the antibiotic subgroup, which is produced by a fungus called *Streptomyces caespitosus*. It blocks DNA and RNA replication by interfering in the G1 and S phases at the late stages of the cell cycle. Local application of MMC to the margins of perforations after myringotomy is reported to delay perforation closure [6, 7].

The aim of this study was to investigate the effects of locally applied MMC on the duration of TM healing and on the expression of bFGF, TGF-β_1, and KGF-1 after perforations induced by myringotomy.

MATERIALS and METHODS

This experimental study was approved by the local animal use committee. Forty healthy (20 males and 20 females) adult Wistar albino rats with intact TMs weighing up to 250–300 g were equally divided into five randomized groups; each group contained four male and four female rats. All animals were anesthetized by the intraperitoneal administration of ketamine (50 mg/kg) and

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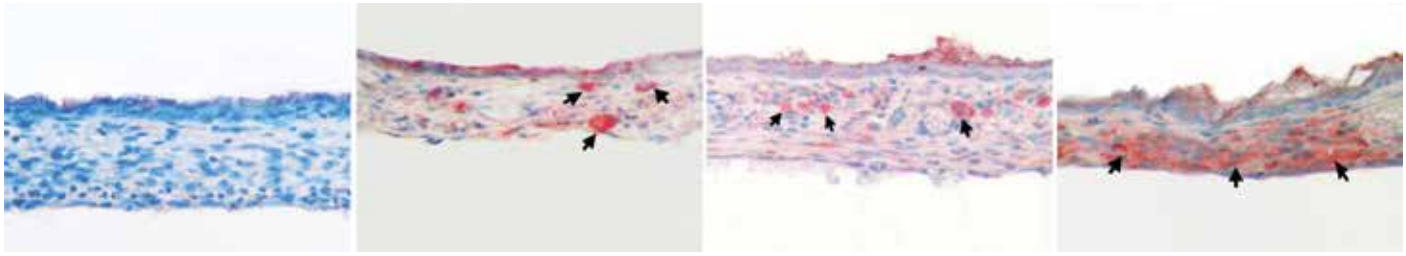

Figure 1 a-d. Expression levels of growth factors in tympanic membrane: (a) no immunostaining, (b) mild immunopositivity (arrows), (c) intermediate immunopositivity (arrows), (d) severe immunopositivity (arrows). Streptavidin peroxidase counterstained with Mayer's hematoxylin. a, b, c, and d: 40×

xylazine (5 mg/kg). The first group was used as a negative control group; bilateral TM specimens were obtained and prepared without performing an intervention. All the remaining rats underwent bilateral myringotomy of 3 mm with electrocautery in the anteroinferior quadrant of TMs using an otomicroscope. Gelfoam soaked in 0.4 mg/mL MMC (Kyowa 10 mg; Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan) solution was applied to the perforations on the right-sided ears for 5 min; similarly, gelfoam soaked in saline solution was applied to the left side. The second, third, fourth, and fifth groups were examined otomicroscopically for TM healing and sacrificed after receiving pentobarbital (80 mg/kg, intraperitoneally) for sequential immunohistochemical examination of the tympanic bullae on days 3, 7, 14, and 30 after perforation. The specimens were fixed and stored in 10% neutral buffered formalin solution. In all groups, the expression of TGF-β_1, bFGF, and KGF-1 in TM epithelia, fibroblasts, and macrophages was evaluated by immunohistochemical staining and compared among the groups. The expression of TGF-β1, bFGF, and KGF-1 was compared immunohistochemically between normal, unperforated TMs, perforated membranes with saline applied, and perforated membranes with MMC applied. The expressing cells (fibroblasts, epithelial cells, and macrophages) were evaluated by two different, uninformed pathologists on a three-grade scale as shown in Table 1 and depicted in Figure 1.

Immunohistochemical study was performed using the streptavidin–biotin–peroxidase staining method and an IHC detection kit (Cat. No: 85-9073; Invitrogen, Camarillo, CA, USA). All tissue samples were embedded in paraffin wax and cut into 5 µm thick sections and these sections were placed onto slides coated with 3-aminopropyltriethoxysilane (APES, Sigma-Aldrich; St. Louis, MO, USA). Following deparaffinization and rehydration, the sections were processed in citrate buffer solution (pH 6.0) with microwaves (800 W, 10 min) to reveal antigenic structures. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 20 min at room temperature. The sections were incubated with 1:100 dilute solutions of primary antibodies to bFGF, TGF-β_1, and KGF-1 (polyclonal rabbit anti-TGF-β_1, Santa Cruz Laboratories, Dallas; TX, USA (sc-146); polyclonal rabbit anti-KGF-1, Bioss Inc., MA, USA (bs-0734R); and polyclonal rabbit anti-FGF2(bFGF), Santa Cruz Laboratories; Dallas, TX, USA (sc-79)) at room temperature for 60 min. Irrigation with phosphate buffer saline solution (PBS) was applied twice before incubation with secondary antibodies that were biotinized according to the primary antibodies for 30 min and incubation with peroxidase-bound streptavidin for 30 min. Then, the sections were exposed to a chromogen solution of 3-amino-9-ethylcarbazole (AEC; Invitrogen Corporation, Paisley, UK) in PBS until coloring was achieved. The sections were counterstained with Mayer's hematoxylin for 15 s. Finally, the sections were dehydrated, covered with a water-based immune adhesive, and evaluated under a light microscope (Nikon Eclipse E600; Nikon Corporation, Tokyo, Japan). In the negative controls, goat or rabbit serum were used instead of the primary antibodies.

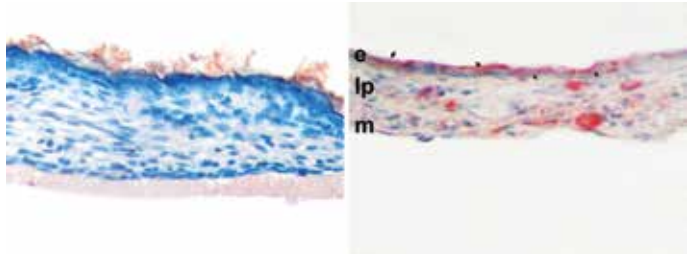

Figure 2 a, b. Expression of bFGF in tympanic membrane epithelia no immunostaining of bFGF in normal tympanic membrane epithelia (arrows) (a), mild immunopositivity in the tympanic membrane epithelia (arrows) in the group with MMC applied (b). Streptavidin peroxidase counterstained with Mayer's hematoxylin. A and b: 40×
e: epidermis; lp: lamina propria; m: mucosa.

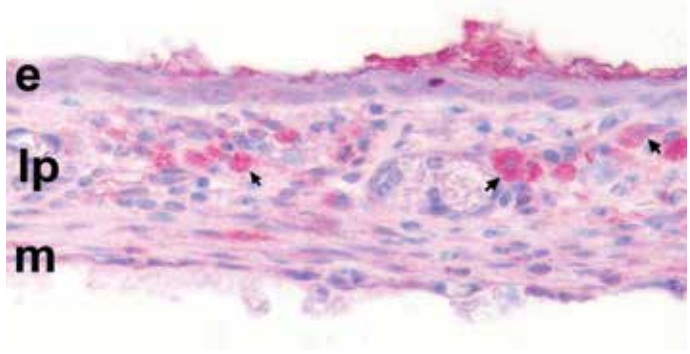

Figure 3. Expression of TGF-β1 in tympanic membrane epithelia. Intermediate immunostaining of the expression of TGF-β1 in macrophage cytoplasm on the 3rd day in the MMC group. Streptavidin peroxidase counterstained with Mayer's hematoxylin. a and b: 40×
e: epidermis; lp: lamina propria; m: mucosa.

Cross tables were created and, owing to the categorical aspects of the data in the study, statistical analyses were performed with Pearson's chi-squared test and Fisher's exact test using 2x2 tables. All the data were described in terms of frequencies and percentages. The SPSS Version 20 program (IBM Corporation; Armonk, NY, USA) was used in statistical analysis and a value of $p<0.05$ was considered to be significant.

RESULTS

Otomicroscopic Findings

On the 3rd day, all 32 rats were examined and complete healing was seen in two rats in the saline group. On the 7th day, the remaining 24

Table 1. Immunohistochemical Scoring: Evaluation of macrophages, epithelial cells, and fibroblasts for the immunostaining of growth factors

Intensity of immunostaining	Number of stained cells
none (–)	No immunostaining
+ (mild)	1–5 cells
++ (intermediate)	6–15 cells
+++ (severe)	>15 cells

Table 2. Number and percentage of completely healed perforations with respect to days after myringotomy (Pearson's chi-squared test and Fisher's exact test)

	No. of complete healing		
Days	Saline	Mitomycin C	p
3 (n=32)	2/32 (6.3%)	0/32 (0.0%)	>0.05
7 (n=24)	6/24 (25%)	1/24 (3.1%)	p=0.035
14 (n=16)	14/16 (87.5%)	3/16 (18.8%)	p<0.001
30 (n=8)	8/8 (100%)	5/8 (62.5%)	>0.05

Table 3. TGF-β1 immunostaining level on the 3rd day (Pearson Chi-squared test and Fisher's exact test)

	Saline	Mitomycin C	
Immunostaining level on:	n=8	n=8	p
Keratinocytes			
-	4 (50%)	8 (100%)	
+	3 (37.5%)	0 (0%)	
++	1 (12.5%)	0 (0%)	
+++	0 (0%)	0 (0%)	p=0.069
Fibroblasts			
-	4 (50%)	0 (0%)	
+	2 (25%)	4 (50%)	
++	2 (25%)	4 (50%)	
+++	0 (0%)	0 (0%)	p=0.069
Macrophages			
-	8 (100%)	0 (0%)	
+	0 (0%)	5 (62.5%)	
++	0 (0%)	3 (37.5%)	
+++	0 (0%)	0 (0%)	p<0.001

TGF-β1: transforming growth factor-β1

rats were examined and complete healing was observed in six rats in the saline group and in one rat in the MMC group. On the 14th day, complete healing was seen in 14 rats in the saline group and in three rats in the MMC group. On the 30th day, only eight rats remained and complete healing was observed in eight rats in the saline group and in five rats in the MMC group. On the 7th and 14th days, complete healing was significantly less in the MMC group ($p<0.05$) (Table 2).

Immunohistochemical Results

bFGF

bFGF immunostaining was more intense in keratinocytes in perforated TMs with saline and MMC applied than in the control group. However, the differences in the findings were insignificant between the saline and MMC groups (Figures 2a, b).

KGF-1

The expression of KGF-1 was greater in both the perforation group with saline and the group with MMC; however, there was no significant difference between these two groups.

TGF-β_1

Immunohistochemical evaluation of TGF-β_1 produced no immunostaining in the saline group on the 3rd day, but mild and intermediate staining were significantly intense in the MMC group ($p<0.001$) (Table 3) (Figure 3).

DISCUSSION

It is well known that closure is delayed with the application of MMC after TM perforation [8-10]. Many studies emphasize the crucial role of growth factors in the wound-healing process [1, 2, 10]. Once an injury occurs, different growth factors play roles in different stages of wound healing. It has also been known that the expression of TGF-β_1, bFGF, and KGF-1 rapidly increases at the margins of a TM perforation [2, 3, 5, 11].

The expression of growth factors is one of the basic factors in wound healing. These factors are secreted from inflammatory cells and released into the environment of the wound during the inflammatory phase. The secretion of growth factors promotes the migration and proliferation of fibroblasts, epithelial cells, and vascular endothelial cells; therefore, proliferation and repair processes are initiated. While the inflammatory cells are diminishing in number, the role of secreting these growth factors is played by fibroblasts, keratinocytes, and endothelial cells. Growth factors also promote the formation of the extracellular matrix and capillary plexus. They participate with collagenases and proteases in well-balanced scar formation in the remodeling phase. There are many studies that refer to the accelerative effect of growth factors on wound healing. In those studies, it is shown that the local application of growth factors leads to upregulation of the wound-healing process [4, 12, 13].

Santa Maria et al. [3] studied the expression of KGF-1 and bFGF in rats after myringotomy and found that there were two peaks on the 3rd and 5th days in the epithelial layer. They also reported peaks in the expression of KGF-1 at the 12th h and on the 3rd day in connective tissue and keratinocytes.

Ishibashi et al. [11] reported that different growth factors were expressed at different levels on different days after myringotomy in a rat model. KGF was found to have peaks on the 1st and 3rd days, whereas bFGF was found to have a peak on the 3rd day and its expression continued on the 7th day.

Somers et al. [2] compared the immunohistochemical distribution of growth factors in intact cadaver TM and live TM tissue that had been perforated for one year and obtained in a myringotomy operation. The distribution of bFGF was found to be similar in intact and

chronically perforated TM. Vascular immunostaining of TGF-β_1 was comparable but no immunostaining was observed in the fibrous and subepithelial layers of intact TM; in contrast, intense immunostaining was present in the layers of chronically perforated TM. They emphasized that the expression of TGF-β_1 was related to the degree of fibroplasia and it played a major role in the formation of a subepithelial scar on the margins of a chronic perforation.

There are several studies that relate the application of MMC to the expression of growth factors, showing an increase in expression with the application of MMC; however, cause and effect in these are controversial [14, 15].

Occleston et al. [14] studied the expression of TGF-β_1 and bFGF after the application of saline and MMC to human eye Tenon's capsules *in vitro*. They found a gradual decrease in levels of growth factors 48 days after the application of saline, but a significant increase in the beginning and a decline at 48 days to the same or slightly higher levels with respect to the saline group after the application of MMC. When the literature is reviewed, the expression of growth factors increases with hypoxia [16] and tissue injury [17] as well as after exposure to ionizing radiation [18]. In that study, the increased levels in the beginning are thought to be secondary to cellular injury that was related to the application of an antiproliferative agent as well as to the autostimulatory potential of these factors. Chen et al. [15] applied MMC at a concentration of 0.4 mg/mL to normal dermal fibroblasts for 4 min *in vitro* in their study and found higher levels of TGF-β_1 and bFGF on days 0, 1, 3, and 5 with respect to the control group.

In this study, we found that the application of MMC delays TM wound healing on otomicroscopy when compared with controls, but similar expression of bFGF and KGF-1 was also found in both groups. The increase in the expression of TGF-β_1 in the MMC group was associated with the role of this growth factor in the sustained prolongation of myringotomy patency in this study by means of its well-known contribution to the wound-healing process. The reason for the increased expression of this growth factor is thought to be autostimulation against the antiproliferative effects of MMC. Future research is needed to verify these findings and to elucidate the contribution and respective roles of MMC and the mentioned growth factors in TM wound healing.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Ondokuz Mayıs University Center for Animal Studies

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