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Extending Myringotomy Patency with Topical Everolimus in Rats

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Everolimus is an immunosuppressant agent that has antiproliferative properties and negative effects on wound healing. The effect of everolimus use to delay the closure time of myringotomy is not known.
Objectives. The aim of the study was to evaluate the impact of topical everolimus on myringotomy patency and to investigate its histopathologic effects on the tympanic membrane.
Material and Methods. Twenty Sprague-Dawley rats were bilaterally myringotomized with a myringotomy knife. Gelfoam soaked in 0.05% everolimus in a microemulsion formulation was applied to the right myringotomy site of the rats for 10 min (the everolimus group). The myringotomy sites of the left ears were treated with sterile saline topically (the control group). The tympanic membranes were routinely examined otomicroscopically every other day for 31 days. The membranes were then harvested and evaluated histologically after 31 days.
Results. All tympanic membranes were closed by the 15th day in the control group, while in the everolimus group the myringotomy remained open in five rats (25%) on day 31. The mean durations of myringotomy patency in the everolimus group and control group were 20.90 ± 7.85 and 10.10 ± 3.14 days, respectively. The difference was found to be statistically significant ($p < 0.01$). In the histopathological examination of the tympanic membranes, there was less fibrosis and less inflammation in the everolimus group than in the control group ($p < 0.01$).
Conclusions. Topical everolimus application is effective in extending myringotomy patency in rat tympanic membranes. Inflammatory reactions and fibrosis in the *lamina propria* were observed to be significantly less when topical everolimus was used (**Adv Clin Exp Med 2016, 25, 1, 5–10**).

Key words: myringotomy, tympanic membrane, everolimus, histopathology, rat.

Myringotomy with tympanostomy tube placement is the most common pediatric surgical procedure in the United States [1]. Otitis media with effusion is the most frequent indication for tympanostomy tube insertion. Other indications include recurrent acute otitis media, acute otitis media with complications such as facial nerve palsy or meningitis, retraction pockets, topical corticosteroid and gentamisin therapy in Meniére's disease, and hyperbaric oxygen therapy [2–5]. There are some well-known complications of tympanostomy tube placement, such as chronic otorrhea, granulation formation, tympanosclerosis and permanent perforations [6, 7].

A myringotomy without use of a tympanostomy tube closes within days because the tympanic membrane heals rapidly. For this reason, topical applications of different drugs have been used to extend myringotomy patency [8–13].

Everolimus (40-0-[2-hydroxyethyl]-rapamycin) is an immunosuppressant and antiproliferative macrolide agent that is used to prevent acute and chronic rejection in allogeneic transplant recipients [14, 15]. Everolimus is a rapamycin derivative. It is evident that rapamycin derivatives have potentially negative effects on wound healing [16–18]. It has also been shown that coating coronary stents with everolimus inhibits local in-stent restenosis [19, 20].

In a previous study by the current authors, everolimus-coated tympanostomy tubes were inserted into the tympanic membranes of myringotomized rats [21]. That study demonstrated that everolimus-coated tympanostomy tubes reduced tube obstruction and led to less fibrosis and inflammatory reaction in the *lamina propria* histologically. The authors hypothesized that topical application of everolimus would extend closure time of myringotomies. The aim of the present study was to investigate the effects of topical everolimus on myringotomy patency and also to examine the tympanic membrane histologically in a placebo-controlled study in rats.

Material and Methods

The study was approved by the Institutional Ethics Committee for the Care and Use of Laboratory Animals of the Marmara University School of Medicine, Istanbul, Turkey, and instituitional guidelines regarding animal experimentation were followed. A 0.05% everolimus microemulsion formulation was prepared from everolimus tablets (Certican®, Novartis, Istanbul, Turkey) by the Marmara University School of Pharmacy, Department of Pharmaceutical Biotechnology, Istanbul, Turkey.

Experimental Design

Twenty healthy adult male Sprague-Dawley rats weighing 300–450 g were used in the study. All animals were obtained from and kept in the Animal Research Laboratory at Marmara University School of Medicine under standard conditions, with free access to food and water. Before the intervention, all ears were examined otomicroscopically and were found to be normal. The rats were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun®, Bayer, Istanbul, Turkey) by intraperitoneal injection. Under sterile conditions and using otomicroscopy, a myringotomy of an approximate diameter of 1 mm was performed on the posterior part of the *pars tensa* with a myringotomy knife through an *aural speculum*.

A small gelfoam sponge (methyl-cellulose) soaked in 0.1 mL of 0.05% everolimus in a microemulsion formulation was applied to the rats' right myringotomy site for 10 min (everolimus group; n = 20). A gelfoam sponge of the same dimensions soaked in sterile saline was applied to the rats' left myringotomy site for 10 min (control group; n = 20).

The tympanic membranes were routinely examined otomicroscopically with ether anesthesia, and myringotomy patency was recorded, as whether the tympanic membrane remained opened or not, every other day for a month. At the end of day 31, the rats were sacrificed by intraperitoneal injections of thiopental sodium (Pentothal®, Abbott, Istanbul, Turkey).

Histopathologic Examination

The tympanic bulla of the rats were dissected and tympanic membranes were obtained, then fixed in 10% formaldehyde solution and decalcified in 10% formic acid solution. The specimens were dehydrated with alcohol baths and embedded in paraffin. The tissue blocks were cut into slides 5 μm thick, stained with hematoxylin-eosin and Masson's trichrome. Masson's trichrome was used to distinguish the connective tissue of the *lamina propria*. The epithelial and mucosal layers were examined histologically under a light microscope at magnifications of ×40, ×200 and ×400 (Olympus U-OCMC 10/100 XY). The parameters were calculated considering one microscopic area under the light microscope (×400). All the histopathological examinations were done by the same pathologist.

Fibrosis was evaluated using a four-point scale comparing the thickness of the tympanic membrane on a qualitative basis: 1) normal tympanic membrane thickness was categorized as "no fibrosis" in the *lamina propria*; 2) a two- to three-fold increase in tympanic membrane thickness was categorized as "slight fibrosis"; 3) a four- to five-fold increase in tympanic membrane thickness was categorized as "moderate fibrosis"; and 4) a more than five-fold increase in tympanic membrane thickness was categorized as "significant fibrosis" in the *lamina propria* [12].

Inflammation was also evaluated using a four-point scale under a light microscope, according to the degree of inflammatory cell infiltration in the *lamina propria*: 1) No inflammatory cell presence was classified as "no inflammation" in the *lamina propria*; 2) up to 25% inflammatory cell infiltration in a microscopic area was classified as "slight inflammation"; 3) 25% to 50% inflammatory cell infiltration in a microscopic area was classified as "moderate inflammation"; and 4) over 50% inflammatory cell infiltration in a microscopic area was classified as "significant inflammation".

Statistical Analysis

Number Cruncher Statistical System 2007 (NCSS) and Power Analysis and Sample Size (PASS) 2008 for Windows (both from NCSS LLC, Kaysville, Utah, USA) were used for the statistical analysis. The Mann-Whitney *U* test was used to

compare myringotomy patency data and to compare fibrosis and inflammation in the *lamina propria* between the everolimus group and the control groups. The comparison of qualitative data was done using Fischer's Exact χ^2 test. A p-value < 0.05 was considered to be statistically significant.

Results

Otomicroscopic Findings

The first closure of myringotomy in the control group occurred on day 5, while the earliest myringotomy closure in the everolimus group was seen on day 7. In the control group, the number of tympanic membranes that closed was highest (n = 6, 30%) on the 9th day; and all tympanic membranes were closed by the 15th day. In the everolimus group, the myringotomy openings were still open in 5 rats (25%) on day 31; the closure of myringotomies was highest on the 21st and 23rd days (n = 3, 15% on both those days). The distribution of myringotomy closures in both groups by day is shown in Fig. 1. The presence of open myringotomies in both groups on day 31 is shown in Table 1. There was a significant difference in the presence of open myringotomies between the two groups (p < 0.05).

The mean duration of myringotomy patency in the everolimus group was 20.90 ± 7.85 days, whereas it was 10.10 ± 3.14 days in the control group. The difference between the two groups was found to be significant (p < 0.05) (Table 1).

Histopathological Findings

There was a highly significant statistical difference between the groups regarding the histological findings of fibrosis in the *lamina propria* (p = 0.0059). In the everolimus group no fibrosis was observed in half of the tympanic membranes, while significant fibrosis was not found in any of the tympanic membranes. In the control group, no fibrosis was observed in four membranes, slight fibrosis was observed in five, and moderate fibrosis was observed in 6 of the 20 tympanic membranes (Table 2).

In the everolimus group 60% of the tympanic membranes showed slight inflammation in the *lamina propria*; no inflammation was observed in two, moderate inflammation was observed in two, and significant inflammation was observed in four of the 20 tympanic membranes. In the control group, slight inflammation in the *lamina propria* was observed in two tympanic membranes, moderate inflammation was observed in three; the remaining 15 tympanic membranes had significant inflammation in the *lamina propria*. The difference between the groups in terms of histological findings of inflammation in the *lamina propria* was statistically highly significant (p = 0.001) (Table 2).

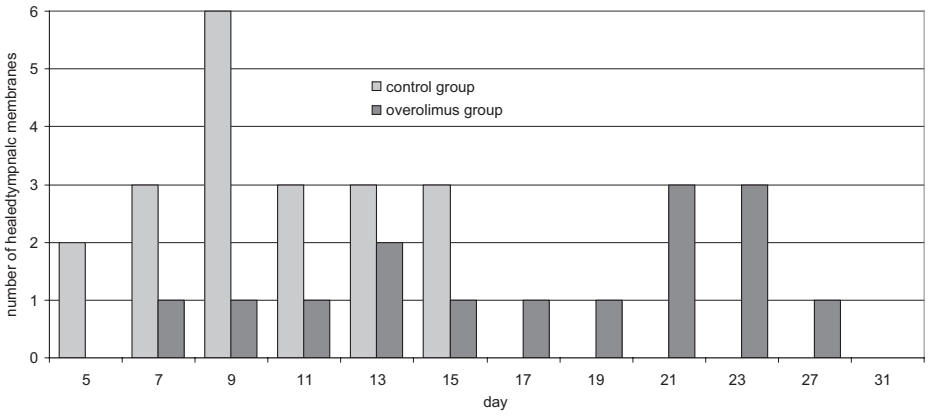

Fig. 1. The distribution of closure of myringotomies by day in the two groups

Table 1. Comparison of myringotomy patency in the two groups at the end of day 31

		Control group (n = 20)	Everolimus group (n = 20)	p-value
Open myringotomy n (%)	yes	0 (% 0)	5 (% 25)	0.047[1]*
	no	20 (% 100)	15 (% 75)	
Myringotomy patency time; mean ± SD (median)		10.10 ± 3.14 (9) days	20.90 ± 7.85 (21) days	0.001[2]**

[1] Fischer's Exact χ^2 Test; [2] Mann-Whitney *U* Test; * p < 0.05, ** p < 0.01.

Table 2. Histological assessment of fibrosis and inflammation in the *lamina propria* of tympanic membranes

Histological assessment		**Control group (n = 20) n (%)**	**Everolimus group (n = 20) n (%)**	**p-value**
Fibrosis	mean ± SD (median)	2.60 ± 1.09 (3)	1.65 ± 0.74 (1.5)	0.0059**[1]
	no fibrosis n (%)	4 (20%)	10 (50%)	
	slight n (%)	5 (25%)	7 (35%)	
	moderate n (%)	6 (30%)	3 (15%)	
	significant n (%)	5 (25%)	0	
Inflammation	mean ± SD (median)	3.65 ± 0.67 (4)	2.40 ± 0.94 (2.0)	0.0002**[1]
	no inflammation n (%)	0	2 (10%)	
	slight n (%)	2 (10%)	12 (60%)	
	moderate n (%)	3 (15%)	2 (10%)	
	significant n (%)	15 (75%)	4 (20%)	

[1] Mann-Whitney *U* test; **p < 0.01.

Discussion

Everolimus is a macrolide immunosuppressant that has potent antiproliferative effects on lymphoid and nonlymphoid cells by limiting cytokine and growth-factor mediated cell signalling [22]. It inhibits cellular proliferation in the late G1 phase of the cell cycle [23]. Everolimus blocks basic fibroblast growth factor (bFGF)-induced proliferation of bovine aortic and human umbilical vein endothelial cells and 3T3 fibroblasts; it also inhibits bFGF and platelet derived growth factor (PDGF)-stimulated proliferation of smooth muscle cells [22, 24].

The antiproliferative and immunosuppressive properties of everolimus have been shown to be useful in preventing rejection in kidney and heart transplantation [14, 15]. Everolimus has been reported to inhibit fibroblast proliferative capabilities and to reduce lung-collagen accumulation in bleomycin-induced pulmonary fibrosis [25]. Everolimus-coated coronary stents have been shown to effectively prevent intimal hyperplasia following stent implantation and to control restenosis [19, 20].

Applying everolimus can severely compromise the restoration of strength in wound healing in experimental studies [16–18]. The effect on the development of wound strength becomes apparent after the third post-operative day and persists for at least four weeks after surgery in experimental anastomoses [16, 18]. Several mechanisms of action have been proposed to explain the negative effects of everolimus on wound healing, including deposition of insufficient collagen, a prolonged proliferation phase, disruption of the remodeling phase, and reduced inflammatory cell infiltration [18, 26].

It has been demonstrated that topically applied everolimus significantly prolonged corneal allograft survival in an experimental keratoplasty model [27]. Local administration of 0.05 or 0.025% everolimus was effective in prolonging the mean survival time of corneal grafts. It has been suggested that everolimus may diminish inflammatory reactions after transplantation by reducing the release of pro-inflammatory cytokine IL-6 and by inhibiting leukocyte migration [28].

Taking all of these data into account, the current study was designed to test the hypothesis that everolimus can enhance myringotomy patency. The dose of everolimus tested in the study is based on the dose used in topical applications of everolimus in experimental keratoplasty [27], and it is far less than the dose used to elicit immunosuppressive effects in rats (1.0 to 3.0 mg/kg/day

orally) [16–18, 25]. The hypothesis was confirmed: In the everolimus group, the mean duration of myringotomy patency was 20.90 ± 7.85 days, which was significantly longer than the control group, which had mean duration of 10.10 ± 3.14 days ($p < 0.05$).

In the study, topical everolimus application resulted in less fibrosis and less inflammation in the *lamina propria*. Fibrosis was not observed at all in the *lamina propria* of the tympanic membranes in half of the everolimus group. In that group, 70% of the tympanic membranes showed either no inflammation or slight inflammation, while 75% of the tympanic membranes in the control group revealed significant inflammation. However, the short follow-up period is a limitation of the study. These histopathological findings are consistent with the current authors' previous study with everolimus, in which everolimus-coated tubes also revealed significantly less inflammatory reaction and fibrosis [21].

The effects of some other topical agents on myringotomy patency have previously been investigated. Mitomycin-C, 5-flourouracil (5-FU), and halofuginone used in myringotomized animal ears were shown to extend the patency of myringotomies [8–13]. With mitomycin-C application the mean closure time of myringotomies was between 6.5 and 9.5 weeks in rats [8–11]. A clinical study demonstrated that mitomycin-C resulted in relatively long myringotomy closure time in patients with otitis media with effusion (mean: 5.3 weeks) and in patients with recurrent acute otitis media (mean: 7 weeks) [29].

Myringotomy patency was maintained up to two to three weeks with topical 5-FU in animal studies [11, 12]. In a clinical study 5-FU ointment applied to patients' myringotomy sites was shown to be effective in prolonging the myringotomy closure time up to four weeks; the average time to myringotomy closure was 20.5 days [30]. Topical halofuginone application extended myringotomy patency in rats, resulting in a mean myringotomy closure time of 19.10 days [13].

In the current study the average myringotomy patency in the everolimus group was shorter than in the studies using topical mitomycin-C, but is similar to the results with 5-FU and with halofuginone. It should be kept in mind that the current study is the first in which everolimus has been used in myringotomy. Five of the everolimus-treated myringotomies remained open at the time of the last observation. The animals were observed for only 31 days. It is possible that these myringotomies would have been patent longer if the animals had been followed for a longer period, which of course would have increased the mean duration of myringotomy patency and the difference between the groups.

Further research would be worthwhile to investigate the effects of everolimus on myringotomy with different concentrations, with repeated applications and also for a longer follow-up period. Future studies will also be needed to determine the effects of everolimus on the middle and inner ear.

This study has demonstrated that topical everolimus application prolonged the patency of myringotomy and led to less fibrosis and inflammatory reaction histologically. Further research appears warranted to confirm these results, to test different concentrations and application regimes, and to evaluate any effects of everolimus on the middle and inner ear.

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