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The Effects of Hyaluronic Acid on Myringoplasty in Rats

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Abstract: Tympanic membrane perforations arise from a variety of causes. Some of them require myringoplasty. In this study, we used hyaluronic acid, a polysaccharide with high-molecular-weight which may be obtained commercially and affects modulations of cell function. An experimental myringoplasty was performed on rats in which a chronic dry tympanic membrane perforation was formed. While physiological saline was embedded in Gelfoam® which was supported

under and over the graft, in group 1, hyaluronic acid embedded gelfoam was used in group 2. Better epithelization, less calcification and scar tissue were established in group 2 myringoplasties. This study shows that hyaluronic acid may be used in myringoplasty especially in myringosclerotic tympanic membrane perforations.

Key Words: Myringoplasty, chronic dry ear, hyaluronic acid.

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Introduction

Tympanic membrane (TM) perforations have various causes: some are secondary to a sudden trauma and others are associated with middle ear diseases. Experimental studies on TM perforations have used acute perforation models. Acute perforations are characterized with spontaneous closure, not similar to chronic TM perforations frequently encountered in humans. After acute perforation, a complex healing process including epithelial migration, increased fibroblastic activity, and vascular proliferation ensues. In chronic perforations, these reparative mechanisms become deactivated and the perforation becomes lined with a mature epithelial rim. Amoils et al. (1) were the first to form chronic TM on the chinchilla and this has been accepted as a model for experimental studies.

Myringoplasty is a widely applied surgical method for closing large dry TM perforations. To date, many materials such as temporal muscle fascia, vein, periotium, perichondrium and dura mater have been used as grafts in myringoplasty.

Hyaluronic acid (HA), 1% hyaluronan, Healon® is an extracellular high-molecular-weight polysaccharide, which is a constituent of many tissues, especially loose connective tissues (2). Many functions of HA can be related to its physicochemical properties (3), although there is an increasing number of reports indicating that HA can also modulate cell functions, e.g., in the inflammatory response (4).

The three main purposes of the present study were: 1. To develop a rat model of chronic TM perforation; 2. To show if rat femoral muscle fascia can be used as autograft in myringoplasty. 3. To explore whether HA has any effect on myringoplasty in rats with experimental chronic dry tympanic membrane perforation.

Materials and Methods

Twenty-five healthy, adult male Sprague-Dawley rats weighing 250-360g were used. Anesthesia was induced using intraperitoneal sodium pentobarbital (35 mg/kg) and supplemented as necessary (15 mg/kg). The right ear of each rat was used. The animals were maintained and the experiments were performed in accordance with the published standards (5).

Creating chronic TM perforations

The ear canal was sterilized with a povidone iodine solution, then irrigated several times with saline under an operation microscope. An aural speculum of suitable diameter was put into the ear canal. Rats that demonstrated signs of otitis media or externa were excluded. In forming TM perforations, the principles established by Amoils et al. (1) who previously formed chronic TM perforation on chinchillas were followed. With the use of a radiosurgery myringotomy loop (Ellman surgitron® TP1) TM perforation was performed. The part close to the manubrium mallei umbo was excised by microscissors. TM excision (myringectomy) rather than incision (myringotomy) was performed. Perforations

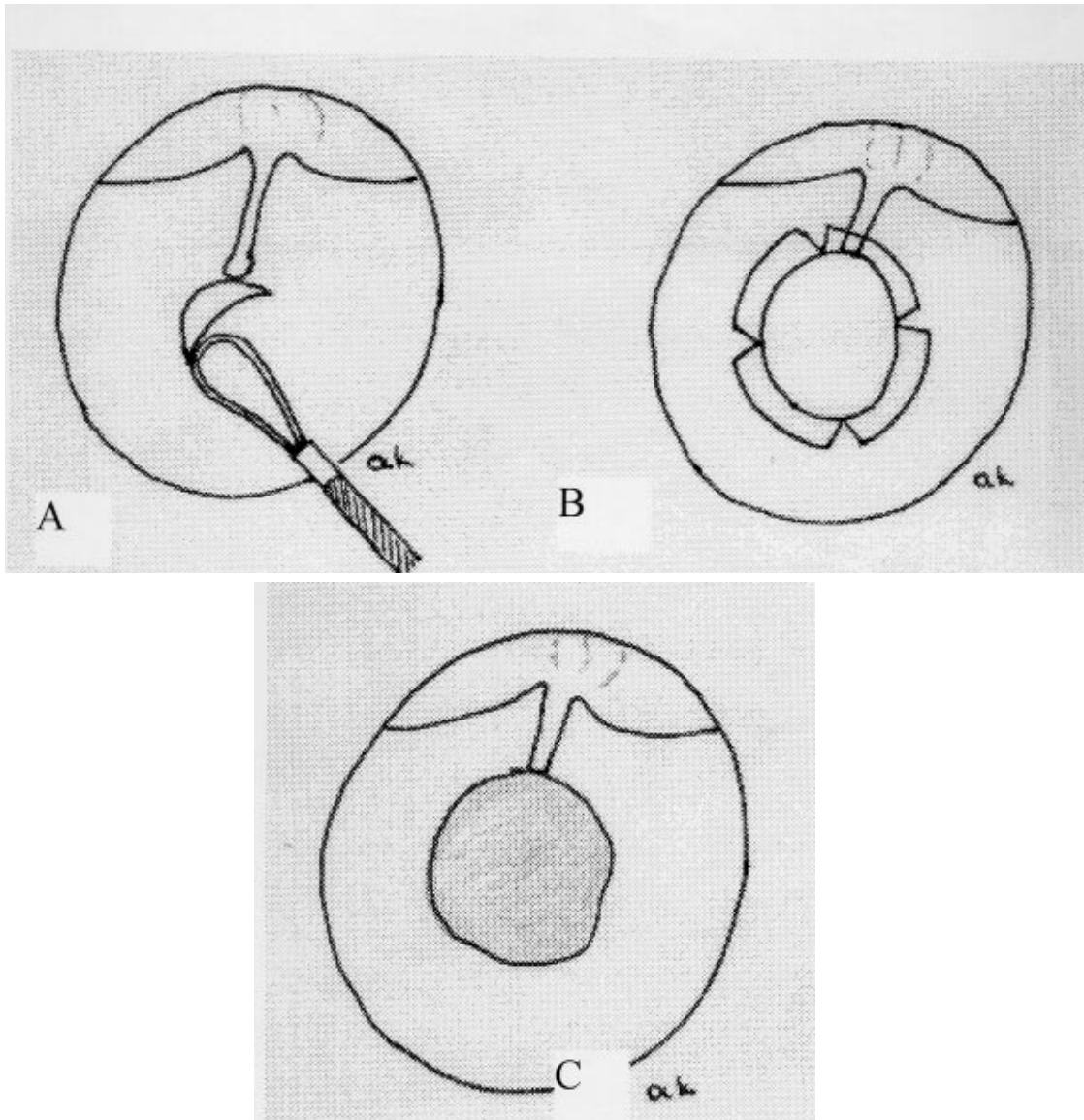


Figure 1. Schematic view of performing subtotal perforation in the rat. A. TM excision (myringectomy) performed with a radiosurgery myringotomy loop. B. Perforation was widened with microflaps to obtain subtotal perforation. C. Chronic dry TM perforation obtained in 8 weeks.

were widened with microflaps to obtain subtotal perforations (70-80 % pars tensa) (Fig.1).

Postoperatively, trimetoprim 40 mg/ml combined with sulfadiazine 200 mg/ml at a dose of 0.125ml/kg were daily injected intramuscularly for 5 days for proflaxy. Perforations were observed for a period of 6-8 weeks. Examinations under anesthesia were performed weekly to detect signs of suppuration and healing. When infection was evident, systemic antibiotic treatment was reinstated, and purulent discharge was cleaned through aspiration. When healing was found, the microflap procedure was repeated. Perforations were kept open

and this was regarded as dry chronic TM perforation. Five rats were excluded from the study, 2 due to uncontrolled infection and 3 due to unformed chronic dry TM perforation.

Myringoplasty

Myringoplasty was performed in 20 rats with chronic dry TM perforation (Fig. 2). After the rats were anesthetized, a transcanal approach was used through a speculum which was as large as possible. The edges of the perforation were denuded, both on the undersurface (using a small hook) and outer surface. Right femoral

muscle fascia in the thigh region was used as the graft (Fig. 3). After the graft was flattened with a vein press, it was placed in 0.4 % formaldehyde solution with pH 6.8 for one minute, washed three times with physiologic saline and left to dry (6).

The graft was placed as to the “underlay technique” and supported in the middle ear by a compressed gelatin sponge saturated with saline. The ear canal was then packed with compressed gelatine sponge saturated with saline solution (Fig. 4). While these procedures were

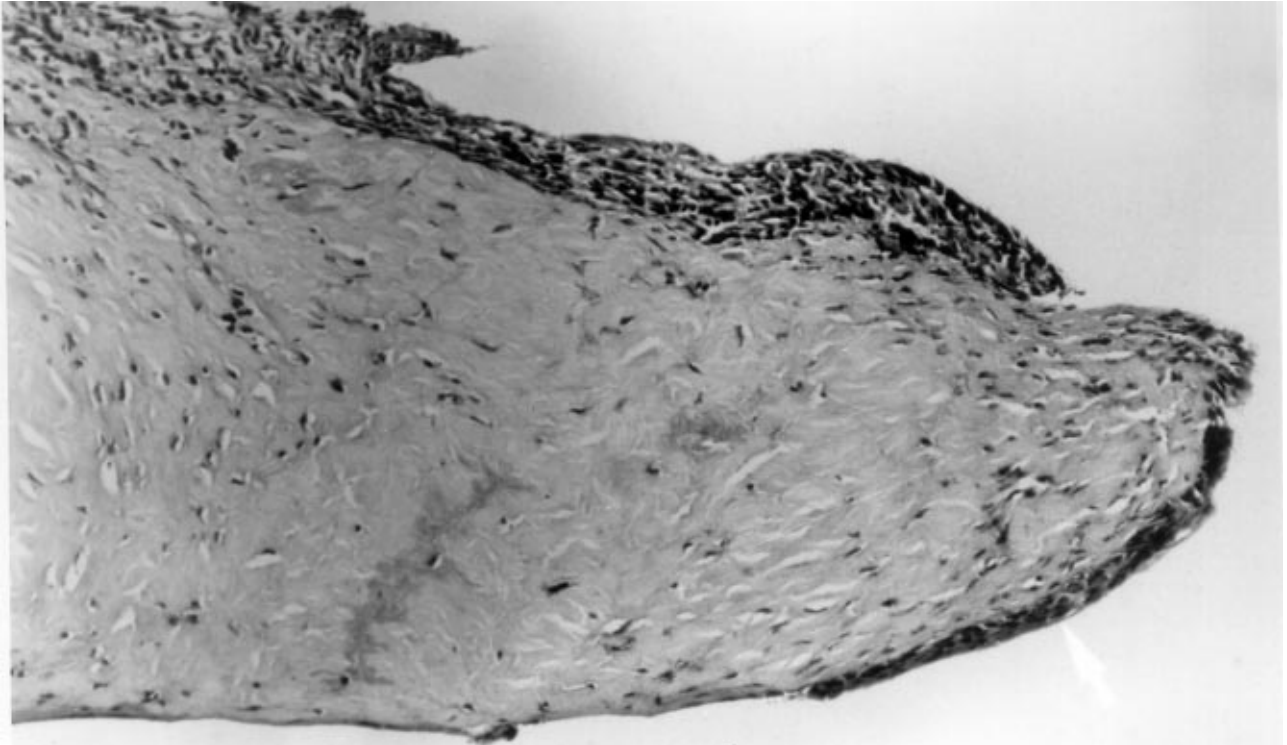


Figure 2 Histologic appearance of the TM 8 weeks after perforation reveals strands of dense connective tissue covered with a thin layer of squamous epithelium. At the edge of the perforation the outer squamous layer extended medially to coapt the inner mucosal layer of the drum (white arrow). Adjacent to the perforation, the squamous layer exhibited marked hyperplasia, which thinned once it rounded the edge, to approximate mucosa. The middle fibrous layer exhibited marked hypertrophy and hyperplasia. (H&E X 50).

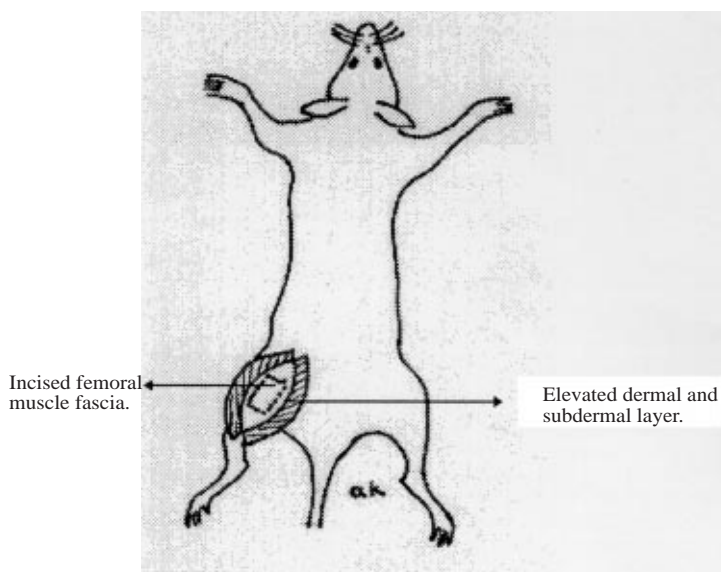


Figure 3. A Schematic diagram of the removal of fascial graft from femoral muscle in the rat.

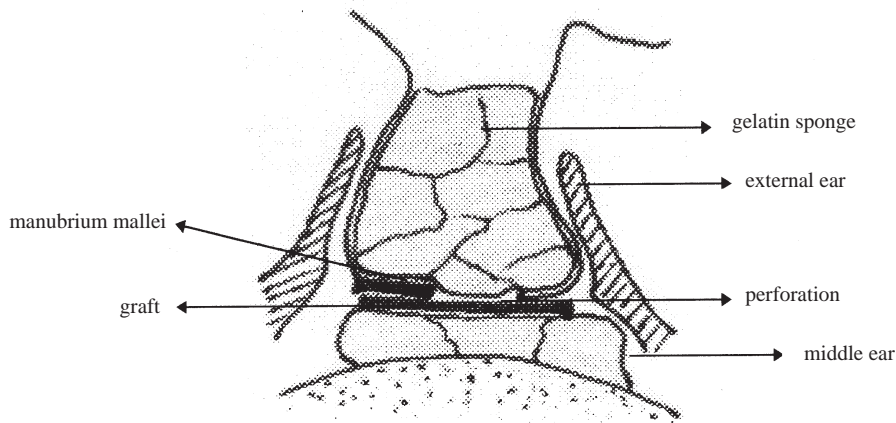


Figure 4. Schematic view of the application of myringoplasty with the underlay technique.

performed on group 1 (10 rats), HA was embedded in Gelfoam® to support the TM graft in group 2 (10 rats), instead of saline solution.

Postoperatively, trimetoprim 40 mg/ml combined with sulfadiazine 200 mg/ml at a dose of 0.125ml/kg daily were injected intramuscularly for 5 days for prophylaxy. All animals were re-anesthetized weekly and their right ears were examined under an operation microscope for control. The animals were sacrificed by ether on postoperative day 28. The otomicroscopic and histopathological findings in both groups were compared.

Temporal bones of the animals were dissected for histopathological evaluation. The TM was microdissected from its annular groove. The isolated TMs were fixed in 10% formalin, prior to routine processing through to a

paraffin-embedded block. Four-micrometer-thick sections were taken parallel to the handle of the malleus and stained by hematoxylin-eosin (H&E). A pathologist made the examination unaware of the experiment groups. t and X² test were used for the statistical analysis.

Results

While graft take was obtained in all myringoplasties in group 1, graft take in group 2 was 90 %. Otomicroscopic examination on day 28, showed calcification in 7 rats in group 1, whereas it was found in only 2 rats in group 2. The difference was statistically significant (p<0.05). Calcareous plaques on the TM were large and wide in group 1 but in group 2 were localized, small and indistinct. Vascularization seen especially on the malleus

Table 1. Otomicroscopic findings (day 28)

Groups	Graft take	Edema	Calcification	Microvascularization	
				good	medium
1 (n=10)	10	5	7	7	3
2 (n=10)	9	7	2	2	8
t value	1.05	1.01	2.6	2.6	2.6

handle edges and developing from the peripher to the centre of the TM in group 1 was better than in group 2 perhaps due to edema (Table 1).

In the histopathological findings, according to the

number of epithelial layers developing over the graft, group 1 myringoplasties showed epithelium with more than 4 layers. But in group 2 myringoplasties, 3-4 layers of epithelium were seen well organized (Table 2). Keratin associated with epithelium was found more commonly in

Table 2. Histopathological findings obtained with light microscopy (day 28)

Groups	Epithelial cell layers			Calcification	Keratin	Inflammatory cells		
	4 ↑	3-4	1-2			Lymphocyte	PMN*	Plasma
1 (n=10)	7	2	1	8	3	2	2	1
2 (n=10)	3	5	2	2	7	3	5	2
	X ² =3.32			t=3.35	t=0.94	t=0.52	t=1.48	t=0.52

*Polymorphonuclear leucocyte

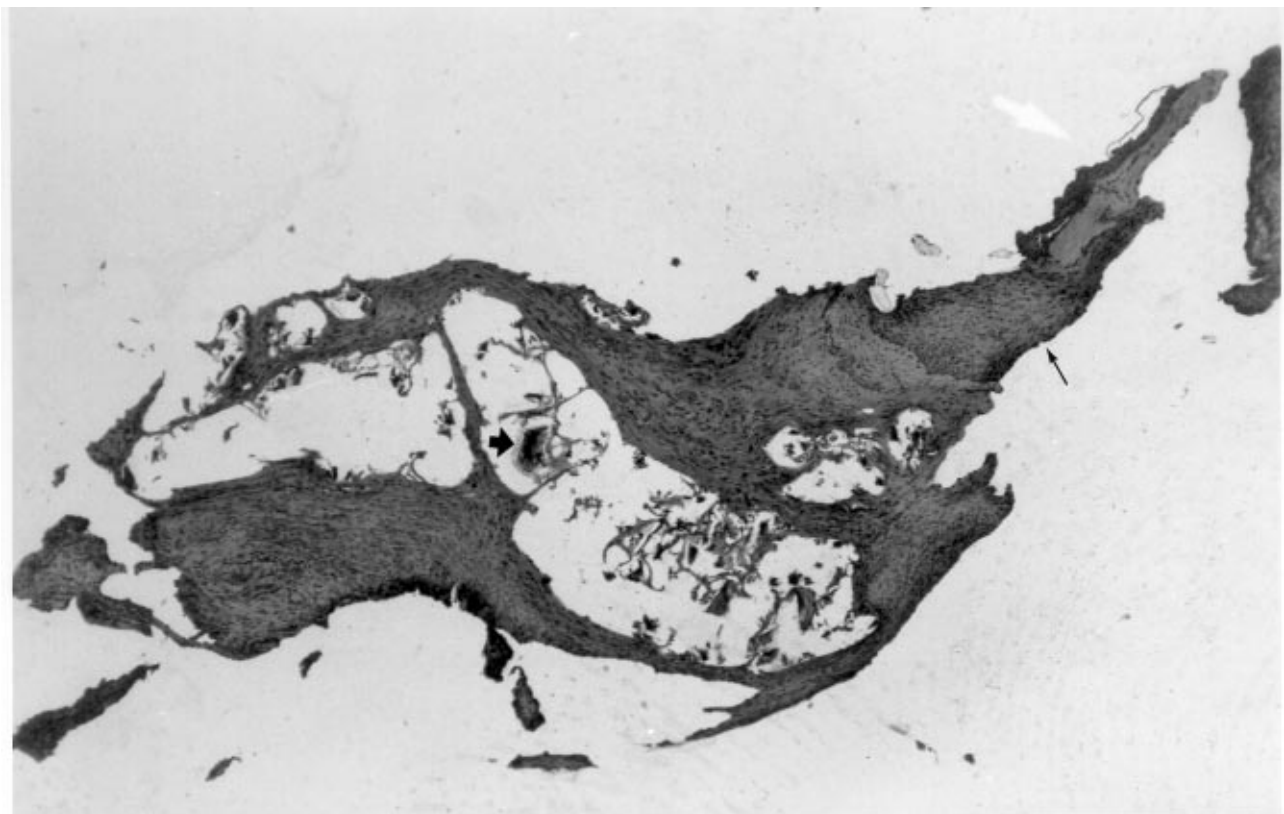


Figure 5. The calcium deposits (black thick arrow) in the graft and two to three layers of stratified squamous cells (white arrow) and eight to ten layers of stratified squamous cells (black thin arrow) are seen (H&E X 25)

group 2. Group 1 myringoplastic calcification was observed in 8 rats in group 1 (Fig. 5), whereas it was observed in 2 rats in group 2 minimally. Inflammatory cell infiltration in group 2 was observed more than group 1.

Discussion

The morphology of the TM is well defined. Three distinct layers can be distinguished in the pars tensa. There is an outer keratinizing stratified squamous epithelium, an inner layer composed of flat epithelial cells and a stratum of collagenous connective tissue interposed between the epithelial layers. The pattern of the specifically oriented collagen fibers differ between species. In man, as well as in the rat, radial fibers predominate (1). The features make the TM unique when considering its mechanism of healing. Unlike other tissues in the body, the TM has no underlying tissue matrix to act as a support for regenerating epithelium and to provide a route of access for reparative cells and nutrients. Despite this paucity of underlying structure, the TM has a remarkable capability for self-repair (7, 8). The second unusual characteristics of the TM is that its outer epithelial layer is normally in a continuous state of migration (5, 9). Re-epithelization of fascial grafts by stable migratory epidermis is essential for successful myringoplasty, and the use of non-migratory split skin in this procedure is unsuccessful (10, 11). Many studies have been performed on epithelial migration (10, 12). Johnson and Hawke (7) demonstrated that in the guinea pig the epidermal surface migrates in a mainly superior direction over the TM at between 0.5 and 1 mm per day.

Myringoplasty refers to a procedure in which a small defect in the drumhead caused by an episode of trauma or infection is grafted without additional surgery on the canal or middle ear. Myringoplasty should be performed in an absolutely dry ear (13). But migration is an important problem in obtaining chronic dry TM perforation in order to perform experimental myringoplasty. Migration may be prevented by thermal injury and myringectomy (5). In our study, myringectomy performed with radiosurgery was applied to prevent migration. Despite this, the rats in which we could not obtain dry TM perforation were excluded from the study. Keratinized stratified squamous epithelium in chronic dry TM perforation indicates that migration may be re-activated with trauma. Hence, re-epithelization has been developed over the graft.

HA is now commercially available for clinical use in veterinary practice and ophthalmic surgery (14). In view of the excellent results with HA as an aid in eye surgery, the question has been raised whether or not HA could be

used in otosurgery. Recently it was shown that HA applied to experimentally induced TM perforations in the rat accelerated the healing rate considerably (9, 15-18). HA appears, in some experimental conditions, to influence cell locomotion (9). In particular, Culp (19) suggested that HA was needed to effect detachment, which allows cells to move forward. These and other cell-to-cell and cell-to-substrate interactions (20) may contribute to the enhanced cellular migration which occurs in hyaluronate-rich matrices. Either the HA acts solely as a mechanical support on which the advancing keratinizing stratified epithelium can slide or it has more direct effects on cell surfaces influencing cell behavior (1). Moreover, the addition of HA to Gelfoam® reduced some of the tissue reactions such as bone formation, known to occur when Gelfoam® is applied alone (21). Application of HA to the perforated area also influences the structural quality of the healed TM. Whereas untreated tympanic membranes still exhibited areas of opaque scar tissue, the HA-treated tympanic membranes were thin, with little scar tissue (1). This seems to occur due to a more rapid reorganization of the fibrous layer involving a faster orientation of the collagen fibers.

In our study, the epithelial organization in group 2 was better than in group 1. Keratin and epithelial cell layers in group 2 were more organized and homogenous. Calcareous plaques in myringoplasty might develop due to trauma, bleeding and infection. The fact that calcareous plaques were observed less in group 2, was regarded as a significant effect of HA. Another histopathological finding in the HA application is that there was more inflammatory cell infiltration than group 1. We believe that this finding may be as a result of modulating cell functions of HA. On the other hand, because HA has no toxicity to structures of the middle and inner ear, especially when it was instilled into the middle ear with the inner ear opened (15, 22-25), it may be used in myringoplasty especially in myringosclerotic TM perforations.

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