

# CHRONIFICATION OF TYMPANIC PERFORATION WITH MITOMYCIN C IN A RAT MODEL

Tomás Esteban<sup>1</sup>, Noemí M. Atucha<sup>2</sup>, José María Moraleda<sup>3</sup>, Joaquín García-Estañ<sup>2</sup>, Carmen L. Insausti<sup>3</sup>, Javier Moraleda-Deleito<sup>3</sup>

Instituto Murciano de Investigación Biosanitaria, Universidad de Murcia,  
Spain

1, Servicio de Otorinolaringología, Hospital General Universitario Santa Lucía, Cartagena, Spain; [tomas.esteban@carm.es](mailto:tomas.esteban@carm.es); 2, Departamento de Fisiología, Facultad de Medicina, 30120 El Palmar, Murcia, Spain; [jgestan@um.es](mailto:jgestan@um.es); [ntma@um.es](mailto:ntma@um.es); 3, Departamento de Medicina, Facultad de Medicina, Universidad de Murcia, Instituto Murciano de Investigación Biosanitaria, 30120 El Palmar, Murcia, Spain; [jmoraled@um.es](mailto:jmoraled@um.es); [caymed@gmail.es](mailto:caymed@gmail.es); [javiermoraled@gmail.com](mailto:javiermoraled@gmail.com)

## ABSTRACT

**Background.** A rat model of chronic tympanic membrane perforation was developed to be used in the search of new materials for the sealing of these perforations.

**Methods.** A longitudinal study was carried out in rats subjected to incisional myringotomy followed by the application of mitomycin C alone or with dexamethasone. Rats were checked at days 3, 7, 10, 14 and weekly thereafter until perforation closure, for up to 6 months.

**Results.** The addition of dexamethasone is a key component in order to obtain a chronic opening. Myringotomies treated with saline had a mean healing time of 8.5 days. At 8 weeks, 70.5% of these remained perforated and at 6 months this number fell to 21.4%.

**Conclusion.** This technique is able to maintain more than 70% of tympanic membrane perforations patent for at least 8 weeks. This rat model is adequate for its use in preclinical or translational research.

**Keywords:** Animal model, chronic tympanic membrane perforation, mitomycin C, myringotomy, dexamethasone.

**Address for correspondence:** Dr. Noemí M. Atucha, Campus de Ciencias de la Salud, Edif. LAIB, 3, 32, 30120 El Palmar (Murcia). Phone: +34-868-884880, E-mail: [ntma@um.es](mailto:ntma@um.es)

## INTRODUCTION

Otitis media is a middle ear pathology that includes a wide range of inflammatory diseases, such as otitis media with effusion, chronic suppurative otitis media with and without cholesteatoma and adhesive otitis media. In chronic suppurative otitis media (CSOM), a tympanic membrane (TM) perforation with persistent drainage from the middle ear is the most common pathology (1). One of the treatments is a surgical repair by placing an underlay support material medial to the TM. This is done after refreshing the edges of the perforation to activate healing and migration of the epithelial layer. Multiple substances have been used as a scaffold in the treatment of TM perforation such as temporalis muscle fascia, perichondrium, cartilage, fat, and spongostan (haemostatic gel foam). Although the rates of

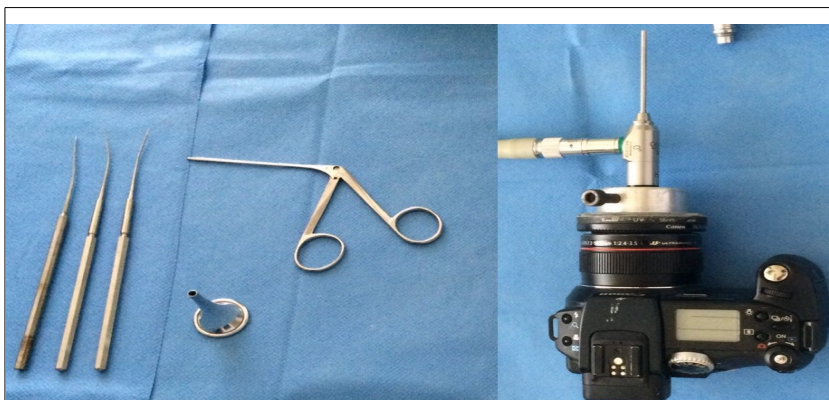
closures after the initial attempt are not bad (2), there is a continuous search for new materials that are more effective in the healing of these TM perforations. Among these, the amniotic membrane could be of interest since it contains stem cells which are endowed with anti-inflammatory, anti-infective and immuno-modulatory properties (3). However, prior to launching human clinical trials to test new materials, a preclinical study is needed using animal models of chronic TM perforation.

Preclinical studies have been performed so far in animal models of TM perforation which involve laser myringotomy and treatment with hydrocortisone or mitomycin C (4, 5), but laser equipment is not a common tool in research centers. Therefore, we, aimed to develop a new model to create a chronic tympanic membrane perforation (CTMP) by myringotomy and instillation of mitomycin C. Previous methods to create a CTMP without a laser equipment, such as amputation of the handle of the malleus (6), and the application of substances such as dexamethasone (7), have not obtained conclusive results. Thus, the main objective of the present paper was to identify whether incisional myringotomy with the application of mitomycin C alone or in combination with dexamethasone may produce a chronic tympanic perforation model in rats. As a secondary objective, we have analyzed whether the amputation of the handle of the malleus with application of mitomycin C before or after incisional myringotomy could influence the duration of tympanic membrane perforation patency in rats.

## MATERIALS AND METHODS

All the research was conducted in the Centre for Experimentation and Biomedical Research (CEIB) of the Universidad de Murcia (Murcia, Spain). All experiments and procedures were approved by the local ethics committee and followed the ethical principles and current legislation on protection of animals for research (8-10). The study was performed in 34 healthy male Sprague Dawley rats of 8 weeks of age and weights ranging from 310 to 370 grams. The environmental conditions were kept constant throughout the study with cycles of 12 hours of light and darkness, a constant temperature of 20°C and a humidity of 48% (11). The animals were anesthetized with a mixture of ketamine (40-90 mg/kg, ip) and Xylazine (5-10 mg/kg) (12).

To study the patency of a perforation and the influence of different agents in creating a chronic perforation we used two substances: mitomycin C and dexamethasone. Mitomycin C is an aminoglycoside antibiotic that has been used as a cytostatic due to its ability to disrupt DNA replication, inhibiting mitosis, and protein synthesis, thus preventing the replication of fibroblast and epithelial cells and eventually prolonging the healing time (13). Dexamethasone is a classical steroid anti-inflammatory drug that inhibits the production of collagen from fibroblasts. The group of controls rats was treated with a 0.9% saline solution.



**Figure 1.** Camera with optical adapter (right) and tympanoplasty instruments used (left).

## Experimental procedure.

Procedures were performed under direct vision with a Zeiss microscope (Germany) with TM photographs being acquired with a digital camera (Canon Power Shot Pro 1, Japan) attached to a 0 degree and 3 mm diameter rigid endoscope via an adaptor (GAES audio test HD) and a Karl Storz cold light source, model 482 (Germany). The surgical instruments consisted of a 4 mm diameter ear speculum, a Hartman clip and a Tympanoplasty set (blunt and sharp straight needle, and a small Rosen ring) as shown in Figure 1. All myringotomies (size of 2.4 x 2.4 mm) were performed in the pars tensa using a sharp punch, amputating the eardrum handle with the same instrument and extracting the bone fragment with a Hartman clip. The concentration of mitomycin C was 0.4 mg/ml and the time of instillation of mitomycin and 0,9 % saline solution was always of 10 minutes.

Rats were checked afterwards to assess the state of healing of the tympanic perforations at days 3, 7, 10, 14 and weekly thereafter until perforation closure, for up to 6 months. The perforations were measured with a small Rosen ring (0.8 mm diameter). The date of the complete closure of the perforation was obtained by averaging the last day that the perforation was seen and the first day that it was found to be closed. The amount of anesthesia was 0.7 ml of ketamine-Xylazine mixture in the first intervention and 0.4 ml in the revision days. Photographs of the eardrums were taken at every intervention and in revisions. Mitomycin C was applied to the TM on spongostan that had been soaked in it (0,4 mg/ml). Dexamethasone (4 mg/ ml) was applied directly in drops given through the external auditory canal until fully filling the middle and the external ear.

The statistical analysis was performed using the software R Core Team 2015, by using an ANOVA test and a p value lower than 0.05 was considered to express a significant difference (14).

**Table 1.** Experimental groups.

Groups	n	Subgroups	Number of Eardrums	1st intervention	2nd intervention	3rd intervention
A	10	A1	10 Left ears	Myringotomy	Mitomycin C	
		A2	10 Right ears	Myringotomy	Saline solution	
B	12	B1	10 Alternate ears	Mitomycin C	Myringotomy	
		B2	10 Alternate ears	Mitomycin C	Myringotomy	
		B3	2 Alternate ears	Saline solution	Myringotomy	
		B4	2 Alternate ears	Saline solution	Myringotomy	
C	14	C1	10 Alternate ears	Mitomycin C	Myringotomy	Dexamethasone
		C2	10 Alternate ears	Mitomycin C	Myringotomy	Dexamethasone
		C3	2 Alternate ears	Saline solution	Myringotomy	Saline solution
		C4	2 Alternate ears	Saline solution	Myringotomy	Saline solution

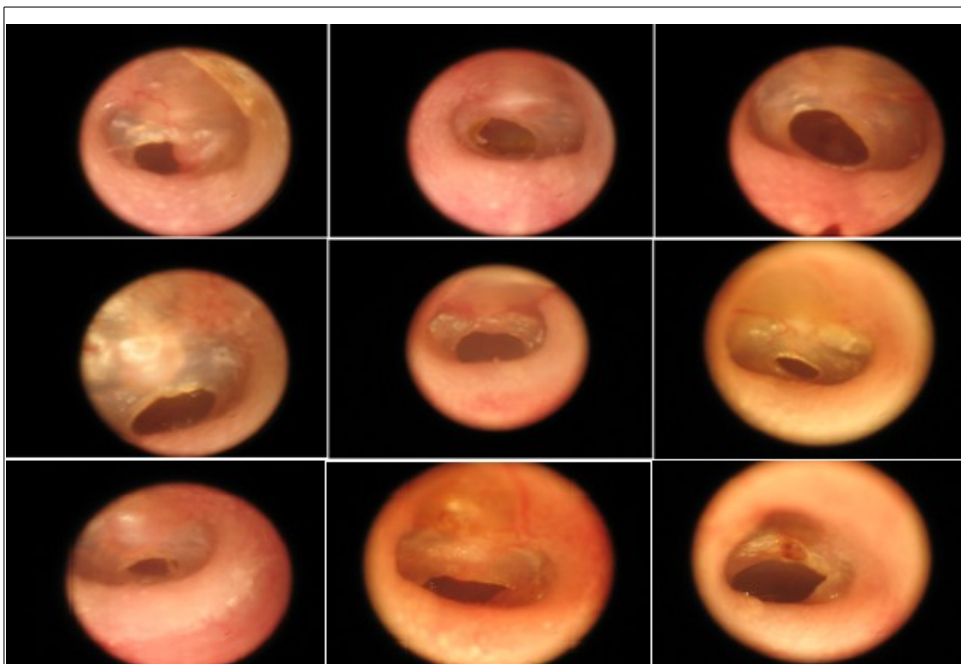
## Experimental groups (table 1).

The 34 rats were divided into three groups (A, B and C) to compare the different interventions and their results. All groups were divided into subgroups according to additional variations in interventions and their controls, as follows:

1. Group A (n=10): rats that underwent myringotomy and amputation of the handle of the malleus and posterior application of mitomycin C or saline. The ears of these rats were later divided into two subgroups (A1 and A2).

1.1. The A1 subgroup consisted of the ten left ears of the rats that underwent instillation of mitomycin C.

- 1.2. The A2 subgroup consisted of the ten right ears of the control rats, that received saline alone.
2. Group B (n=12), consisted of rats that were equally divided into four subgroups depending on the instillation of mitomycin C or saline before myringotomy and with or without amputation of the handle of the malleus. In this group, we alternated between left and right ear for further randomization. The four subgroups were:
- 2.1. Subgroup B1 consisted of 10 ears in which we applied mitomycin C prior to myringotomy but without amputating the handle of the malleus.
  - 2.2. The B2 subgroup was formed by the 10 contralateral ears of B1 animals, which had the same treatment plus amputation of the malleus handle.
  - 2.3. The B3 subgroup were 2 rats in which we applied saline followed by incisional myringotomy without amputation of the handle of the malleus.
  - 2.4. The B4 subgroup was formed by the contralateral ears of B3 which had the same intervention along with amputation of the handles of the malleus.
3. Group C (n=12): rats that were also divided into 4 subgroups to assess the effect of the instillation of dexamethasone (4 mg/ml) in three revisions after the myringotomy with or without amputation of the handle of the malleus. The four subgroups were:
- 3.1. The C1 subgroup was formed by 10 rat ears that were instilled with mitomycin C and then underwent myringotomy without amputating the handles of the malleus. In the first 4 revisions (days 3, 7, 10 and 14) dexamethasone was administered.
  - 3.2. Subgroup C2 was formed by the 10 contralateral ears of C1, in which the only modification was the additional amputation of the malleus handle.
  - 3.3. The C3 subgroup consisted of 2 rats in which we started by applying saline instead of mitomycin C prior to myringotomy without amputation of the handle of the malleus. An additional modification was that saline solution was applied instead of Dexamethasone at their four reviews.
  - 3.4.



**Figure 2.** Perforated eardrums at 8 weeks.

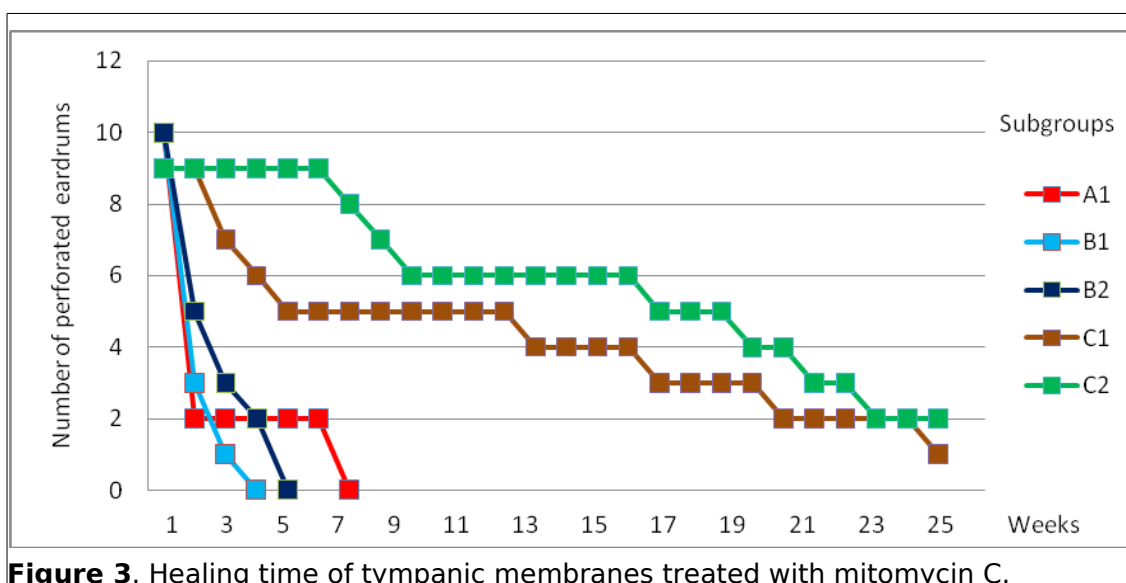
The C4 subgroup consisted of the contralateral ears of C3 that underwent the same protocol with the additional amputation of the handle of the malleus.

## RESULTS

Myringotomies treated with saline solution (table 2) had a mean healing time of 8.5 days. In group C3, one ear healed at 8.5 days and the other one, at 5 days. Those treated with mitomycin C and/or dexamethasone are shown in table 3. At 8 weeks, 70.5% of these TMs remained perforated and at 6 months this number fell to 21.4% (figure 2). Figure 3 shows the three TMs that remained persistently perforated at 6 months. Laterality (the influence of treating the right or the left ear of the rat) did not significantly affect healing time ( $p > 0.6$ ). With regard to healing time, the comparison of subgroups A1 and B2 did not show a significant difference when mitomycin C was applied before or after myringotomy ( $p > 0.8$ ). We subsequently compared groups A1 and A2, showing a significant prolongation of the healing time with mitomycin C compared to saline ( $p < 0.03$ ). The administration of mitomycin C and dexamethasone (C1 and C2) was significantly different ( $p < 0.006$ ) to the groups with saline (C3 and C4). Regarding amputation, there were no differences between subgroups C1 and C2. Finally, the use of mitomycin C alone or in combination with dexamethasone had a longer duration of tympanic permeability in the ears treated with dexamethasone ( $p < 0.001$ ), figure 4.

**Table 2.** Tympanic permeability duration in myringotomies treated with saline solution.

Subgroups	Number of eardrums	Treatment	Permeability (days)
A2	10	Saline solution	8.5
B3	2	Saline solution	8.5
B4	2	Saline solution	8.5
C3	2	Saline solution	8.5 (n=1), 5.0 (n=1)
C4	2	Saline solution	8.5



**Figure 3.** Healing time of tympanic membranes treated with mitomycin C.

## DISCUSSION

The tympanic membrane perforations in experimental animals are considered chronic when they are maintained open between 8 and 15 weeks (15). Since the 1990s, numerous studies have attempted to establish an animal model of chronic TM perforations using different animals and substances. Wang et al. (16) performed a review of the literature and, out of 37 studies, only 23 of them were able to achieve chronic TM perforations. The

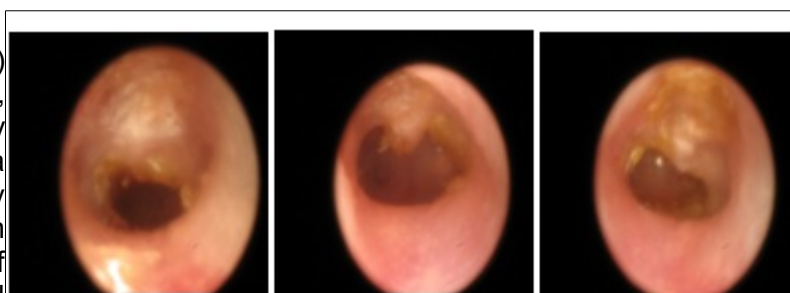
chinchilla was the animal most commonly used in these studies, followed by the rat and guinea pig. In our study, we used Sprague Dawley rats because they were easy to handle and have eardrums with histological characteristics similar to humans (17).

**Table 3.** Tympanic permeability duration in myringotomies treated with Mitomycin C.

Sub-groups	Treatment	Number of eardrums	Permeability (days)	Unhealed Myringotomies at 6 months	Mean permeability (days)	Discarded eardrums	Mean permeability by groups (days)
A1	Mitomycin C	8	12.0		18.6		
		2	45.5				
B1	Mitomycin C	5	8.5		12.6		15.1
		2	12.0				
		2	17.5				
		1	24.5				
B2	Mitomycin C	2	8.5		17.5		
		3	12.0				
		2	17.5				
		1	24.5				
		2	31.5				
C1	Mitomycin C + Dexamethasone	1	17.5	1	95.0	1 Exitus 2 Otorrhea	112.7
		1	24.5				
		1	31.5				
		1	87.5				
		1	143.5				
		1	178.5				
C2	Mitomycin C + Dexamethasone	1	45.5	2	130.5	1 Exitus 2 Otorrhea	
		1	52.5				
		1	136.5				
		1	150.5				
		1	164.5				

Also we decided to use a dose of 0.4 mg/ml of mitomycin C. This was based in the study of Jassir et al. (4) that compared mitomycin doses of 0.2, 0.4 and 2 mg/ml showing that at 8 weeks 50% of myringotomies treated with 0.4 mg/ml remained patent. Babu et al. (18) recommended the use of gel foam soaked in mitomycin C. These authors performed a comparative study between the direct application of mitomycin C and application with a gel foam, evaluating the toxicity by measuring auditory brainstem responses. The application with gel foam completely prevented toxicity, justifying its use.

Strem et al. (19) conducted a study in 60 rats, producing myringotomies by laser and mitomycin C at a concentration of 2 mg/ml. They established several groups with different times of application of mitomycin C and concluded that the patency rate did not improve with an application longer than 10 minutes nor by



**Figure 4.** Perforated eardrums at 6 months (one of subgroup C1 and two of C2)

repeating the dose. In the study by Jang et al. (20) on the effects of mitomycin C in tympanic membrane fibroblasts in vitro, using different concentrations and times of exposure, the lowest fibroblasts viability was obtained when applying mitomycin C for 10 minutes at a concentration of 0.4 mg/ml. According to these studies, we applied mitomycin C by spongostan at a concentration of 0.4 mg/ml for 10 minutes. Our data shows that the addition of dexamethasone to mitomycin C treatment prolongs the patency rate of myringotomies. These results agree with those of Kaftan H et al. (7) in a rat model in which

they used mitomycin C at a concentration of 2 mg/ml applied for 10 minutes before producing the tympanic perforation. In the revisions, they applied dexamethasone at a concentration of 4 mg/ml, with a mean patency of the perforation of 17.5 days when they used mitomycin C alone and 32 days when associated with dexamethasone. In our study we obtained a similar average patency of 15.1 days when treating rats with myringotomies after application of mitomycin C alone (group B), while in Group C (application of mitomycin C and dexamethasone) we obtained an average permeability of 112.7 days, significantly higher than that achieved by Kaftan et al (7). Previously published studies of incisional myringotomy with mitomycin C did not succeed in obtaining enough chronic tympanic membrane perforations. Only the laser myringotomy associated with mitomycin C treatment was able to produce chronic perforations (21). However, the laser technology is not available to the majority of experimental laboratories.

Recent studies by Wang et al (22) have used a similar method although they obtained perforations of insufficient duration to establish and constitute a chronicity model. The difference is probably related to the method used since these authors employed a higher dose of mitomycin, different from the one recommended in the Jassir et al (4) work. Another important difference, in our opinion, is the application of dexamethasone, which they apply only once. We think that the application of dexamethasone in every revision is essential to succeed with the chronic lesion.

In this study, we have established a rat model of chronic tympanic membrane perforation by combining mitomycin C (0.4 mg/ml for 10 minutes) prior to incisional myringotomy and adding dexamethasone (4 mg/ml) on four additional doses. The addition of dexamethasone to mitomycin C is a key component for obtaining a good rate of tympanic opening in this experimental animal model. Our technique is able to maintain 70.5% of tympanic membrane perforations created by incisional myringotomy and mitomycin C treatment patent for at least 8 weeks. This model performed in Sprague Dawley rats is robust, reproducible and convenient for preclinical as well as translational research of this pathology.

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