Title: Rat model of chronic tympanic membrane perforation: A longitudinal histological evaluation of underlying mechanisms

Article Type: Full Length Article

Keywords: Tympanic membrane perforation, chronic, animal model, rat, ventilation tube, histology

Abstract: Objective: To evaluate histologically the progressive development and underlying mechanisms of chronic tympanic membrane perforation (TMP) in a rat model using a two-weeks ventilation tube (VT) treatment combined with topical application of mitomycin C/dexamethasone (VT-M/D), compared with normal tympanic membrane and acute TMPs.

Methods: Fifty male Sprague-Dawley rats were divided into three experimental groups: a normal control group (n = 5), an acute TMP group (n = 5) (i.e. 3 days post-myringotomy) and a VT-M/D group (n = 40). The TMs were regularly assessed by otoscopy. The normal control animals were sacrificed on day 0 and the acute TMP group was sacrificed 3 days post-myringotomy for histological and immunohistochemical evaluations. The VT-M/D group was sacrificed at various time points - 14 and 17 days, 3, 4, 6, 8 and 10 weeks.

Results: On longitudinal histological examination, compared with normal TM and acute TMP, the perforation edges at the later time points illustrated thickened stratified squamous epithelium rimming around the edges, significant increase in keratin and collagen deposition, increased macrophage infiltration as well as reduced cellular proliferation. Three phases of TMP healing process were identified - the acute healing phase (3-17 days), the transition phase (3-4 weeks) and the chronic phase (6-10 weeks).

Conclusion: Based on the histological results of this study, the progressive development of chronic TMPs appeared to be associated with increased epidermal thickening, collagen and keratin deposition, macrophage infiltration and reduced cellular proliferation. After the 3-4 weeks of transition phase, the TMPs seemed to have transformed into a non-healing chronic TMP between 6 and 10 weeks.
Professor Robert Ruben  
Editor-in-Chief  
International Journal of Pediatric Otorhinolaryngology  

14th December, 2016  

Dear Professor Ruben,  

RE: Rat model of chronic tympanic membrane perforation: A longitudinal histological evaluation of underlying mechanisms (IJPORL-D-16-00768R1)  

Thank you for reviewing our manuscript and allowing us the opportunity to undertake second revision. We appreciate the constructive comments and suggestions of the reviewers and feel the revised manuscript has been enhanced both in content and clarity by these revisions.  

We have modified the manuscript in which we address all of the suggestions of the reviewers. A detailed list of changes and comments is also submitted. New text is underlined in the re-submission.  

Yours sincerely,  

Dr Allen Yu-Yu Wang, MBBS MS PhD  
Ear Sciences Centre, School of Surgery, the University of Western Australia, Perth, Australia  
On behalf of all co-authors  

Corresponding Author:  
Dr Allen Yu-Yu Wang  
Ear Sciences Centre, School of Surgery, The University of Western Australia  
M507, 35 Stirling Highway, Nedlands, Perth, WA 6009, Australia  
Email: allenwang_@hotmail.com
Reviewers’ Comments:
Reviewer: 1

(1) 2.32: 'shown ... on a rat model from another group [11, 19]' -> 'shown by another group [11, 19] ... in a rat model'
The sentence has been modified accordingly.

(2) 8: The information in the table provided in the authors' response is very useful and should be included in the manuscript, perhaps in a table with 3 columns: group; total number in group; and number (and %) patent.
A new table (Table 1) has been included in the manuscript illustrating the number of patent and closed tympanic membrane perforations of different longitudinal groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total No. of animal (n = 50)</th>
<th>No. of TMP patent (n = 42)</th>
<th>No. of TMP closed (n = 8)</th>
<th>Proportion of patent TMP at each time point (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Acute TMP (3 days)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>14 days (VT-M/D)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>17 days (VT-M/D)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>3 weeks (VT-M/D)</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>71</td>
</tr>
<tr>
<td>4 weeks (VT-M/D)</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>71</td>
</tr>
<tr>
<td>6 weeks (VT-M/D)</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>83</td>
</tr>
<tr>
<td>8 weeks (VT-M/D)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>10 weeks (VT-M/D)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80</td>
</tr>
</tbody>
</table>

(3) 8.2-21: Thickening is discussed in this section on otoscopic observation, suggesting that we should be able to judge the presence or absence of thickening in column 1 of Fig. 2, but we cannot. The references to Fig. 2 are awkward to follow because of the inconvenient panel labelling, which has not been changed. The discussion is confusing because the 14-day group (referred to as two weeks, which does not help) is discussed before the 3-day group. The statement about 'no obvious thickening' at 14 days cannot be judged from the histological image in column 2. Thickening is evident in the 3-day histological image but is not mentioned. The 17-day histological image does not show the wide perforation well so it is difficult to judge the thickening. In few of the images is it easy to judge whether the TM is 'thickened around the perforation edge' compared with its thickness away from the edge. (In particular, what are we seeing at the position of the "*" in column 2 for the 8-week group?) In any case, none of this seems relevant to the section heading. At the end of the section, 'stable size' seems to mean stable across time within an individual rat. The wording of the sentence suggests that we should be able to see this in Fig. 2 but we cannot. How was the size determined? If we are not shown the measurements, we should at least have a more quantitative description of the observation.
- The references to Fig. 2, 3, 4 have been modified as per Reviewer (1) in order to improve the ease to follow by the readers – now identified by row (A to I) & column (1 to 2 or 3).
- To avoid confusion, we have removed the descriptions of thickening around the edges of TMP on otoscopic observation from section 3.1.
- The name of 2-week group has been changed to 14-day group.
- The description of thickening of perforation edges evident in the 3 & 14 days of H&E histological image have been added onto section 3.2.1.

- The description of thickening of perforation edges in 17 days of H&E histological image have been removed from section 3.2.1.

- In column 2 for 8-week Fig. 2H (ii), the position of '*' indicates a clot/debris sitting right between the perforation gap which is a separate entity on high power Fig. 2H (iii). This statement has been added at the end of paragraph of 3.2.1.

- The reviewer (1) is correct in stating that the 'stable size' means stable across time within an individual rat. The size of the TMP was determined relatively to the scale bar on right bottom corner (column (i) 600 µm at 4× magnification and column (ii) 200 µm at 20× magnification).

(4) Fig. 2: Some of the black boxes in column 2 do not correspond with what is seen in column 3 (especially W & X). The caption still does not specify that (or if) columns 2 & 3 illustrate the same rat as shown in column 1. In the caption, 'sections of the nine' -> 'sections from the nine'.

The black boxes in column 2 of figure 2 have been modified so that they correspond better to what is seen in column 3. The labelling of Fig. 2 of the rows as well as the captions have been changes to improve clarity.

“Fig. 2. Representative otoscope images (i) and corresponding H&E histology images (ii & iii) of one selected rat TMP from the nine different longitudinal groups. A. Normal TM; B. 3-day TMP; C. 14-day TMP; D. 17-day TMP; E. 3-week TMP; F. 4-week TMP; G. 6-week TMP; H. 8-week TMP; I. 10-week TMP. All images in (iii) are actual magnified images of the black boxes in (ii). Scale bars: 600 µm at 4× magnification and 200 µm at 20× magnification. White asterisks on otoscope images indicate the actual TMP sites. Black asterisks on H&E sections indicate cross-sectional perforation defects. The black boxes correlate locations of the higher 20× magnification in third column. H&E = Hematoxylin and eosin staining, TMP = tympanic membrane perforation; VT-M/D = ventilation tube insertion in conjunction with mitomycin C & dexamethasone, MEC = middle ear cavity; TM = tympanic membrane; M = handle of malleus; EAC = external auditory canal.”

(5) 13.19: The wording is still incorrect. It is the 'procedure', not the TMP’s themselves, that mimic (or not) the clinical causes.

The sentence has been modified as per the reviewer – “Firstly, the VT insertion method only mimic one of the causes of chronic TMP in clinical settings (i.e. long term VTs), but not other possible causes including Eustachian tube dysfunction and chronic middle ear infection.”

(6) 13.32: The revised wording still tells us nothing about what Santa Maria et al. (2011) were able to observe about rat TM healing using microarrays.

The sentence has been modified to – “Santa Maria et.al [43] have identified a few potential markers of acute TMP healing such as Stefin A2 and Natriuretic peptide precursor type B. Therefore, further studies would be valuable to determine whether these genes are involved in chronic TMP development and to identify novel molecular markers.”

(7) In their responses the authors appear to have provided the additional information requested by reviewer 2, but none of that information has been added to the manuscript.
Additional information points (1), (2), (3) requested by reviewer 2 previously has been added to the manuscript
- Under the discussion section, 4th paragraph (see underlined).

“For the acute TMP group, we have selected 3 days as the timeline to sacrifice the animal. An acute TMP group needs to be in the situation of an ongoing healing process before closure. Acute TMPs in rats almost all closed by 7 days [38]. Thus, the acute time frame has to be less than 7 days. We eventually decided on a ‘middle’ time of day 3, which it has had a few days to allow acute healing response to be activated but not too late so that it has closed. For the VT-M/D group, we decided the various time frame of sacrifice from our previous publication developing this rat model [37]. The initial ‘acute phase’ post VT removal is crucial, thus the initial time frame of 14 days, 17 days, 3 weeks. Thereafter, we wanted regular time frame samples between the 3 weeks and the final time point of 10 week, thus the following time frame of 4, 6, 8, 10 weeks. The study was initially designed as purely a histological descriptive experimental studies so power was not calculated. Normal control and acute TMP groups were each assigned with 5 rats. The rest of the VT-M/D groups had 40 rats in total - 7 time points (14, 17 days, 3, 4, 6, 8, 10 weeks)."
Rat model of chronic tympanic membrane perforation: A longitudinal histological evaluation of underlying mechanisms

Allen Y. Wang a,b,c,*, Lawrence J Liew a,b, Yi Shen a,b,d, Jeffrey T. Wang a, Magnus von Unge e,f, Marcus D. Atlas a,b,c, Rodney J. Dilley a,b

a Ear Sciences Centre, School of Surgery, The University of Western Australia, Perth, Western Australia, Australia
b Ear Science Institute Australia, Perth, Western Australia, Australia
c Department of Otolaryngology, Head and Neck, Skull Base Surgery, Sir Charles Gairdner Hospital, Perth, Western Australia, Australia
d Department of Otolaryngology, Head and Neck Surgery, Ningbo Lihuili Hospital (Ningbo Medical Centre), Ningbo, Zhejiang, China
e Akershus University Hospital and University of Oslo, Norway
f Center for Clinical Research Västerås, University of Uppsala, Sweden

* Corresponding author: Dr Allen Yu-Yu Wang, Ear Sciences Centre, School of Surgery M509, The University of Western Australia, Nedlands, Western Australia 6009, Australia. Tel.: +618 6151 0993.
E-mail address: allen.wang@earscience.org.au, allenwang@hotmail.com (Allen Y. Wang)

Allen Y. Wang and Lawrence J Liew are co-first authors
Abstract

**Objective:** To evaluate histologically the progressive development and underlying mechanisms of chronic tympanic membrane perforation (TMP) in a rat model using a two-weeks ventilation tube (VT) treatment combined with topical application of mitomycin C/dexamethasone (VT-M/D), compared with normal tympanic membrane and acute TMPs.

**Methods:** Fifty male Sprague-Dawley rats were divided into three experimental groups: a normal control group (n = 5), an acute TMP group (n = 5) (i.e. 3 days post-myringotomy) and a VT-M/D group (n = 40). The TMs were regularly assessed by otoscopy. The normal control animals were sacrificed on day 0 and the acute TMP group was sacrificed 3 days post-myringotomy for histological and immunohistochemical evaluations. The VT-M/D group was sacrificed at various time points - 14 and 17 days, 3, 4, 6, 8 and 10 weeks.

**Results:** On longitudinal histological examination, compared with normal TM and acute TMP, the perforation edges at the later time points illustrated thickened stratified squamous epithelium rimming around the edges, significant increase in keratin and collagen deposition, increased macrophage infiltration as well as reduced cellular proliferation. Three phases of TMP healing process were identified - the acute healing phase (3–17 days), the transition phase (3–4 weeks) and the chronic phase (6–10 weeks).

**Conclusion:** Based on the histological results of this study, the progressive development of chronic TMPs appeared to be associated with increased epidermal thickening, collagen and keratin deposition, macrophage infiltration and reduced cellular proliferation. After the 3–4 weeks of transition phase, the TMPs seemed to have transformed into a non-healing chronic TMP between 6 and 10 weeks.

**Keywords:** Tympanic membrane perforation, chronic, animal model, rat, ventilation tube, histology
1. Introduction

Tympanic membrane perforation (TMP) is a common clinical pathology worldwide, usually resulting from otitis media, trauma or ventilation tube (VT) treatment. A majority of acute TMPs heal spontaneously within 10 days [1, 2] but those that fail to heal and stay patent are defined as chronic. In cases of chronic TMPs, surgical grafting is an option for treatment. In order to evaluate novel graft or repair treatments, it is essential to develop a chronic TMP animal model that would mimic the clinical condition of chronic TMPs.

The current level of evidence in the literature on chronic TMP animal models has been recently brought to attention [3-5]. Various animal species have been utilized in the creation of models for chronic TMPs including chinchilla [6, 7], guinea pig [8, 9], rat [10, 11], dog [12] and mouse [13]. Numerous techniques have been trialed including infolding methods [6, 14], thermal injury [15, 16], re-myringotomy [17, 18], application of topical agents (e.g. mitomycin C [11, 19], steroids [10, 20]) and by genetic modification [13]. Controversies and discussions have been generated regarding both the reproducibility and validity of previously reported methods [21-26]. Topical applications of mitomycin C and dexamethasone (M/D) have been shown by another group [11, 19] in creating chronic TM perforations in a rat model. However, our research group recently found that topical M/D delayed healing but not enough to produce a chronic TMP in rats [26].

A strong association of increased chronic TMP incidence in patients with long-term VT treatment (i.e. several years) compared with short-term VT treatment (i.e. 8–14 months) has been reported in the literature. Previously reported rates of persistent TMPs in patients after long-term VT treatment ranged between 10 and 30% [27-36]. Based on this clinical observation, our research group embarked on a rat study and found that chronic TMPs staying patent for up to 10 weeks were successfully created by VT treatment only. The success rate, however, was only 20%. When VT treatment was combined with topical application of M/D (VT-M/D) the success rate at 10 weeks was increased to approximately 70%, which is a rate suitable for experimental evaluation of potential preventions or remedies [37]. We also showed that these persistent TMPs depicted the classic histological features of chronic TMPs as described in the literature [6].
Acute TMPs have been investigated for healing mechanisms at histological [38-42] and genetic levels [43, 44], however, the mechanisms involved in delayed or arrested healing of a TMP leading to the formation of chronic TMPs in patients are still not clear. Why do certain TMPs close spontaneously yet others stay patent as chronic TMPs? Twenty years ago, a pioneering study by Spandow et al. [45] investigated the perforation edges histologically in chronic TMP from 25 patients selected for myringoplasty. Nevertheless, further experimental mechanistic studies on the formation of chronic TMPs have not yet been described in the literature.

The aim of this study was to evaluate longitudinally the histological development of chronic TMPs in a rat model utilizing VT-M/D at various time points to assess the underlying mechanisms in the progressive development of chronicity, in comparison with normal controls and acute TMPs.
2. Materials and methods

2.1. Animals

Fifty male Sprague-Dawley rats, weighing 250–300 g, were obtained from the Animal Resources Centre (Murdoch, Western Australia, Australia). Experiments were approved by the Animal Ethics Committee of The University of Western Australia (No. 100/1239). Experiments were performed in accordance with the National Health and Medical Research Council of Australia Code of Practice for Care and Use of Animals for Scientific Purposes. Rats were maintained in a room with twelve-hour light/dark cycles and provided with food and water *ad libitum*.

2.2. Materials

Fluoroplastic Collar Bobbin VTs were utilized with an inner diameter of 0.75 mm (Olympus, Australia; Fig. 1A). Five mg of Mitomycin C (Sigma-Aldrich, USA) was dissolved in sterile water to a concentration of 0.5 mg/ml. Dexamethasone solution (5 mg/ml) was purchased from Ilium, Australia. Gelfoam® was purchased from Ethicon Inc (Somerville, USA). Microsurgical instruments (Karl Storz Ltd., Germany) were autoclaved routinely before use. The otomicroscope was Stativ S3 from Zeiss (Sydney, Australia) and the digital video-otoscope was from MedRX (USA).

2.3. Experimental design

A total of fifty rats were assigned to three treatment groups: a normal control without any intervention (n = 5), an acute TMP group (n = 5) (i.e. day 3 post-myringotomy) and a VT-M/D group (n = 40). Only the right TM of each animal underwent a procedure while the left TM was untouched. Post-operatively, all ears were observed with otoscopy regularly. The normal control group animals were sacrificed on day 0 while the acute TMP group animals were sacrificed on day 3 post-myringotomy for histological and immunohistochemical evaluations. In the VT-M/D group, three to four animals with
patent TMPs were sacrificed longitudinally at each of the various time points – 14 and 17 days, 3, 4, 6, 8 and 10 weeks. Animals with closed TMPs on routine otoscopy at each of various time points were excluded from the study.

2.4. Surgical procedure

Before commencement of experiments, both ears were examined with an otomicroscope to exclude middle ear disease. Debris from the external auditory canal was removed when needed. The animals were put under general anesthesia with isoflurane (Bomac, New Zealand) (4% induction, 2% maintenance in 100% oxygen) throughout all surgical procedures. Unilateral right side myringotomy was performed via a transcanal approach using a Wullstein needle. The posterior half of the pars tensa was perforated to an approximate diameter of 0.8 mm, gauged using the tip of the Wullstein needle. The primary author (AYW) performed all surgeries to ensure consistency.

2.4.1. Normal control group

The TMs underwent no intervention at all and animals were sacrificed on day 0.

2.4.2. Acute TMP group

After myringotomy, there was no further intervention (Gelfoam was not applied to the TMP), allowing the TMPs to start spontaneous healing. The animals were sacrificed on day 3 post-myringotomy.

2.4.3. VT-M/D group (ventilation tube and mitomycin C/dexamethasone)

Immediately following myringotomy on day 0 (Fig. 1B), a small piece of Gelfoam soaked with mitomycin C (0.5 mg/ml) was placed over the TMP for ten minutes and then removed. Next, a second small piece of Gelfoam soaked with dexamethasone (5 mg/ml) was placed over TMP for ten minutes and then removed. Immediately thereafter, a VT was inserted into the TMP (Fig. 1C) using a Wullstein needle. On day 14, immediately after removal of the VT (Fig. 1D), a second dose of M/D was topically applied.
on TMP according to the same protocol as described above. The animals were sacrificed at various time points - 14 and 17 days, 3, 4, 6, 8 and 10 weeks.

2.5. Otoscopy

All rats underwent regular otoscopic observation under general anesthesia postoperatively until the time of sacrifice using an otomicroscope and a digital video-otoscope. The TMPs were judged as either closed or patent. Digital images were recorded using Aurisview software (Ear Science Institute Australia, Australia).

2.6. Tissue harvest

Animals were euthanized by intraperitoneal pentobarbitone injection (160 mg/kg). After sacrifice, the external ears were separated at the osteocartilaginous junction, and the TMs along with the malleus and the bony annulus were removed from the tympanic bulla. The harvested specimens were fixed in 10% neutral buffered formalin for 24 h, followed by decalcification in 14% ethylenediaminetetraacetic acid solution (pH 7.2) at 40°C. The decalcified TMs were dehydrated in a series of graded alcohols, embedded in paraffin wax and transversely sectioned at a thickness of 5 µm.

2.7. Histological evaluation

The sections were stained with hematoxylin and eosin (H&E) for light microscopy and digitally scanned using an Aperio ScanScope XT automated slide scanner (Aperio Technologies Inc., CA).

2.8. Histochemistry
2.8.1. Pancytokeratin, CD68 and Ki67 immunohistochemistry

Following rehydration, sections were treated in 3% H₂O₂ in methanol for five min and Rodent Block R (Biocare Medical, USA) for 30 minutes at room temperature (RT). The sections were then incubated with mouse anti-pancytokeratin antibody (Biocare Medical, USA; 1:100) at RT for 1 h or mouse anti-CD68 antibody (AbD Serotec, UK; 1:100) at RT for 2 h or anti-Ki67 antibody (Biocare Medical, California, USA; 1:100) at RT overnight to detect cytokerains, macrophages and cellular proliferation respectively. A secondary antibody, Mouse-On-Rodent HRP-polymer (Biocare Medical, USA) was applied to the sections for 30 minutes at RT. Diaminobenzidine was applied for up to ten minutes, followed by counterstaining with hematoxylin.

2.8.2. Picrosirius red stain

Picrosirius Red Stain Kit (Polysciences, USA) was used to stain collagen I and III according to the manufacturer’s protocol.
3. Results

3.1. Otoscopic observation

Table 1 illustrates number of patent and closed TMPs of different longitudinal groups. The otoscopic findings were consistent between the two evaluators. Figure 2, column (i) illustrates the representative otoscopic images obtained at the end of each time point. The normal TM (Fig. 2A (i)) showed an intact and transparent pars tensa. In the acute TMP group, harvested on day 3 post-myringotomy (Fig. 2B (i)), the tissue surrounding the TMP appeared inflamed but the TMP size appeared smaller/reduced, indicative of a progressive healing process. In comparison, immediately after VT removal at 14 days (Fig. 2C (i)), the large TMP could be seen with mild hemorrhage and erythema around it. By contrast, in the VT-M/D group harvested at 17 days to 10 weeks post-myringotomy (Fig. 2D-I (i)), no signs of local infection such as erythema or discharge was observed.

3.2. Histologic evaluation

3.2.1. Hematoxylin and eosin examination

The normal control TMs had a thin, trilaminar pars tensa membrane (Fig. 2A (ii & iii)). Increased neutrophilic staining was observed at 3, 14 and 17 days (Fig.2B, C & D (ii & iii)). Moderately increases in thickness of the epidermal layer between the vicinity of the handle of the malleus and the perforation could be seen at day 3 and 14 days (Fig.2B, C (ii & iii), but not rimming around the edge. The later time points of 3 to 10 weeks (Fig. 2E-I (ii & iii) showed significantly thickened lamina propria with prominent macrophage infiltration. Furthermore, the stratified squamous epithelium continued from the lateral surface growing around the perforation edge to join with the mucosal layer of TM on the medial side. In column 2 for 8-week, Fig. 2H (ii), the position of '*' indicates a clot/debris sitting right between the perforation gap which is separate entity on high power Fig. 2H (iii).

3.2.2. Squamous epithelial layer and keratin accumulation (pancytokeratin stain)

The squamous epithelial layer in the normal TM displayed a uniformly thin keratin deposition (Fig. 3A (i)). The perforation edges at 3, 14, 17 days (Fig.3B. C & D (i)) showed a thickened epithelial
layer that eventually terminated in the vicinity of the handle of the malleus rather than rimming around the edges. From 3 to 10 weeks (Fig. E-I (i)), an accentuated epithelial layer thickness was seen rimming around the perforation edges with increased intensity of keratinocyte activity.

3.2.3. **Collagen deposition (picrosirius red stain)**

The perforation edges at the earlier time points of 3, 14 and 17 days, 3 weeks (Fig. 3B-E (ii)) showed mildly increased collagen deposition in the fibrous layer close to the handle of malleus, as compared to normal TM (Fig. 3A (ii)). From 4 to 10 weeks (Fig. F-I (ii)) there was a dramatic increase of collagen deposition in the lamina propria that appeared structurally disorganized and disrupted. The deposition tended to increase somewhat from 4 weeks onwards.

3.2.4. **Macrophage infiltration (CD68 stain)**

The perforation edges at the earlier time points of 3, 14 and 17 days, 3 weeks (Fig. 4B-D (i)) showed a mild degree of macrophage infiltration surrounding the handle of the malleus. At the later time points of 4 to 10 weeks (Fig. 4F-I (i)), the perforation edges showed a dramatic increase in CD68 positive macrophage infiltration in a granulomatous pattern within the lamina propria at the perforation edges.

3.2.5. **Cellular proliferation (Ki67 stain)**

The earlier time points of 3, 14 and 17 days, 3 and 4 weeks (Fig. 4B-F (ii)) showed raised cellular proliferations in the perforation edges in both the epithelial and fibrous layers. In comparison, the perforation edges at later time points from 6 weeks onwards (Fig. G-I (ii)) showed an overall reduction in cellular proliferation in both the epithelial and fibrous layers. In a more close-up view, compared with the increased cellular proliferation in the acute phase of 3 and 17 days (Fig. 5A, B), the chronic phase at 8 and 10 weeks (Fig. 5C, D) showed an obvious reduction in cellular proliferation throughout.
4. Discussion

In 1965, Taylor and McMinn [46] stated “further work is needed to see whether chronic perforations can be produced experimentally, so that the cell behaviour at the margins of acute and chronic lesions can be compared.” Fifty years have passed and the mechanisms resulting in persistent patency of chronic TMPs remain poorly understood. We recently developed this chronic TMP rat model [37] as a valuable experimental platform for testing new remedies for chronic TMP. Here we used it to study mechanisms of TMP chronicity.

The wound healing pathway is a complex multi-scale hierarchical system [47] that involves complex interplays between numerous cell types (e.g. epithelia, fibroblasts, neutrophils, macrophages), intercellular messengers (e.g. cytokines, chemokines, hormones, growth factors), synthesized matrix products (e.g. collagen, proteoglycans), and enzymes (e.g. matrix metalloproteinases, plasmin). However, it remains unclear why some TMPs escape the normal rapid healing seen in the majority of acute TMPs and turn chronic. Presumably, the chronic TMPs develop as a result of defective regulation of one or more of the complex molecular and biological events that occur in acute healing.

The current model consists of three key elements - (1) 14-day (2 weeks) VT insertion and topical applications of (2) mitomycin C and (3) dexamethasone. The 14-day duration of VT insertion is most likely crucial in inducing chronicity because it prolongs the patency of the TMP beyond the initial acute healing ‘window’ of 5 to 10 days, a time interval well known for acute TMPs to spontaneously heal in humans [1] and rodents [38]. The VT insertion may also have disrupted the anatomy of the healing TMP by misdirecting epithelial growth medially into the middle ear rather than across the perforation to bridge the defect. Our previous study showed that a 14-day VT insertion alone was capable of creating chronic TMPs with similar histological features but at a lower success rate [37]. Mitomycin C is an alkylating anti-neoplastic antibiotic which most likely acts by interrupting DNA replication and mitosis [48], leading to inhibition in the proliferation of fibroblasts and epithelial cells. This application may explain the relatively sparse Ki67 staining in the outer epithelial layer in the studied group soon after VT removal.

Dexamethasone is a long-acting steroid that may disrupt the healing at the TMP edge by immunosuppression, delaying macrophage chemotaxis and cellular proliferation [49].
For the acute TMP group, we have selected 3 days as the timeline to sacrifice the animal. An acute TMP group needs to be in the situation of an ongoing healing process before closure. Acute TMPs in rats almost all closed by 7 days [38]. Thus, the acute time frame has to be less than 7 days. We eventually decided on a 'middle' time of day 3, which it has had a few days to allow acute healing response to be activated but not too late so that it has closed. For the VT-M/D group, we decided the various time frame of sacrifice from our previous publication developing this rat model [37]. The initial 'acute phase' post VT removal is crucial, thus the initial time frame of 14 days, 17 days, 3 weeks. Thereafter, we wanted regular time frame samples between the 3 weeks and the final time point of 10 week, thus the following time frame of 4, 6, 8, 10 weeks. The study was initially designed as purely a histological descriptive experimental studies so power was not calculated. Normal control and acute TMP groups were each assigned with 5 rats. The rest of the VT-M/D groups had 40 rats in total - 7 time points (14, 17 days, 3, 4, 6, 8, 10 weeks).

In our previous study [26], a single topical application of M/D (without ventilation tube or second dose of M/D) only delayed TMP closure but not enough to create chronic TMPs. The healed TMs at 8-week histologically showed a mild thickening in the lamina propria but the outer epithelial layer had normal thickness with moderate collagen deposition. In comparison to the current study, the 8-week histology (Fig. 2H (ii & iii)) showed a stratified epithelial layer around the perforation edge with prominent thickening and significant collagen deposition in the lamina propria. The histological disparities between the two models at the same time point of 8-week strongly suggest that the VT insertion plays a crucial role in producing a chronic TMP.

The stratified squamous epithelium forming around the TMP edge is an important histological feature observed from 3 weeks onwards (Fig. 2E-I (ii & iii)). This is a classic histological characteristic of a chronic TMP that has been described in the literature [3, 6, 9, 12, 37, 45, 50, 51]. In acute TMP healing the epithelium would migrate across the perforation edge to bridge the defect, whereas in a non-healing chronic TMP, a stratified epithelial rim forms around the perforation edge which seems to act as a structural barrier impeding the migration of keratinocytes [6, 52]. The cessation of keratinocyte migration seen in chronic TMPs could be due to contact inhibition. It is defined as a reduction in mitotic rate and/or
migration when epithelial cells at a wound healing front contact each other to form a monolayer [53].

When epithelial cells reach still higher densities, crowding can promote anoikis or cell death by loss of adhesion-based cell-survival signaling. Other potential contributing factors to the crowding are the physical barrier created by the VT insertion as well as the anti-proliferative effect of M/D.

Increased collagen deposition with disorganized morphology of the TMP edges was seen at the later time frames toward chronicity (Fig. 3F-I (ii)). Disordered and misoriented collagen seen within the residual lamina propria of chronic TMP edges appeared to be a form of scarring which is different from the subsequent collagen deposition of new lamina that usually occurs in healing of acute TMPs, although previous studies have shown that scar tissue after closure of acute TMP may prevail for a long time [54, 55]. In the current study, collagen deposition started to dramatically increase between 4 and 10 weeks, suggestive of increased collagen production by the fibroblasts causing excessive scar formation as part of the ‘becoming chronic’ process of TMPs. Similar findings of extensive fibrosis were exhibited in the histological study by Spandow et al. [45] of the edges of chronic TMPs in patients, where extensive fibrosis was seen. Studies of chronic skin wounds have shown that fibroblasts are altered [56] such that they exhibit premature senescence that disrupts normal functioning, with impaired migration capacity and reduced response to growth factors. Li et al. [57] described a chronic inflammatory response involving fibroblasts being attracted by macrophages and over time leading to an increase production of collagen and fibrosis that results in an altered structure and loss of function.

In this study, increased level of macrophages were observed in the later time points of 4 to 10 weeks (Fig. 4F-I (i)), with the unique granulomatous pattern on CD68 staining that is absent in the acute healing phase, indicating that a sustained chronic inflammation and infective process (e.g. bacterial colonization) within the perforation edge is likely to play a role in the failed healing of chronic TMPs. The abundant macrophage infiltration seen could be responsible for the chronic inflammation of non-healing chronic TMPs. A persistent chronic TMP is possibly locked into a state of chronic inflammation characterized by inflammatory cells such as macrophages and neutrophil infiltrations with associated reactive oxygen species and destructive enzymes [58].

The TMPs from the early acute healing time points (i.e. 3–17 days) have increased cellular
proliferation (Fig. 5A, B). In comparison, at the later time points of 6 to 10 weeks, there was an overall reduction in the level of cellular proliferation within the perforation edges (Fig. 5C, D) suggestive of an ‘under-healing’ response involving impairments in keratinocyte proliferation and migration leading to the chronicity of the TMP. For a chronic wound to heal, it needs to be forced to leave the chronic the inflammatory phase, enter a proliferative phase and eventually enter a remodeling stage. Due to the excessive amount of inflammation, it is possible that the chronic TMPs have failed to progress from inflammatory phase to a predominantly proliferative phase [47]. The anti-mitotic effect of mitomycin C and the anti-proliferative effect of dexamethasone might also play an important role.

Overall, our histological results can categorize the progress of development of chronic TMP into three chronological phases illustrated by Figure 6 - (1) the acute healing phase (3, 14 and 17 days), (2) the ‘transition’/delayed healing phase (3 to 4 weeks) and (3) the chronic phase (6 weeks and onwards). The acute healing phase demonstrated an expected active healing process including inflammation, proliferation and re-epithelialization. The 3-to-4-week time frame appeared to be a ‘transition’/delayed healing phase where acute healing started to gradually transform into chronicity with a steady increase in keratinocyte layer thickening, collagen deposition and macrophage infiltration as well as a reduction in cellular proliferation of the middle fibrous layer. From 6 weeks onwards, the histological features are suggestive of becoming chronic, indicating that the healing of TMP edges has been halted with a dramatic increase in keratinocyte thickness, disorganized collagen production, granulomatous macrophage pattern and overall ‘shutting down’ of cellular proliferative activities.

While our current study found several chronological features of chronic TMP development, it is not without limitations. Firstly, the VT insertion method only mimic one of the causes of chronic TMP in clinical settings (i.e. long term VTs), but not other possible causes including Eustachian tube dysfunction and chronic middle ear infection. Nevertheless, valuable information can still be obtained utilizing this model for studies on the genesis of TMP chronicity. A second limitation is the solely histological point of view in the investigation. Santa Maria et.al [43] have identified a few potential markers of acute TMP healing such as Stefin A2 and Natriuretic peptide precursor type B. Therefore, further studies would be valuable to determine whether these genes are involved in chronic TMP development and to identify...
novel molecular markers. This would assist in the development of innovative therapies to improve the healing process of chronic TMPs.
5. Conclusion

Based on the results of this study, progressive development of chronic TMPs appeared to be associated with various histological features. The formation of stratified squamous epithelium and thickened keratinocyte layer around the chronic TMP edges may act as a structural barrier to closure. The increased collagen deposition is indicative of excessive scar formation and fibrosis. The increased macrophage infiltration is suggestive of a chronic inflammatory response while the reduction in cellular proliferation portrays an overall slowing down of keratinocyte activities.

Allen Y. Wang and Lawrence J Liew are co-first authors

Conflicts of interest

The authors have no conflict of interest to disclose.

Acknowledgments

The authors wish to thank the animal house staff (Carmel McLeod, Neill Wilson, Sandra Goodin) and Karl Storz Ltd. for the sponsorship and supply of microsurgical instruments.

Funding

This study has been funded by a scholarship to AYW from The University of Western Australia; by the Garnett Passe and Rodney Williams Memorial Foundation and by the National Health and Medical Research Council of Australia (1056589). MvU was supported by the Raine Medical Research Foundation Visiting Professorship.
References


perforations heal without significant loss of strength, Otol Neurotol. 26 (2005) 1100–1106.


**Figure legends**

**Fig. 1.** (A) Photo of the Collar Bobbin ventilation tube, made of fluoroplastic, manufactured by Olympus, with an inner diameter of 0.75 mm. Otoscope images of a rat TM immediate post-myringotomy on day 0 (B), ventilation tube inserted into the TMP on day 0 (C) and immediately after removal of VT on day 14 (D).

**Fig. 2.** Representative otoscope images (i) and corresponding H&E histology images (ii & iii) of selected rat TMP from the nine different time points. A. Normal TM; B. 3-day TMP; C. 14-day TMP; D. 17-day TMP; E. 3-week TMP; F. 4-week TMP; G. 6-week TMP; H. 8-week TMP; I. 10-week TMP. All images in (iii) are actual magnified images of the black boxes in (ii). Scale bars: 600 µm at 4× magnification and 200 µm at 20× magnification. White asterisks on otoscope images indicate the actual TMP sites. Black asterisks on H&E sections indicate cross-sectional perforation defects. The black boxes correlate locations of the higher 20× magnification in third column. H&E = Hematoxylin and eosin staining. TMP = tympanic membrane perforation; VT-M/D = ventilation tube insertion in conjunction with mitomycin C & dexamethasone, MEC = middle ear cavity; TM = tympanic membrane; M = handle of malleus; EAC = external auditory canal.

**Fig. 3.** Representative pancytokeratin (i) and picrosirius red (ii) histology images of rat TMP at different time points. A. Normal TM; B. 3-day TMP; C. 14-day TMP; D. 17-day TMP; E. 3-week TMP; F. 4-week TMP; G. 6-week TMP; H. 8-week TMP; I. 10-week TMP. Scale bars: 200 µm at 20× magnification. TMP = tympanic membrane perforation; VT-M/D = ventilation tube insertion in conjunction with mitomycin C & dexamethasone.

**Fig. 4.** Representative CD68 (i) and Ki67 (ii) histological images of rat TMP at different time points.
A. Normal TM; B. 3-day TMP; C. 14-day TMP; D. 17-day TMP; E. 3-week TMP; F. 4-week TMP; G. 6-week TMP; H. 8-week TMP; I. 10-week TMP. Scale bars: 200 µm at 20× magnification. TMP = tympanic membrane perforation; VT-M/D = ventilation tube insertion in conjunction with mitomycin C & dexamethasone.

**Fig. 5.** Ki67 immunohistochemical staining of the TMPs from 3 and 17 days (acute phase) and 8 and 10 weeks (chronic phase) from top to down (A, B, C, D, Scale bars: 200 µm at 20× magnification). High levels of cellular proliferation are shown in stratified epithelial layer in 3 and 17 days (black arrowhead). In comparison, reduced level of cellular proliferation can be seen in 8 and 10 weeks. TMP = tympanic membrane perforation; VT-M/D = ventilation tube insertion in conjunction with mitomycin C and dexamethasone.

**Fig. 6.** Flow diagram illustrating the concept of the 3 chronological phases in the development of chronic tympanic membrane perforations.
### Table 1

Number of patent and closed tympanic membrane perforations of different experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total No. of animal (N = 50)</th>
<th>No. of TMP patent (N = 42)</th>
<th>No. of TMP closed (N = 8)</th>
<th>Proportion of patent TMP at each time point (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Acute TMP (3 days)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>14 days (VT-M/D)</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>17 days (VT-M/D)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>3 weeks (VT-M/D)</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>71</td>
</tr>
<tr>
<td>4 weeks (VT-M/D)</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>71</td>
</tr>
<tr>
<td>6 weeks (VT-M/D)</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>83</td>
</tr>
<tr>
<td>8 weeks (VT-M/D)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>10 weeks (VT-M/D)</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80</td>
</tr>
</tbody>
</table>

TMP – tympanic membrane perforations; VT-M/D – ventilation tube in conjunction with mitomycin C/dexamethasone
Figure(s)
Click here to download high resolution image
Acute healing phase
(3, 14, 17 days)

Transition / delayed healing phase
(3–4 weeks)

Chronic phase
(6, 8, 10 weeks & onwards)