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Myringotomy in the *Junbo* mouse model of chronic otitis media alleviates inflammation and cellular hypoxia

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Nothing to declare

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Abstract

Objective

Ventilation of the chronically inflamed middle ear is a key outcome in functional middle ear surgery. Grommets eliminate middle ear effusion, but there is also evidence that they downregulate inflammation. The reason for this is not understood, but there is little to suggest alteration in Eustachian tube ventilatory capacity. Previous work has shown that the *Junbo* mouse model of chronic otitis media has hypoxic middle ear mucosa and bulla fluid leucocytes. Here we explore if surgical ventilation may alleviate chronic otitis media through downregulation of hypoxia.

Study Design

Surgical intervention on a mouse model of disease.

Methods

We established patency of myringotomy incision as 5 days in wild-type mice. We performed unilateral myringotomy on three cohorts of mice: 10 wild type controls, 12 *Junbo* mice, and 15 *Junbo* mice with additional removal of middle ear effusion. A small cohort of these mice were labeled in-vivo by intraperitoneal injection of pimodinazole to identify tissue hypoxia. Tissues were assessed for mucoperiosteal thickening and pimodinazole labeling, comparing operated to non-operated ears.

Results

Ventilation of the inflamed *Junbo* middle ear revealed significant reduction in inflammatory thickening associated with loss of pimodinazole labeling, suggesting resolution of cellular hypoxia.

Conclusion

Surgical ventilation may achieve therapeutic effect through alleviation of cellular hypoxia in the chronically inflamed middle ear. Targeted molecular therapy of hypoxia signaling may offer future alternative therapy for chronic OM.

Key words:

Otitis media
Grommets
Ventilation
Hypoxia
Junbo mouse model

Level of Evidence

N/A

Introduction

The middle ear evolved when our evolutionary predecessors moved from an aquatic to a terrestrial existence. The development of a gas-filled pocket next to the cochlea overcame air-fluid impedance mismatch, and so enabled audition of air-borne sound¹. In mammals maintenance of this gas pocket is primarily achieved through gaseous exchange across the mucosal epithelium of the postero-superior middle ear cleft². The Eustachian tube can also help to equilibrate gross pressure alterations, but likely plays a small role in minute-to-minute ventilation of the middle ear³.

Chronic middle ear inflammation can lead to sub-atmospheric middle ear pressure, tympanic membrane retraction, and/or middle ear effusion, and thus disable the middle ear gas pocket. Re-establishment of middle ear ventilation is critical to the success of functional middle ear surgery⁴⁻⁶. The most common operation for restoration of hearing is the insertion of a grommet (ventilation tube), which reliably eliminates effusion in glue ear (chronic otitis media with effusion, COME). The therapeutic effect of this procedure is thought to be rheological, whereby creation of a vent reduces fluid inertia, allowing effusion to be cleared down the Eustachian tube by the ciliary apparatus of the protympanum. Aspiration of effusion at the time of grommet insertion is not necessary⁷.

However, a number of lines of evidence suggest that grommets moderate the inflammatory process in addition to, or in place of, any rheological effect that may occur. Performing myringotomy and aspiration without intubation of the tympanic membrane leads to a rapid reaccumulation of effusion in children with COME⁸. Recurrence of effusion still occurs in 20-30% of children once grommets extrude⁹⁻¹², but retrospective case series suggest that the risk of recurrence is inversely related to the duration of ventilation^{13 14}. There is also

endoscopic¹⁵ and histological^{16 17} evidence that resolution of middle ear inflammation is proportional to the duration of ventilation.

We have previously suggested that some, or even the main therapeutic effect of middle ear ventilation may be through alleviation of tissue hypoxia¹⁸. Hypoxia is a common finding in chronically inflamed microenvironments^{19 20}, coordinated through the transcription factor hypoxia inducible factor (HIF). Our group has explored the role of tissue hypoxia in the chronically inflamed middle ear by exploitation of the genetically altered *Junbo*²¹ and *Jeff*²² mouse models of chronic otitis media¹⁸. We have shown that systemic administration of pimodiazole (PIMO, a marker of tissues with an oxygen tension below 10 torr²³) labels leucocytes in the exudate of the inflamed middle ear of both *Junbo* and *Jeff* mice, and also the middle ear mucosa in the *Junbo* mouse. Fluorescence-activated cell analysis confirms hypoxia in viable and apoptotic polymorphonuclear cells in the effusion, and transcriptome and proteome analysis of effusion reveals upregulation of a number of HIF responsive genes, notably in the signaling protein vascular endothelial growth factor (VEGF), a key executor of HIF response.

Here we use the *Junbo* mouse model to investigate our hypothesis that the medium to long-term beneficial effect of middle ear ventilation may be through alleviation of cellular hypoxia in the chronically inflamed middle ear. Specifically, we demonstrate that surgical myringotomy leads to reduction of mucoperiosteal inflammatory thickening, and loss of PIMO labelling in operated ears.

Materials and Methods

Mice

Wild type (WT +/+) and heterozygote *Junbo* (*Jbo*/+) mice (hereafter referred to as *Junbo* mice) on a congenic C3H/HeH background were generated and maintained as previously described. *Junbo* phenotype is characterised by the spontaneous development of chronic inflammatory disease that is anatomically restricted to the ME¹⁸.

Establishing duration of myringotomy patency in the mouse

Surgical myringotomy has not previously been reported in the mouse. We performed myringotomy on WT C3H/HeH mice (aged 6-9 weeks) to establish normal duration of patency. Mice were anaesthetized by intraperitoneal injection of 10mg/kg xylazine and 100mg/kg ketamine. The posterior pars tensa of the left ear was incised using a disposable myringotome (Exmoor plastics, UK) under direct vision with an operating microscope. We always operated on the left ear because this was technically easier for our right-handed surgeon. Anaesthetic was reversed with 5mg/kg atipamezole hydrochloride. Mice were subsequently euthanized with an overdose of intraperitoneal barbiturate at time points of 3, 5, 7, and 10 days post-operatively (three mice for each time point). The tympanic membranes were assessed using a binocular microscope.

Myringotomy in three mouse cohorts

We subsequently operated on the left ear of three cohorts of mice, all aged 6-10 wks-old:

- a) 10 WT controls who underwent myringotomy only
- b) 12 *Junbo* mice who underwent myringotomy only
- c) 15 *Junbo* mice who underwent myringotomy and removal of effusion using absorbent endodontic paper points.

The right ear was left unoperated to act as a control. Prior to operation all *Junbo* mice were assessed for bilateral visible evidence of tympanic membrane opacity, which we have previously shown to be a reliable marker of inflammatory effusion in the murine middle ear²⁴.

At intervals after myringotomy groups of mice were euthanized and skinned heads were fixed for 48 hours in 10% neutral buffered formalin, decalcified with Immunocal (Decal Corp) for 72 hours, and embedded in paraffin wax. 3 μ m sections were stained with haematoxylin and eosin. Middle ear mucoperiosteal thickness was measured as previously described²⁴ and compared between the operated left ear and the unoperated right ear using a paired sample t-test.

Five days after left-sided unilateral myringotomy 3 WT and 3 *Junbo* mice were labeled for 3 hours *in vivo* by i.p. injection with 60 mg/kg pimonidazole (PIMO) dissolved in 100 μ l of sterile PBS. The mice were euthanized and 4 μ m histological sections of the middle ear were immunostained using anti-PIMO rabbit polyclonal PAb2627AP primary antibody at 1:200 dilution at room temperature for 60 mins (Hypoxprobe-1 Omni Kit; hpi Hypoxyprobe Inc., Burlington MA) followed by goat-anti rabbit IgG HRP conjugate secondary antibody at 1:50 dilution at room temperature for 30 mins (Dako P0448) according to the manufacturer's instructions and with the following modifications. Antigen retrieval was by incubation at 60°C overnight in Vector high pH buffer (Vector Laboratories H-3301) to prevent detachment of tissue from the electrostatically charged slide; both primary and secondary antibodies were diluted in Dako antibody diluent (S0809); and visualisation was carried out using Liquid DAB+ (Dako K34811).

Ethical approval

Full details of these studies were reviewed and approved by MRC Harwell ethical review committee. The humane care and use of mice in this study was carried out under the authority of the appropriate UK Home Office Project License.

Accepted Article

Results

We established that myringotomy in WT mice was reliably patent at up to five days post-operatively (table 1) and used this time point in later experiments in *Junbo* mice. There were no significant complications or identifiable adverse effects from myringotomy. In each cohort data for mucoperiosteal thickness were normally distributed (Kolmogorov-Smirnov test).

In WT mice (cohort a, n=10) there was no evidence that myringotomy induced mucosal inflammation. The difference in mucoperiosteal thickness between left operated ($19.9 \pm 2.1 \mu\text{m}$; mean and standard error of mean) and right unoperated ($16 \pm 1.5 \mu\text{m}$) ears in WT mice was not significant (figure 1). There was no visible effusion in any of these ears.

In *Junbo* mice (cohort b, n=12) there was histological evidence of reduced middle ear inflammation in response to myringotomy, although effects were variable. Effusion volume was difficult to quantify precisely. The cellularity of bulla fluid varied and histological processing resulted in uneven shrinkage of bulla fluid profiles. Nevertheless the qualitative impression was of reduced effusion in the operated compared to the unoperated ear, and in many cases appeared to have resolved completely. Mucoperiosteal thickness in this cohort was less in the left operated ear ($62.1 \pm 9.1 \mu\text{m}$) than the right unoperated ear ($87.1 \pm 9.1 \mu\text{m}$), $p < 0.05$ (figure 1).

In *Junbo* mice that underwent myringotomy with fluid removal (cohort c, n=15) we again found a variable response to surgery on qualitative histological assessment. However, in the majority of cases (12/15 cases) there was no evidence of reappearance of effusion in the operated ear after bulla fluid removal at time of surgery, and there was a reduction in

inflammation, sometimes markedly so (figure 2). Mean mucoperiosteal thickness in the left operated ear was significantly less ($84.6 \pm 9.3 \mu\text{m}$) than in the right unoperated ear ($104.6 \pm 8.3 \mu\text{m}$), $p < 0.05$ (figure 1).

When data for cohort b and c were combined (i.e. all $n=27$ mice undergoing myringotomy with or without removal of effusion, analysed as a group) the mean mucoperiosteal thickening in the operated ear was significantly less ($75.0 \pm 6.9 \mu\text{m}$) compared to the unoperated ear ($97.1 \pm 6.3 \mu\text{m}$), $p < 0.003$ (figure 1).

In the three WT mice PIMO labeling was only present in the healing myringotomy site, a feature also seen in the *Junbo* mice (figure 3). Two of three *Junbo* mice showed PIMO staining for hypoxia in the unoperated ear, labeling both middle ear mucoperiosteum and bulla exudate macrophages. In both of these cases in the contralateral operated ear there was very little effusion and no PIMO labeling of the mucoperiosteum (figure 3). The other *Junbo* mouse had persistent effusion in both ears despite myringotomy, and here the macrophages in the effusion showed bilateral PIMO staining (but without PIMO labeling of the mucoperiosteum).

Discussion

Macroscopic otitis media in *Junbo* heterozygote mice is evident from the accumulation of middle ear bulla fluids resulting in tympanic membrane opacity. In ($n=54$) 8-wk-old *Junbo* mice the incidence of bilateral fluids was 78%, unilateral fluids 13% and there was no overt bulla fluid in either ear of 9% of mice¹⁸.

In the current experiments *Junbo* mice were assessed pre-operatively for the presence of bilateral fluids. The important assumptions in our experimental design were that myringotomy itself did not induce middle ear inflammation and bulla fluid effusion; that *Junbo* mice had pre-operative bilateral middle ear inflammation (and the bulla effusion was sufficiently cellular to drive middle ear hypoxia); and that myringotomy with or without fluid removal would significantly reduce bulla effusion volume and cellularity.

We have shown here that myringotomy did not itself induce middle ear bulla effusion or mucoperiosteal thickening in WT mice but there was a proliferative epithelial repair response at the site of myringotomy incision. There was a statistically significant reduction (~23%) in inflammatory thickening of the mucoperiosteum in the *Junbo* mouse five days after surgical myringotomy (with or without removal of effusion), the period in which the incision is patent. We also found that myringotomy alone resulted in a qualitative reduction in the space occupied by bulla effusion, and that myringotomy with fluid removal resulted in a 80% reduction in the occurrence of bulla fluid five days post surgery. The results of a small-scale *in vivo* labeling experiment with PIMO to identify sites of tissue and cellular hypoxia provide preliminary evidence that mucoperiosteal labeling was reduced in two of three operated *Junbo* middle ear bullae. In these cases mucoperiosteal thickening was reduced and effusion was almost entirely removed. In

the third *Junbo* mouse effusion was present in both the operated and unoperated ear, and here PIMO labeling of the effusion was present bilaterally.

These data are consistent with our hypothesis that myringotomy reduces middle ear mucoperiosteum inflammation and leads to resolution of bulla effusion, and that an important biological mechanism of middle ear ventilation may be through alleviation of cellular hypoxia in chronically inflamed middle ear tissues. However the reduction of mucoperiosteal thickening and resolution of effusion was not universal after myringotomy and it is possible that more prolonged ventilation could improve the response to surgery in the *Junbo* mouse model.

In the non-inflamed middle ear the partial pressures of oxygen, carbon dioxide, and nitrogen mirror those of venous blood, as a result of trans-mucosal gaseous exchange²⁵⁻²⁸. In our mouse models the chronically inflamed middle ear is hypoxic¹⁸ and human data also supports hypoxia pathway activation in COME, with elevated levels of VEGF reported in two studies^{29,30}.

Hypoxia is a common finding in inflamed environments. Inflammation increases cellular energy demands, but simultaneously distances inflammatory cells from blood vessels due to cellular oedema and mucosal hyperplasia and extravasation of leucocytes into the bulla lumen. Transcriptional regulation through HIF-VEGF pathways acts to compensate for the hypoxic environment, and restore tissue homeostasis to enable cellular survival under stress. However, persistent hypoxia signaling is known to be maladaptive, and can contribute to ongoing inflammation and tissue damage^{31,32}.

Grommets expose the middle ear space to the relative hyperoxia³³ of atmospheric oxygen, and this would presumably reverse tissue hypoxia, both of cells in the effusion and ultimately mucosal cells. This may reduce inflammation and hence lead to the eventual resolution of middle ear effusion. However, it is interesting to note that hypoxia pathways also have direct transcriptional activity on mucin production. The *MUC5AC* gene, which encodes one of the major mucins found in COME³⁴, contains a highly conserved HIF binding site that acts as a transcriptional promoter. Experimental disruption of this binding site abolishes stimulated mucin secretion³⁵. Middle ear ventilation may therefore downregulate hypoxia pathways, which in turn eliminates one major driver for the transcription of the mucins that are the hallmark feature of COME.

Alleviation of hypoxia may be an important adjunct to rheological effect of grommets. It is noteworthy that grommets have not been shown to affect the ventilatory function of the Eustachian tube in the short³⁶, medium^{15 37 38}, or long-term³⁹, and so a purely physical action of grommets would seem an inadequate explanation as to why prolonged ventilation affects subsequent disease severity or recurrence.

In this study we have used the *Junbo*²¹ mouse model of chronic otitis media. The *Junbo* mouse spontaneously develops a highly penetrant chronic otitis media by 28 days of age, with a neutrophil and macrophage rich effusion. *Junbo* carries a point mutation at the *Mecom* locus (also known as the *Mds1-Evi1* cluster), which may affect its interaction with TGF- β ^{40 41} JNK⁴² or NF- κ B⁴³ pathways. Mouse models have made a considerable contribution to the experimental investigation of otitis media, because of their easy husbandry and the repertoire of techniques available to manipulate their genome, leading

to the recovery of several mouse models of chronic otitis media (reviewed elsewhere^{44,45}).

However mouse models do have limitations⁴⁶, including species differences in inflammatory response, which could limit the applicability of these models to human disease.

Nevertheless, hypoxia pathway activation has been reported as a feature of human COME²⁹

³⁰, and this compels us to believe that the *Junbo* mouse model is valid for exploration of human pathobiology.

An extension of our study may be to look at the biological effects of a more extended duration of ventilation. This could be achieved by laser myringotomy⁴⁷⁻⁵⁴ or by application of mitomycin-C⁵⁵⁻⁵⁹ to the incision, but these methods probably only slightly prolong patency of myringotomy and may in themselves contribute to inflammation.

Conclusion

We have undertaken the first animal study to investigate the biological effects of ventilation in chronic otitis media. Using the *Junbo* mouse model, we have shown that surgical ventilation reduces inflammatory thickening of the middle ear mucoperiosteum, and that this may be due to alleviation of tissue hypoxia in the middle ear. Induction of chronic otitis media in larger species through bacterial challenge or genetic engineering may enable our studies to be repeated and extended to include intubation of the tympanic membrane.

Grommets are the only treatment known to reliably lead to resolution of effusion in COME¹¹.

If, as we propose, their therapeutic effect is through alleviation of tissue hypoxia, it suggests that in the future their therapeutic benefit could be replaced with targeted molecular therapy based on hypoxia pathways. Indeed, targeting hypoxia pathways with VEGF

receptor inhibitors moderates hearing loss in our mouse models of chronic otitis media¹⁸.

The findings presented here suggest this may be a fruitful avenue to pursue in man, in place of surgical ventilation.

Accepted Article

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Table 1:

Macroscopic patency of myringotomy in WT mice up to 10 days after incision. Unilateral surgery was performed on three mice for each time-point of two, five seven, and ten days post surgery.

Days post myringotomy	Proportion patent
2	3/3
5	3/3
7	1/3
10	0/3

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Figure 1:

Mucoperiosteal thickness in the operated (myringotomy) and non-operated ears of the mice: WT mice (cohort a), *Junbo* mice (cohort b) and *Junbo* mice treated with myringotomy and removal of effusion (cohort c). p-values refer to a paired sample t-test. ns = not significant.

Figure 2:

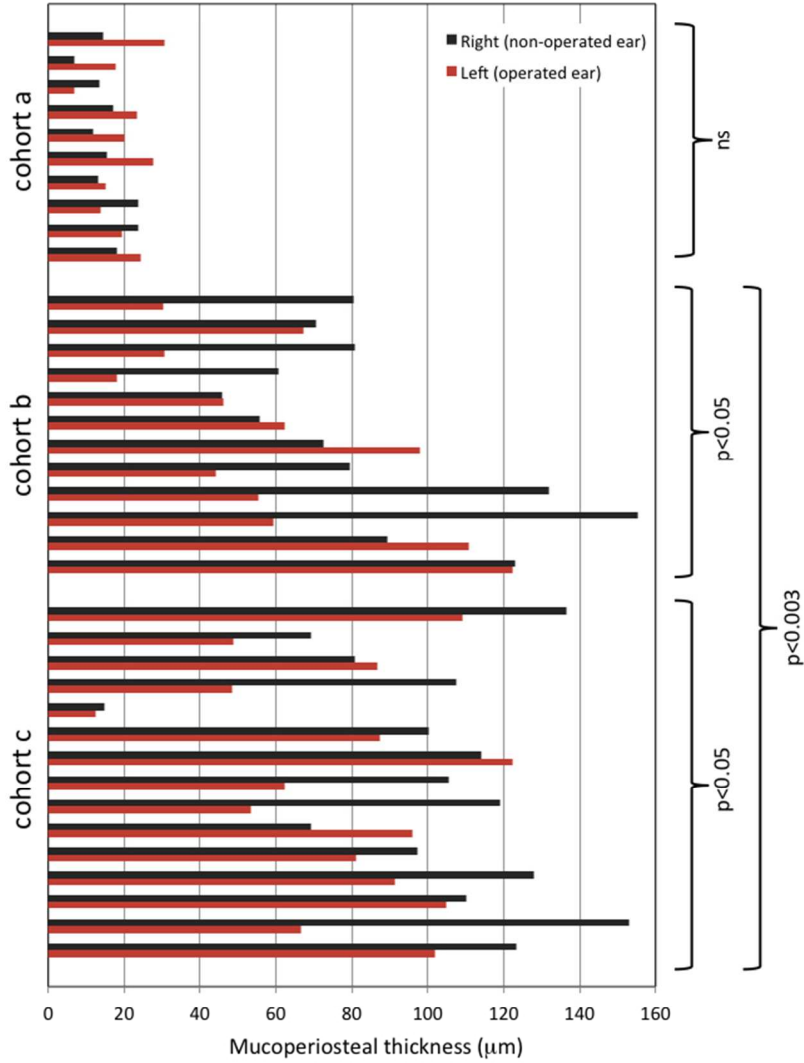
The histology of the middle ear of *Junbo* mice five days post myringotomy and removal of the bulla effusion.

(A) In the majority of cases there is minimal recurrence of effusion (e); (B) in a minority of cases myringotomy did not effectively reduce bulla effusion. Note thickening of tympanic membrane results from epithelial and stromal hyperplasia at the site of surgical myringotomy (s). A and B scale bar = 500 μ m

Figure 3:

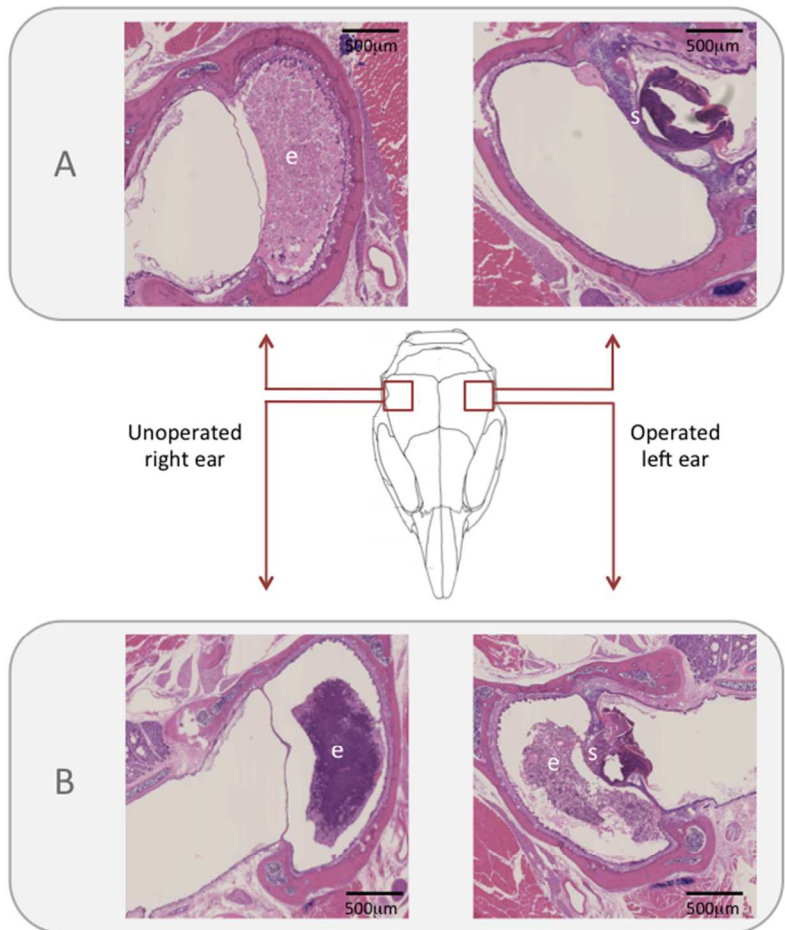
Hypoxia labeling with PIMO of the middle ear in the *Junbo* mouse is reduced five days post myringotomy and removal of effusion.

(A and C) In two *Junbo* mice the operated ear has much reduced effusion (e) and reduced inflammatory thickening of the mucoperiosteum (m) compared to (B and D) the corresponding contralateral unoperated ear where both the mucoperiosteum and leucocytes within the effusion are labeled with the hypoxia marker PIMO (arrows indicate cells with brown DAB staining). (E and F) The healing surgical myringotomy site is labeled with PIMO in both WT (E) and (F) *Junbo* mice. A-D scale bar = 100 μ m; E and F scale bar = 200 μ m.



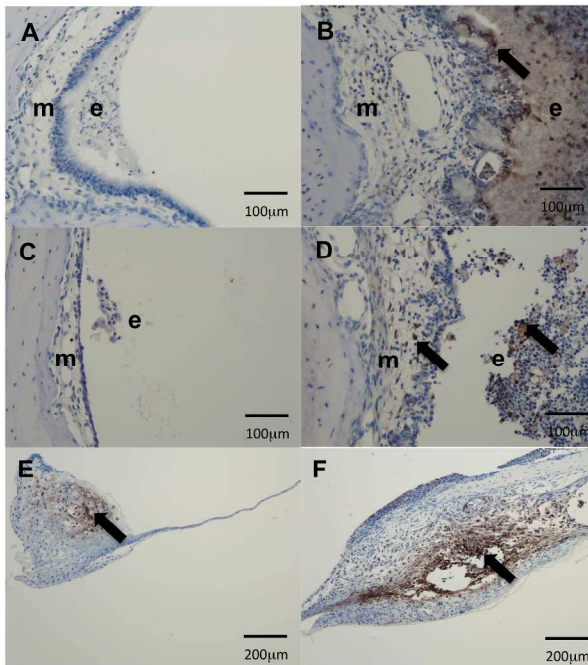
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