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A Melting Method for RNA Extraction from the Mucosal Membrane of the Mouse Middle Ear

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Purpose: There is much confusion surrounding the methods of RNA extraction from the middle ear mucosa of mice. In this study, we worked to develop a "melting method," which is faster, purer, and more reliable than other methods in common use. **Materials and Methods:** Thirty-two ears were used for this study. Light microscopy with hematoxylin-eosin staining of the bullae, scanning electron microscopy (SEM), spectrophotometer analysis, and reverse transcription polymerase chain reaction were performed before and after melting the half lateral bullae, which were detached from the temporal bone by using a lateral retroauricular approach. **Results:** Each resected half bulla contained a well distributed mucosal membrane. After a TRIzol melting duration of 10–30 minutes, only mucosal marker (MUC5AC) was expressed without bony marker (total osteocalcin). The same results were determined from SEM. **Conclusion:** This melting method, compared with stripping and irrigation methods, is effective and offers an easier, more robust approach to extracting RNA from the middle ear mucosal membranes of mice.

Key Words: Melting, RNA extraction, middle ear mucosa, mouse

INTRODUCTION

Otolaryngologic research commonly involves animals as experimental models, especially guinea pigs and mice.[1] Knowledge about the middle ear anatomy of these animals and methods for performing animal-based experiments have become valuable for such research. Anatomical descriptions of these animals' ear structures, found in veterinary anatomy textbooks, are scarce and merely schematic; they also do not go beyond a rough enumeration of the structures, often with no data on topography and variability.[2] With few publications dealing with the anatomy of the mouse middle ear, many important experiments at the RNA level are performed with unreliable methods, such as stripping and washing. In our studies, we have discovered a "melting method," which is faster, purer, and more reliable than other methods in common use.

MATERIALS AND METHODS

Thirty-two ears (16 left and 16 right, 9 male and 7 female specimens) derived

from 16 white mice (kist; ICR, Boston, USA), weighting about 150–200 mg, were used for this study. Animals were handled in accordance with Institutional Animal Care and Use Committee-approved protocol. To control for variations in surgical technique, all procedures were performed by only one surgeon. Each animal was deeply anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg) and then positioned on a wooden block on its side (lateral ventral approach).[2]

Light microscopy

Six bullae (middle ears) were completely dissected from the temporal bone to locate the mucosal membrane in each bulla and to compare locations. It was possible to harvest them easily and completely. Subsequently, temporal bones were fixed overnight at 4°C in a buffered solution of paraformaldehyde (Merck ultra pure 4005, Germany). Thereafter, temporal bones were decalcified for 5 days at 4°C in a Tris-hydrochloride buffer (0.1 mol/L, pH 7.4) (Merck), ethylenediamine tetra-acetic acid (EDTA) (10% weight/volume) (Merck), and polyvinylpyrrolidone (7.5%) (Serva, Heidelberg, Germany).[3] Decalcified bones were rinsed in the same buffer, except without EDTA (4 hours, 4°C). Each specimen was then embedded in paraffin and cut according to the standard histologic technique and stained with hematoxylin and eosin.

Melting method

To justify the melting method, 26 bullae were extracted from the mice repeatedly using the same method. Under sterile conditions, a postauricular incision measuring 3 cm long was made in the skin and extended ventrally to the rostral neck skin until the subcutaneous connective tissues were separated to expose the deep structures (Fig. 1). Access to the tympanic bulla was achieved by means of blunt dissection of the digastric muscles. If the digastric muscle was dissected carefully along the lateral surface of the bulla, the soft tissues around it were separated to expose the half portion (lateral wall) of the bulla clearly. After the bulla was exposed, the external auditory canal with the tympanic membrane was separated from the bulla in order to see the inside of bulla cavity. With sharp microscopic scissors, the exposed bulla was cut anterolaterally as one piece.

To collect an adequate amount of RNA from the bulla, we gathered two pieces of tissue into one tube (except the

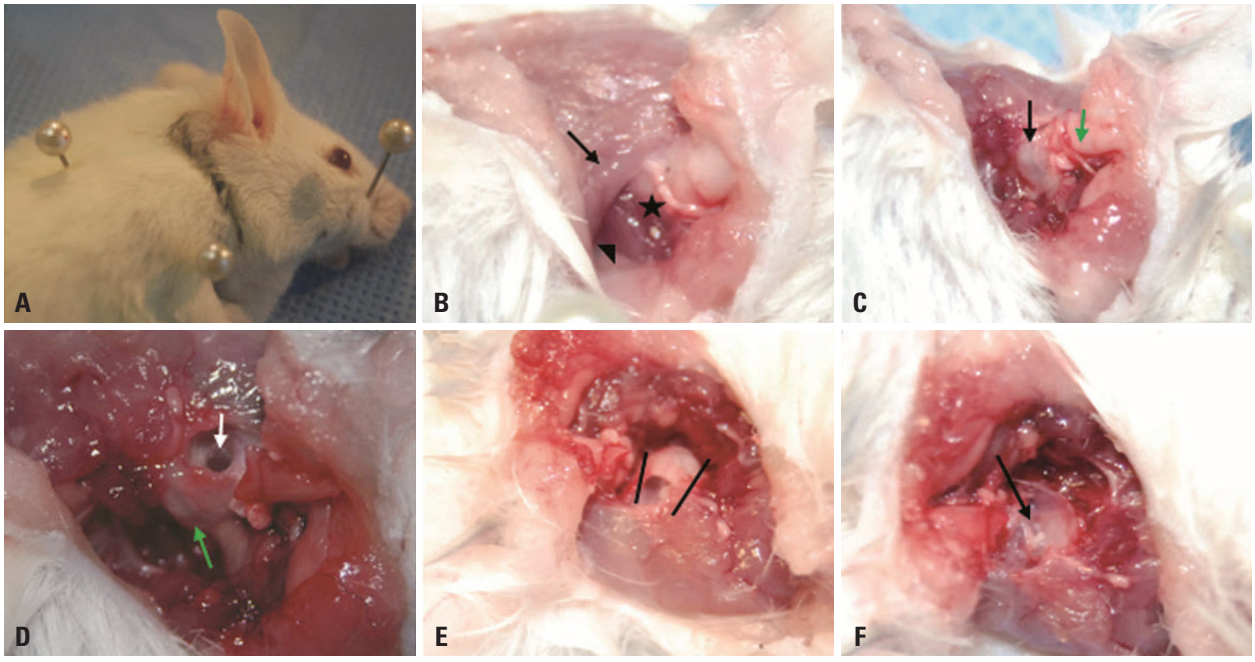

Fig. 1. Lateral postauricular approach to the mouse middle ear (bulla). (A) In each animal, a postauricular incision (black line marker) was made and extended ventrally to the rostral neck skin. The subcutaneous connective tissues were separated to expose the deeper structures. (B) The incision was deepened to the underlying temporalis and cervical muscles. Subsequently, the submaxillary gland was carefully dissected out and retracted, working across the sternocleidomastoid muscle (black arrow) and the digastric muscles (arrowhead). The bulla (star) is the posterior insertion of the digastric muscle into the jugular process of the occipital bone. (C) Access to the tympanic bulla was achieved by means of a blunt dissection slightly lateral to the insertion of the digastric muscle. The figure shows the bulla (black arrow) connected to the EAC (green arrow). (D) The EAC was resected with the tympanic membrane using a probe that was inserted into the EAC and bulged toward the dissection. The previously exposed bulla (green arrow) is inferior to the resected tympanic membrane, showing the middle ear cavity inside (white arrow). (E) Looking through the hole of the bulla upside the mouse, the half-exposed clean bulla (black lines) was resected with sharp micro-scissors, leaving no fragments. (F) After the resection, the medial wall of the bulla, including the promontory (black arrow), remained. EAC, external auditory canal.

number 3 tube) and prepared six tubes. Subsequently, we put the two membranes attached to the resected bulla bones into 1 mL of RNA stabilization reagent solution (RNA later, Qiagen, Cat. No. 76104) and then transferred these sequentially into tubes of 800 μL TRIzol Reagent (Invitrogen, Cat. No. 15596-018) for 10 minutes, 30 minutes, 1 hour, and 3 hours, respectively, at room temperature. After each time interval passed, we transferred only the TRIzol solution into another clean tube, without the bulla bone pieces. The total RNA was extracted according to the manufacturer's instructions.

Scanning electron microscopy (SEM)

Parts of the specimens, prepared as described for the "melting method," were processed for scanning electron microscopy. For this purpose, after fixation, specimens were dehydrated in a graded series of ethanol and critical point drying using liquid CO_2. The distribution of epithelial cells was studied with a Philips scanning electron microscopy (SEM) 525 M scanning electron microscope after mounting and coating samples with gold in a Balzers MED10 Sputter-Coater (Balzers Union, Balzers, Liechtenstein).[4]

Reverse transcription-polymerase chain reaction (RT-PCR)

Mucin gene expression analysis was determined using standard reverse transcription-polymerase chain reaction (RT-PCR) techniques. RNA integrity was assessed with optical density (OD) measurements via Nano-Drop (ND-3300, NanoDrop Technologies, USA). Quantity and quality assessments using a UV/VIS spectrophotometer were performed at multiple wave lengths at 240 nm (background absorption and possible contaminations), 260 nm (specific for nucleic acids), 280 nm (specific for proteins), and 320 nm (background absorption and possible contaminations). OD 260 was used to determine quantity, an OD 260/280 ratio was used to assess quality, and an OD 260/240 or 260/320 ratio was used to verify the purity and extraction performance. An OD 260/280 ratio greater than 1.75 is usually considered an acceptable indicator of good RNA quality.[5]

Primer sets used in PCR for MUC5AC (mucosal primer) and total osteocalcin (bone primer) to discriminate bone contamination from the RNA of the mucosal membrane were: for total osteocalcin (amplicon: 371 bp), 5'-CAAGTCCCAC ACAGCAGCTT-3' and 5'-AAAGCCGAGCTGCC AGAGTT-3';[6] and for MUC5AC (amplicon: 518 bp), 5'-CACCATCTCTACAACCCAAA-3' and 5'-TGAGGTC CAGGTCTTTGTGTCTCT-3'.[7] PCR parameters of both total osteocalcin and MUC5AC were 94°C for 4 minutes, followed by 30 cycles at 94°C for 30–45 seconds, 58°C for 30–45 seconds, and 72°C for 2 minutes, followed by a final extension at 72°C for 15 minutes.

The extracted RNA was treated with amplification grade deoxyribonuclease I (Invitrogen) prior to cDNA synthesis. DNase I-treated RNA (2 μg) was converted to cDNA by the addition of GeneAmp® RNA PCR Kit components (MMLV-reverse transcriptase, RNase Inhibitor, and Random Hexamers; Applied Biosystems Inc., NJ, USA) to give a total volume of 25 μL. After reverse transcription, RT-PCR was performed in a MyCycler™ thermal cycler system (Bio-Rad Laboratories, Inc., CA, USA). The PCR reactions were run on a 2% agarose gel at 100 V. The PCR product was visualized with Gelstar (Cambrex, Bio Science Rockland).

RESULTS

Light microscopy demonstrated the presence of middle ear

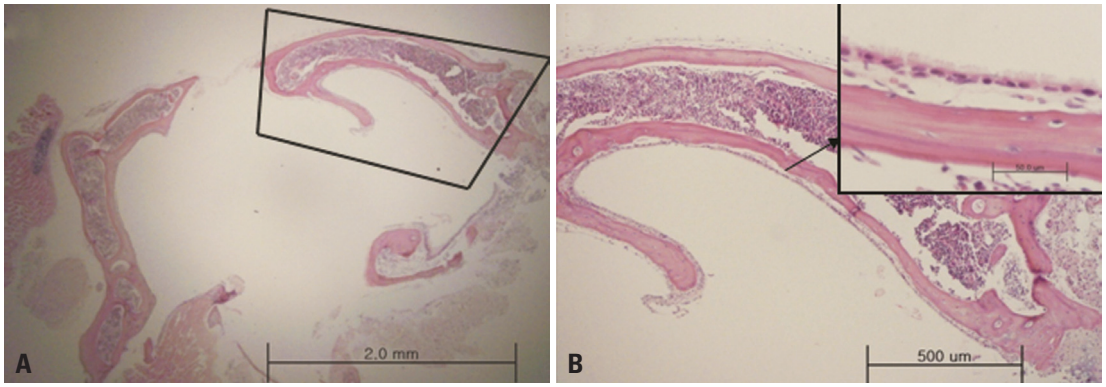

Fig. 2. Light micrographs of the middle ear mucosa in the whole bulla. (A) Inside the bulla bone, ciliated mucosal membranes were evenly distributed. A lateral approach exposed the half lateral bulla (black box) (×10). (B) The resected lateral bulla had securely attached mucosal membranes (×20, ×400).

mucosa that was pseudostratified, had a cuboidal or cylindrical epithelium, and contained an abundant number of ciliated cells and several goblet cells (Fig. 2). The mucosal membrane was distributed evenly and attached inside the bulla. Though it was carpeted with a mucosal membrane layer around the Eustachian tube (anteromedial wall of the bulla) and beyond the promontory (medial wall of the bulla), there were enough to obtain pure and healthy mucosal membrane specimens of the middle ear in the lateral wall of the bulla.

Before melting a resected lateral piece of the bulla, SEM showed ciliated mucosal cells well-distributed on the surface of the bony bulla (Fig. 3A and B). After melting the membrane into the TRIzol solution for 10 minutes, there were almost no mucosal cells on the surface and nearly all mucosal cells of the bulla had been melted, without exposing bony surfaces (Fig. 3C and D). On the other hand, stripping of the mucosal membrane was seen to remove mucosal membrane wholly with some bony destruction (Fig. 3E and F).

Amounts of RNA extractions from the cells in the melting method were sufficient enough for all measurements. The mean volume of the resected bullae was 92.8 ng/μL

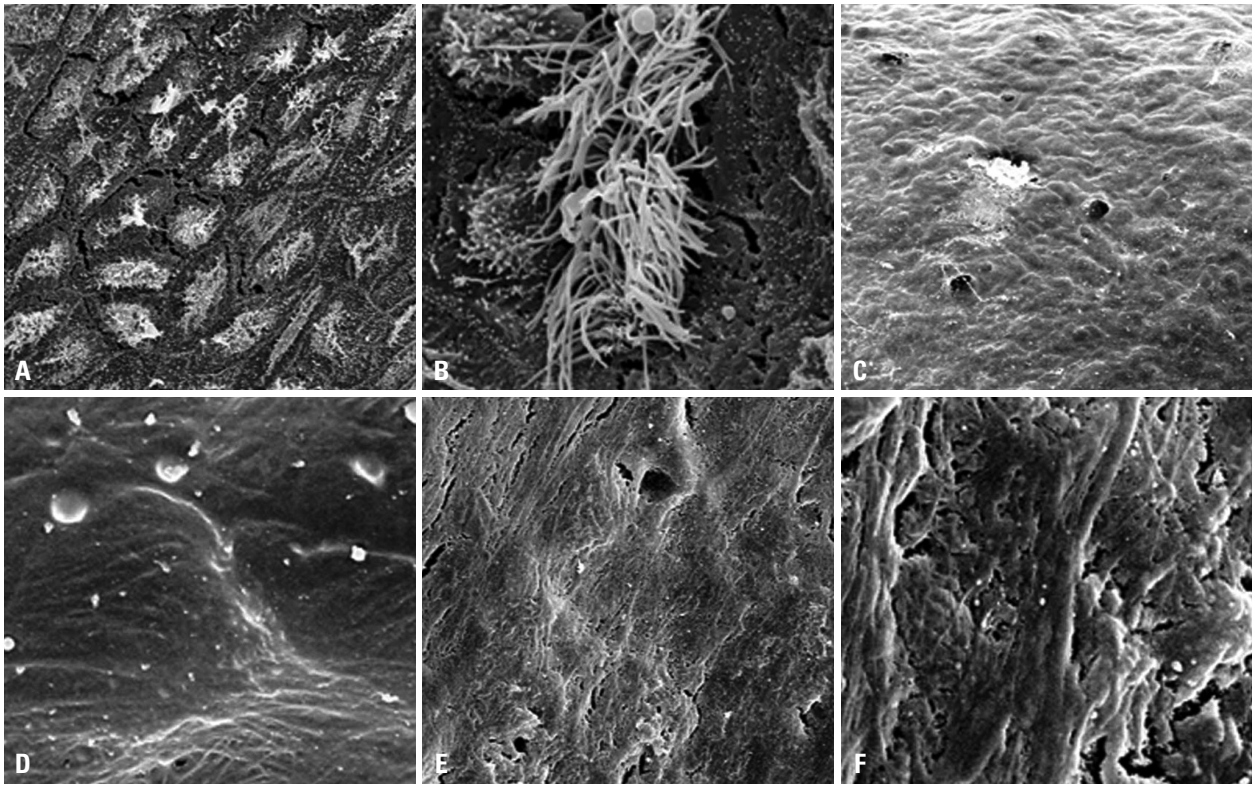

Fig. 3. Scanning electron micrographs of the middle ear mucosa in the resected bulla. (A) Before melting, the picture shows ciliated mucosal cells regularly distributed on the surface of bony bulla (×1000). (B) A bundle of cilia on the hexagonal cell (×10000). (C and D) After melting the membrane into the TRIzol solution for 10 minutes, it showed uneven surfaces and no ciliated cells (×1000, ×10000). There were very few mucosal cells on the surface, and it melted almost all mucosal cells of the bulla, not exposing bony surfaces. (E and F) After stripping, the bony surface was exposed with some bony scratching (×1000, ×10000).

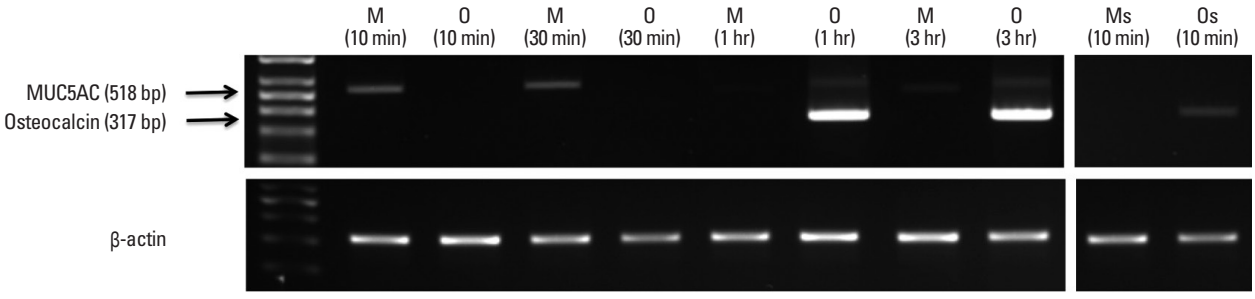

Fig. 4. Expression of mucosal marker (MUC5AC) and bony marker (osteocalcin) in mouse middle ear epithelium by RT-PCR; 518-bp and 371-bp fragments amplified on the RT-PCR probed by MUC5AC and total osteocalcin primers. MUC5AC was detected in the cells within 1 hour, while MUC5AC and osteocalcin were simultaneously detected over a period of 1 hour. RT-PCR, reverse transcription-polymerase chain reaction; M, MUC5AC primer in the melting method; O, osteocalcin primer in the melting method; Ms, MUC5AC primer in the stripping method; Os, osteocalcin primer in the stripping method.

(OD 260/240=1.84). The RT-PCR products (MUC5AC for mucosal marker and total osteocalcin for the bulla bone) are shown in Fig. 4. MUC5AC mRNA expression was detected in the mouse middle ear mucosa at 10 minutes and 30 minutes, yet osteocalcin was not. MUC5AC and osteocalcin were simultaneously detected over a period of 1 hour. Stripping of the mucosal membrane largely impaired the results of RNA purification and consequently showed only osteocalcin with no mucosal marker (MUC5AC).

DISCUSSION

The middle ear of mouse is similar to those of the human middle ear located in the interior the tympanic bulla, which forms part of the skull base and is the equivalent to the human tympanic cavity.[8] Thus, RNA extraction techniques from the middle ear are important to many researchers working with the middle ears of mice. Currently two different methods are used for RNA extraction: stripping and irrigation. The stripping method involves detaching the mucosal membrane from the bony bulla carefully under microscope.[9,10] The bulla of the mouse is very small (2 mm), making it difficult to manipulate despite using a stereomicroscope, and due to the long treatment time, RNA from the middle ear cells are easily damaged. In this study we stripped the mucosa from the bone within 3 minutes and melted it in TRIzol for 10 minutes. Though all mucosal membranes were completely removed from the bulla bone in SEM, the RNA was impaired (OD 260/280=1.66) by the Nano-drop to the degree that MUC5AC was not detected in the RT-PCR. However, the results of RNA extraction from the cells by stripping can yield better results if done by skilled experimenters. The other method for RNA extraction is called "irrigation." The middle ear cavity is injected transbullarly with 50 mL of lysis buffer containing guanidine isothiocyanate from an Rneasy Mini Kit (Qiagen, Cat. No. 74106) and incubated for 15 seconds to lyse the middle ear mucosa. This process is repeated three times, and the lysates are aspirated, pooled, and stored at -70°C until RNA extraction.[11,12] In the middle ear cavity, there are many micro-structures, such as ossicles, muscles, and the tympanic membrane. We are doubtful of the assumption of this method that transbullar irrigation contains only mucosal cells. Soft tissues around the bulla or secretions including blood can contaminate the irrigation solutions, resulting in RNA extractions from the wrong target organs.

In the melting method, the anatomy of the mouse is very important, as the tiny structures around the bulla disturb access to the middle ear. A retroauricular lateral incision allowed for the clearest and fastest access possible to the lateral part of the bulla, without the destruction of any middle ear structures, including the tympanic membrane.[8,13] After careful dissection of the digastric posterior belly, the lateral bulla could be exposed without damaging any of the surrounding soft tissues.[2] Leaving the ossicles, stapedius muscle, and the medial wall of the bulla, the promontory lateral wall, which is made up of mucosal membranes attached to the half bony bulla, could be resected with sharp microscissors. The specimen contained both mucosa and bone. This lateral surgical approach can make the melting method reliable for RNA extraction.

In several ways, the "melting method" was found to be very useful. Within 30 seconds of separation from the temporal bone, the mucosal membrane was transferred to the TRIzol solution. After 10–30 minutes at room temperature (10 and 30 minutes of melting showed no differences in the RT-PCR results; thus, we concluded that 10 minutes would be better than 30 minutes for RNA stabilization), sufficient samples of pure mRNA (about 100 ng/μL per two bulla, 48 ng/μL per one bulla) from the mucosal membrane of the middle ear were extracted without any problems. In view of SEM and RT-PCR results, the bulla bone did not appear to be melted within 30 minutes. However, the SEM results after 10 minutes showed that nearly all mucosal cells were melted, including ciliated and goblet cells. The uneven surface of the bulla bone had no cell figures or bony fragments. After stripping the mucosal membrane, SEM indicated that some scratches were made on the bone, and the RT-PCR indicated that bony fragments were contained in the RNA extractions. On the other hand, TRIzol solutions after 30 minutes seemed only to melt the mucosal layers, although after 1 hour, the bone fragments could be melted by TRIzol. Though RNA is impaired by long time–room temperature conditions, RT-PCR results can be positive, as some non-impaired mRNA samples are duplicated beyond count through the RT-PCR process.[5] Thus, the finding that mucosal markers and bony markers were simultaneously expressed after 1 hour could be meaningful.

In conclusion, due to the increase in the number of experiments involving manipulation of the middle ear RNA of mice *in vivo*, it is important to take note of this robust, easy approach to RNA extraction from the middle ear mucosal membrane of the mouse, through the half lateral wall of the

tympanic bulla. Considering that this melting method can obtain sufficient samples of pure mucosal RNA, we believe that it can be safely and widely used for RNA molecular work *in vivo*.

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