Microscopic techniques for application in experimental pathology of the middle and inner ear

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Experimental ear research is crucial for the understanding of intimate mechanisms of hearing physiology and ear diseases. While studies in humans are limited by anatomical features, studies in experimental models of disease allow for a wide range of electrophysiological, analytical and morphological determinations. In the case of morphological analysis many microscopy techniques are useful, from the conventional light microscopy stainings and immunohistochemical methods to the most advanced electron microscopy and confocal laser scanning methods. This chapter shows application of light microscopy, immunohistochemistry, transmission and scanning electron microscopy and environmental scanning electron in the study of middle ear infections, biomaterials compatibility, inner ear damage and detailed anatomical features in experimental models in the rat and the guinea pig.

Keywords inner ear, middle ear, immunohistochemistry, light microscopy, electron microscopy, hearing loss

1. Introduction

Hearing loss is a condition affecting humans worldwide. It is highly prevalent in the western countries due to increasing ageing of population, industrial sources of intense noise and employment of drugs for severe diseases that may damage the inner ear. Otitis media is an important health care pediatric problem all around the world and can also lead to hearing deficits. Many questions regarding infection pathophysiology and control, prevention of damage by noise and ototoxics and regenerating the ageing inner ear remain unknown. Approaches to the hearing organ in the past were mainly based in morphological descriptions of temporal bones of patients at necropsies. Technical advances have allowed detailed electrophysiological studies of the hearing in the living. However, studies in humans are limited by the fact that the inner ear is enclosed in a bony capsule (the otic capsule) and sensory cells immersed in a fluid with a highly specific electrolytic composition. This fact makes tissue sampling unattainable in the living. Therefore, extensive research in middle and inner ear pathology is being done in animal models and will still be necessary in the future. Animal models of ear disease allow a precise timing of the inflicted damage (whether toxic, traumatic or immunologic), electrophysiological studies to determine effects over hearing function and finally detailed morphological studies of the alterations caused.

The preferred species in hearing research are rodents: guinea pigs, rats, chinchillas, gerbils, mice and rabbits. The preference for a particular model is dependent on the advantages that it may have in answering particular scientific questions. Rat and chinchillas are extensively used in otitis media research. Rats are also employed in inner ear research as well as mice (many inbreds are available) and guinea pigs, with an anatomically prominent cochlea that makes manipulation of inner ear easier.

2. Light microscopy

2.1 Hematoxylin and eosin stain

This is the most commonly employed stain in medical diagnosis and still of great interest in ear research. It allows the study of middle and inner ear structures in a variety of experimental pathology models such as experimental otitis and biomaterial implantation. Destruction of middle ear ossicles of infectious origin is commonly treated with bone reposition or implantation of prostheses made of polymers or metal. Rat has been widely employed to study interactions between middle ear tissues and biomaterials in healthy ears [1], following obstruction of the Eustachian tube [2,3] and in models of infection [4]. Conventional light microscopy stains provide information about reactive inflammation, changes in bony structures, mucoperiosteal covering of middle ear cleft, integration of biomaterials, and other pathological features in middle ear [5] and inner ear [6].
Fig. 1 A Plastipore fragment implanted for 30 days in the middle ear of an animal inoculated with Pseudomonas aeruginosa. A fibroepithelial interface (arrow) can be observed covering the prosthesis with penetration of fibrous tissue within the pores. Irregular shape (d) and loss of some granules (l) can be observed (Hematoxylin&eosin x20).

Fig. 2 Polymorphonucleated cells (arrow) in contact with a fragment of Gore-tex® (G) and penetrating the small pores of the biomaterial only in the periphery. Sacrifice at 30 days after implantation and inoculation with Pseudomonas aeruginosa (Hematoxylin&eosin x50)

Interesting anatomical features can also be visualized with this staining. A longitudinal section of the stapes, a middle ear ossicle, can be seen in figure 3. This small bone is located in the oval window, opening into the basal turn of the cochlea. The stapedial artery can be found between the crurae of the stapes in the rat model.
The whole structure of the stapes can be appreciated in this image, including the head (h), crurae (c) and footplate (f). The stapedial artery is usually an embryological structure in the human and other mammals, but it persists in the rat after birth (ea). Note the annular ligament between the footplate of the stapes and the rim of the oval window (arrows). Hyperplasic mucoperiosteum can be seen surrounding the bony structures, as this image was taken from a rat inoculated with Pseudomonas aeruginosa 15 days before (Hematoxylin&eosin x50).

2.2 Masson trichrome

Masson's trichrome is a three-colour staining protocol used for distinguishing the connective tissue in the samples. It is used preferentially for analysing connective tissue changes in the middle ear in animal models of otitis media.

2.3 Immunohistochemistry

This technique allows identification and precise localization of specific proteins in the tissue sampled. In the case of experimental inner ear pathology, this is a technique that has been employed in localizing specific markers of apoptosis, neural tissue, different signaling molecules in regeneration processes, receptors, etc. As an example, it is possible to study apoptotic changes caused in the rat cochlea after injection of cisplatin, an antitumoral drug widely employed in the treatment of solid tumors and associated with hearing loss in a high percentage of treated patients [7]. Caspase-3 activity in different cell populations of the organ of Corti can be determined by immunohistochemistry. Figure 4 shows increased expression of caspase-3, which is related to the intrinsic pathway of pro-apoptotic signalling within the rat cochleae [8].

**Fig. 3**  The whole structure of the stapes can be appreciated in this image, including the head (h), crurae (c) and footplate (f). The stapedial artery is usually an embryological structure in the human and other mammals, but it persists in the rat after birth (ea). Note the annular ligament between the footplate of the stapes and the rim of the oval window (arrows). Hyperplasic mucoperiosteum can be seen surrounding the bony structures, as this image was taken from a rat inoculated with Pseudomonas aeruginosa 15 days before (Hematoxylin&eosin x50).

**Fig. 4**  Immunostaining of active caspase-3 in the nuclei of inner ear cells from rats 7 days after a single dose of cisplatin (5 mg kg⁻¹). Nuclear caspase-3 immunostaining is seen in several cell populations after administration of cisplatin. OHCs, outer hair cells; IC, interdental cells of the spiral limbus. Magnification × 400.
Fig. 5 Immunostaining of heat shock protein 70 (HSP-70) in the stria vascularis of the rat cochlea after cisplatin injection (arrows). HSP-70 might be a reparative molecule expressed after cellular damage.

2.4 Cytocochleogram

A cytocochleogram is a graphic representation of the anatomical state of the hair cells along the complete width and length of the organ of Corti from which morphometric information can be obtained. It was first described by Engstrom and coworkers in 1966 for producing surface preparations of the organ of Corti and a 2-dimensional graphic representation of damaged hair cells along the entire length of the basilar membrane [9]. It is then possible to relate structural damage with the frequency-related localization. This was first performed manually and was time-consuming. Some authors have described computer-assisted methods to assess hair cell damage and produce a graphical representation [10].

3. Transmission Electron Microscopy (TEM)

This technique is capable of visualizing objects to the order of a few angstrom (10⁻¹⁰ m) therefore allowing the study of very small details in the cell populations of the organ of Corti. Different cell types can be analysed as well as organelles and cell junctions. Immunohistochemistry is also applicable to this technique. Similarly, ultrastructural studies of explanted prostheses of middle ear can also be performed. Figure 6 shows an example of fibrocyte of the lateral wall of guinea pig cochlea. Ultrastructural features suggest that this is a type IIb fibrocyte. These cells are located between root cells in the external sulcus and type I fibrocytes and are related to active pumping of K⁺ to endolymph from perilymph (Na/K ATPase) through external sulcus cells, which is crucial in electrophysiological processes of hearing function [11, 12].

Fig. 6 View of a type II fibrocyte in the lateral wall of guinea pig cochlea by means of a transmission electron microscope (x5000).
4. Scanning Electron Microscopy (SEM)

SEM is a commonly employed high resolution technique that allows topographical study of surfaces. It provides spectacular images of biological and non-biological tissues and therefore is a popular technique in the field of ear and hearing research. Immunohistochemical methods are available for this technique.

Panoramical views of the cochlea are available with this technique for determining the areas with better conservation of the organ of Corti (figure 8) and ulterior visualization at higher magnifications (figure 9). This allows anatomical studies in healthy organs as well as determination of pathological changes after inflicting a damage to the inner ear of immunological or toxic nature [13]. Changes in sensory cells as well as supporting cells can be observed. In fact, supporting cells are believed to play a more active role in inner ear homeostasis and function than traditionally considered [14, 15]. In middle ear pathology, SEM allows visualization of cholesteatoma debris, ciliated cells in the respiratory mucosa, etc.
Fig. 9 In this closer view of the sensory hair cells the three rows of outer hair cells (OHC) can be observed with the characteristic V-shaped stereocilia. The single row of inner hair cells is separated from the OHC by the tunnel of Corti (original magnification x1800).

Fig. 10 The monocellular layer of the Reissner membrane can be fully appreciated in this image.

Fig. 11 Closer view of the inner hair cells (IHC) row of the organ of Corti after immunomediated damage in a guinea pig model of immuno-mediated disease of the inner ear. Balloon-like protrusions correspond to “bleb” formation in the cuticular region of IHC. Blebs have been have been associated with cell injury, although they have also been observed in healthy cells during particular stages of the cell cycle and play a role during embryogenesis.
5. Environmental Scanning Electron Microscopy

Environmental scanning electron microscopy (ESEM) is a descendant of the conventional scanning electron microscopy that allows visualization of biological samples in their natural state. This technique is capable of imaging samples without vacuum in the sample chamber and therefore biological samples do not need dehydration and conductive coating for visualization, thus avoiding distortion of structures and artifacts. ESEM can be applied to the study of biofilms, bacterial communities enclosed in a self-produced matrix or glycocalyx that protect them from biocidal attacks. Biofilms have been associated with infectious diseases of the middle and inner ear and may explain difficulty to eradicate middle ear infections in some patients. Experimental models of biofilm infection in the middle ear are being developed and ESEM plays a crucial role in identifying presence of biofilms and analysing glucocalyx features (figure 12).

![Micrograph of colonization by biofilms of Pseudomonas aeruginosa (*) in an experimental model of biofilm infection in the rat.](image)

Fig. 12 Micrograph of a fragment of middle ear mucosa with colonization by biofilms of Pseudomonas aeruginosa (*) in an experimental model of biofilm infection in the rat. Note the smooth surface of non-colonized mucosa surrounding the biofilm (original magnification 2000x, pressure 4.4 Torr).

6. Confocal Laser Scanning Microscopy

This microscopy technique is currently considered a gold standard in the study of biofilms. It allows the differentiation between living and death cells within the biofilms as well as identification of species by use of different dyes.

References


