HAIR CELL LOSS AND REPAIR PROCESSES IN MAMMALIAN VESTIBULAR SENSORY EPITHELIA

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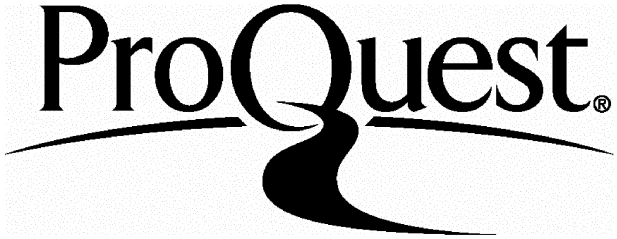

ABSTRACT

The sensory hair cells of the inner ear transduce the mechanical stimuli involved in hearing and balance. Loss of hair cells has been thought to be irreversible in mammals and is a major cause of human deafness and vestibular disturbances. In this thesis, scanning electron microscopy (SEM), transmission electron microscopy (TEM), fluorescence microscopy and immunohistochemistry have been used to examine the progression of hair cell loss and subsequent repair processes in the vestibular sensory epithelia of guinea pigs following injury induced by ototoxic aminoglycoside gentamicin. It is found that hair cell recovery may occur in the mammalian vestibular tissues.

Hair cell loss in the utricular maculae and cristae was apparent after chronic, systemic gentamicin treatment. With topical application of drug, the saccular macula was also affected. Damaged hair cells undergoing degeneration showed morphological features characteristic of apoptosis. The lost hair cells were replaced by expansion of supporting cells. In longer survival animals, SEM showed there were many cells with immature hair bundles within the areas where hair cells were originally lost. Thin sections of equivalent areas showed the presence of immature hair cells. This suggested that replacement of hair cells occurred spontaneously after gentamicin induced hair cell loss. Studies of this phenomenon over a period of 33 weeks after gentamicin treatment demonstrated that the immature hair cells continued to develop towards structural maturity. The number of hair bundles assessed by SEM of utricles and saccules showed that an initial decrease in number was followed by an increase, confirming recovery of hair cells. However, the hair cell density was lower than in controls, suggesting recovery was still incomplete.

A technique for the maintenance of explants of the utricles and saccules from adult guinea pigs and gerbils in organotypic cultures was developed. Exposure of cultures to gentamicin resulted in progressive hair cell loss in a pattern similar to that seen in vivo. The degenerated hair cells again showed morphological features of apoptosis. In situ end labelling (ISEL) method was applied to detect apoptotic cells in the cultured utricles. Immature like hair cells were occasionally identified in cultures exposed to gentamicin. The results suggest that organotypic culture system of the mature mammalian vestibular epithelia is a useful model for examination of ototoxicity and recovery processes.

Proliferative activity was identified in the damaged vestibular sensory tissues by immunohistochemical bromodeoxyuridine (BrdU) labelling, but the extent was insufficient to account for the recovery of hair cell numbers. Mechanisms other than proliferation may also be invoked after gentamicin induced hair cell loss in the vestibular sensory epithelia of the mammalian inner ear. Nevertheless, these results show that the potential for replacement of hair cells after loss may exist in the vestibular sensory organs of mature mammals.

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CHAPTER 1: GENERAL INTRODUCTION

Sensory hair cells of the vertebrate auditory and vestibular organs transduce mechanical stimuli into electrical activity that is transmitted to the brain via the cranial nerves. It had generally been believed that hair cells in the mammalian inner ear cannot be regenerated after they have been lost. Loss of hair cells can occur after intense noise exposure, infections, drug ototoxicity, head trauma, Ménière's disease, genetic defects, or as consequence of the normal ageing process, thus causing hearing impairment and balance disorders. The aminoglycoside antibiotics have been the major group of ototoxic agents that drew great attention to the problem of drug induced acquired sensorineural hearing loss and vestibular balance disturbances since they were discovered in the 1940's.

The initial aims of this work were to characterise the mechanisms of the hair cell death induced by aminoglycoside gentamicin and the subsequent processes of lesion repair effected by the supporting cells in the guinea pig vestibular organs, compared with the organ of Corti (Forge, 1985; McDowell et al., 1989; Raphael and Altschuler, 1991a; 1991b). Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and fluorescence microscopy have been used to examine the vestibular sensory epithelia of guinea pigs following hair cell loss induced by the aminoglycoside gentamicin both in vivo and in vitro. During the course of the work, evidence was obtained suggesting the possibility of hair cell regeneration in the mammalian vestibular sensory epithelia (Forge et al., 1993). The characterisation of this phenomenon became the focus of further work and immunohistochemical labelling of bromodeoxyuridine (BrdU) has been used to detect the proliferative activity in the mammalian inner ear.

1.1 ANATOMY OF THE MAMMALIAN INNER EAR

The mammalian ears are peripheral, compound organs including external ear, middle ear and inner ear, which consists of the auditory system and the vestibular system. The inner ear is also called the labyrinth. It lies within the petrous part of the temporal bone in the higher primates and humans, whilst in most other mammals, it is enclosed in a bony capsule called the bulla. The inner ear comprises a membranous labyrinth within a protective bony shell. The bony labyrinth is divided into the vestibule, the semicircular canals, and the cochlea (Figure 1.1). There are the utricle and saccule within the bony vestibule; three membranous semicircular ducts within their corresponding bony canals; and the cochlear duct (the scala media) in the bony cochlea. Between the bony and membranous labyrinths is the perilymph fluid, which resembles normal extracellular fluid having a high Na^+ to K^+ ratio. Within the sacs (the utricle and the saccule) and ducts (semicircular ducts and the cochlear duct) is the endolymph fluid. The endolymph has a high concentration ratio of K^+ to Na^+ ions, similar to body intracellular fluid. The structure of the sensory epithelia of the auditory and vestibular systems in the inner ear are broadly similar in all vertebrates, although certain differences may exist in detailed ultrastructural compositions. It is with the sensory epithelia of the vestibular and cochlear organs, that this chapter is principally concerned.

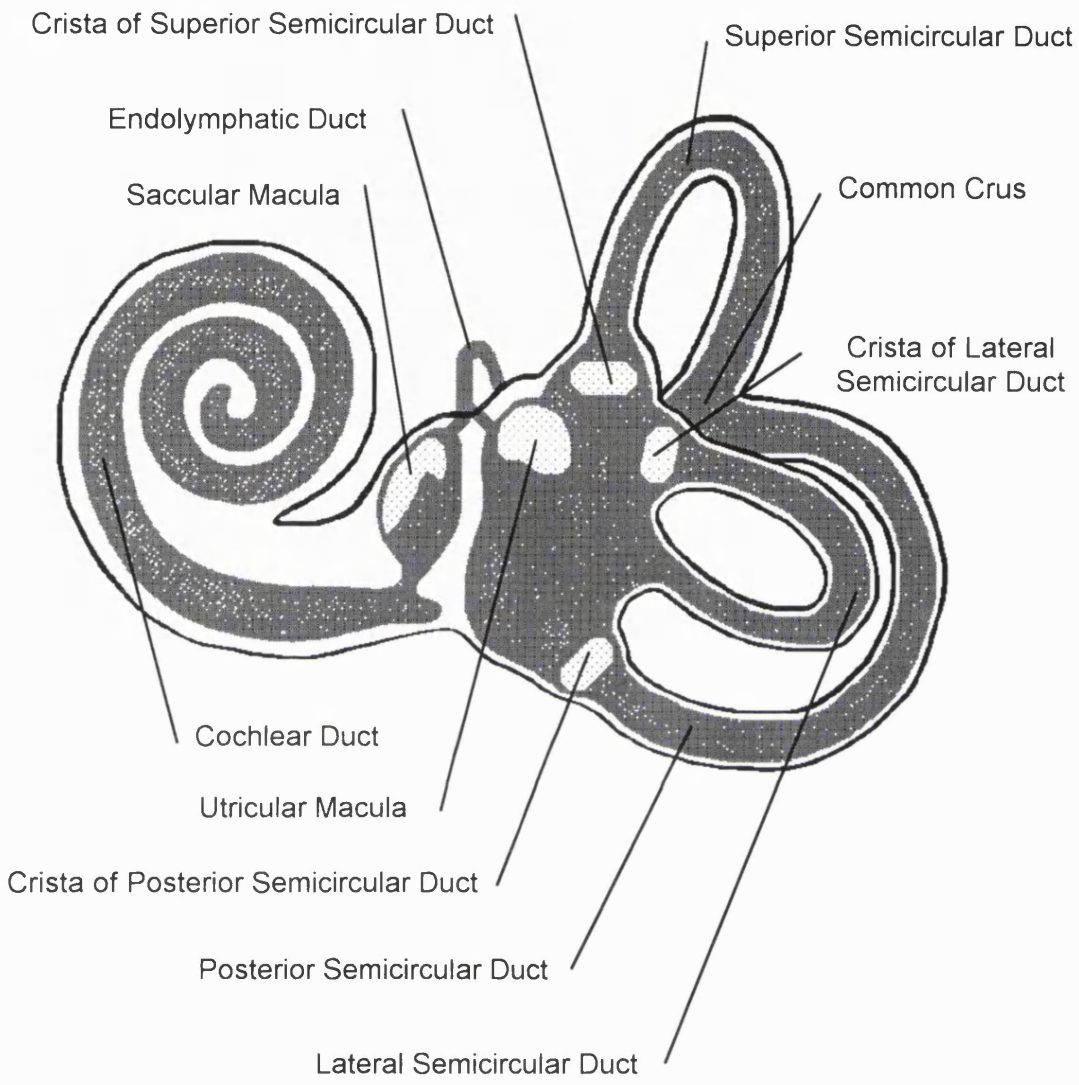

Figure 1.1 Diagram of the Inner Ear

The membranous labyrinth is enclosed within the bony labyrinth in the inner ear. The cochlear duct is seen spiralling within the bony cochlea. The utricle and saccule are positioned within the vestibule and the three semicircular ducts are inside their respective canals. The sensory epithelia of the inner ear vestibular system are the utricular macula, saccular macula and three cristae. The sensory epithelium of the auditory system, organ of Corti, is located inside the cochlear duct.

1.1.1 The Vestibular System

The vestibular system of the inner ear contains the balance end-organs. There are five sensory receptors that are located in the utricle (utricular macula); the saccule (saccular macula); and each of the three semicircular ducts (crista) in mammalian animals. The capacity of the maculae and cristae to function as sensors of linear and angular accelerations depends on their anatomic configurations. In the utricle, saccule, and three semicircular canals, it is possible to recognise the sensory epithelium towards the endolymph and the connective tissue towards the perilymph. A basement membrane is interposed between them. Beneath the sensory epithelium, the connective tissue layer is thicker and a large number of myelinated nerve fibres and a particularly rich network of fine capillaries are located in the connective tissue of these areas.

The Otolithic Organs

There are two otolithic organs: the utricle and the saccule in the mammalian vestibular system. The utricle is an irregular membranous sac and connects to the membranous semicircular canals via five openings. Its sensory epithelium, known as the macula utriculi, is generally shell shaped in mammals and is oriented in a horizontal plane. The hook-shaped sensory epithelium of the saccule, the macula sacculi, is oriented perpendicular to the macula utriculi. The saccule communicates with the endolymphatic duct by the saccular duct and with the cochlea by the ductus reuniens. Upon the utricular macula and the saccular macula are large numbers of crystalline otoconia which contain calcium carbonate ($CaCO_3$). Otoconia are embedded in a layer of gelatinous substances overlying the macular sensory epithelia, and together constitute the otolithic membrane (Lim, 1971). There are sensory "hair" cells, supporting cells and neural elements in the vestibular

macular epithelia. On a morphologic basis, each macula may be divided by a narrow curved zone. This zone had been termed the striola by Werner in 1933. The striolae of the utricle and the saccule are generally arranged in C and L shapes respectively (Figure 1.2). The striola is an important landmark for morphologic and physiologic polarisation of hair cells. It is in the striola where sensory hair cell orientation changes. The maturation of the sensory hair cells starts from the striolar region in the maculae (Harada, 1988). It is along the striolar region of the maculae where the sensory hair cells are first affected by the ototoxic aminoglycoside drugs (Lindeman, 1969a; 1969b; Wersäll et al., 1973; Harada, 1988; Yan et al., 1991).

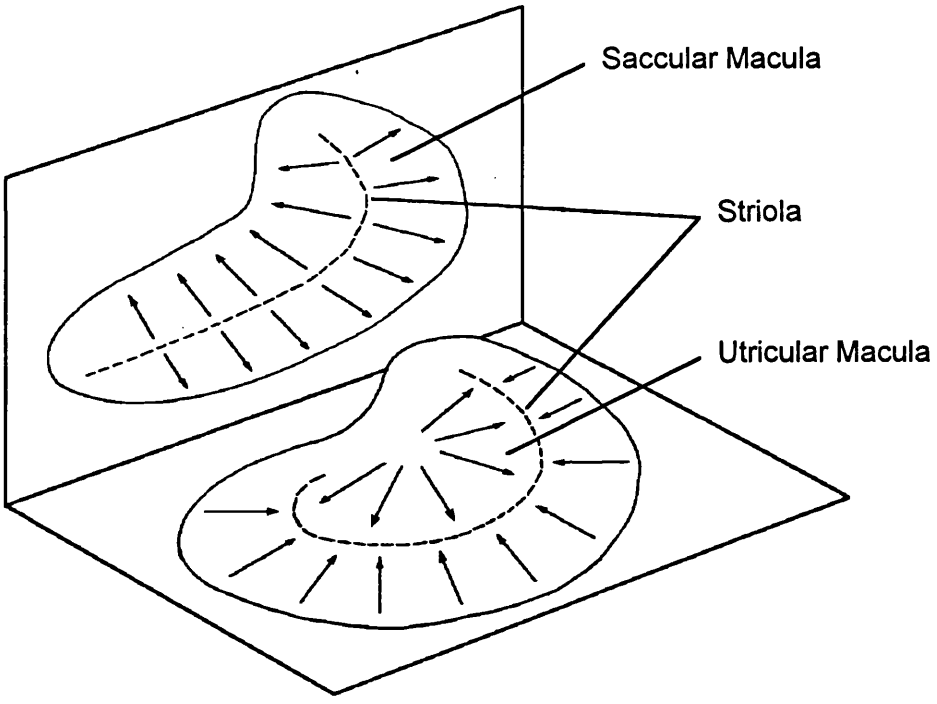

Figure 1.2 Diagram of the Vestibular Utricular and Saccular Maculae

The morphological polarisation of the utricular and saccular hair cells is indicated by the arrow direction. The kinocilium and tall stereocilia of hair cells are oriented towards the striola in the utricular macula and oriented away from the striola in the saccular macula.

The Semicircular Canals

The three semicircular canals, the lateral, superior and posterior, each occupies slightly more than half a circle and they are arranged at around 90° degree angles to each other. One end of each semicircular canal has an enlargement, called the ampulla. The sensory epithelium, called the crista, is located in the ampulla of each of the lateral (horizontal), superior and posterior semicircular ducts. The shape of the crista is best described as a saddle, which is covered by a dome shaped gelatinous structure, called the cupula. Morphological evaluation of the cupula is difficult because the gelatinous substance that forms the cupula frequently shrinks and comes off during the process of preparation (i.e. fixation and dehydration). The general structure of the crista is identical to that of the maculae of the utricle and saccule. In many mammalian species, such as humans, monkeys, rabbits and guinea pigs, there is little morphological difference in the cristae of the vertical (superior and posterior) and the horizontal (lateral) semicircular ducts. The sensory cells are evenly distributed over the crista sensory epithelium.

1.1.2 The Cochlea

Whilst the work to be described relates predominantly to the vestibular system, the auditory organ, the cochlea, inevitably also needs to be considered because of the intimate relationship of the hearing and balance organs of the inner ear. The mammalian cochlea is a coiled tube. The number of turns to the coil varies with species. Humans have two and three quarters turns, while guinea pigs have four. The cochlea is divided by the basilar membrane and the Reissner's membrane into three parallel canals, namely the scala vestibuli, the scala tympani and the scala media. The surface of the sensory epithelium of the cochlea, the organ of Corti, is bathed in endolymph. The organ of Corti is an epithelial

formation situated on the spirally arranged basilar membrane that runs along the length of the cochlea. The cellular constituency of the organ of Corti is a collection of sensory 'hair' cells, supporting epithelial cells and neural elements, similar to sensory epithelia of the vestibular system. A layer of fibrillar, acellular substance, called the tectorial membrane, extends to cover the apex of the hair bundles of the cochlear sensory cells.

1.1.3 The Vestibular Dark Cell Region and the Stria Vascularis

Apart from the sensory epithelia, there are specialized ion transporting epithelia in the vestibular organs and the cochlea. The ion transporting epithelia, the dark cell area in the vestibular organs and the stria vascularis in the cochlea, are involved in inner ear homeostasis. Functionally, the production and maintenance of the endolymph fluid, which bathes the apical surfaces of the sensory epithelia, take place in the vestibular dark cell region and in the cochlear stria vascularis (Dohlman, 1965; Kerr et al., 1982; Kimura, 1969; Nakai and Hilding, 1968). There is no dark cell layer in the saccular epithelium (Kimura, 1969). The blood supply to the membranous labyrinth of the inner ear is derived from the labyrinthine artery, which is a terminal artery in that it has no anastomosis with other surrounding arteries. Oxygen and nutrients to the sensory epithelia must be diffused from vessels in the surrounding connective tissues.

1.2 THE SENSORY HAIR CELLS

The characteristic feature of all mechanoreceptive cells like those of the inner ear is an organised bundle of actin filled projections from their apical surfaces, the "stereocilia", from which the sensory cells derive their common name 'hair' cells (Figure 1.3). In addition to the stereocilia, the hair bundle in most hair cells (except mammalian cochlear hair cells) possesses a single eccentrically positioned "kinocilium", a true cilium formed of

microtubules organised in typical "9+2" arrangement. A thick network of cytoskeletal filaments in the apical portion of the hair cell is called the cuticular plate, which provides an anchorage for the rootlets of the stereocilia. The cuticular plate does not cover the whole upper end of the cell but leaves a small space on one side of it free. The space contains the basal body of the kinocilium. The kinocilium from the mature hair cells (excluding the mammalian cochlear hair cells) is thick and tall, and determines the polarity of the hair cell (Lowenstein and Wersäll, 1959). The number of stereocilia varies on each sensory cell and they are arranged in rows of increasing length towards the position of the kinocilium (Figure 1.3 A). The taller stereocilia are proximate to the kinocilium while shorter stereocilia are located farther away from the kinocilium. The stereocilia are thinner than the kinocilium, narrower at the base and penetrate into the cuticular plate. The stereociliary membrane is continuous with the surface membrane of the cell and stereocilia may be considered as enlarged, specialized microvilli containing actin filaments in paracrystalline assay (Sobin and Flock, 1983, Tilney et al., 1986). Interconnections, namely lateral cross links and tip links, among the adjacent stereocilia, stereocilia and the kinocilium have been found on mammalian hair bundles (Osborne et al., 1984; Pickles, et al., 1984; Ernstson and Smith, 1986). Stereocilia are linked together by fine extracellular filaments and these links play an important role in the mechanical transduction system of the hair cells (Pickles et al., 1984; Hackney and Furness, 1995).

The cytoplasm of the hair cell is rich in cell organelles including the nucleus, endoplasmic reticulum, microtubules, ribosomes, lysosomes, Golgi apparatus and numerous mitochondria. The nucleus of the hair cell is located in the lower part of the cell and is round or oval in shape. Hair cells have a well developed cytoskeletal framework responsible

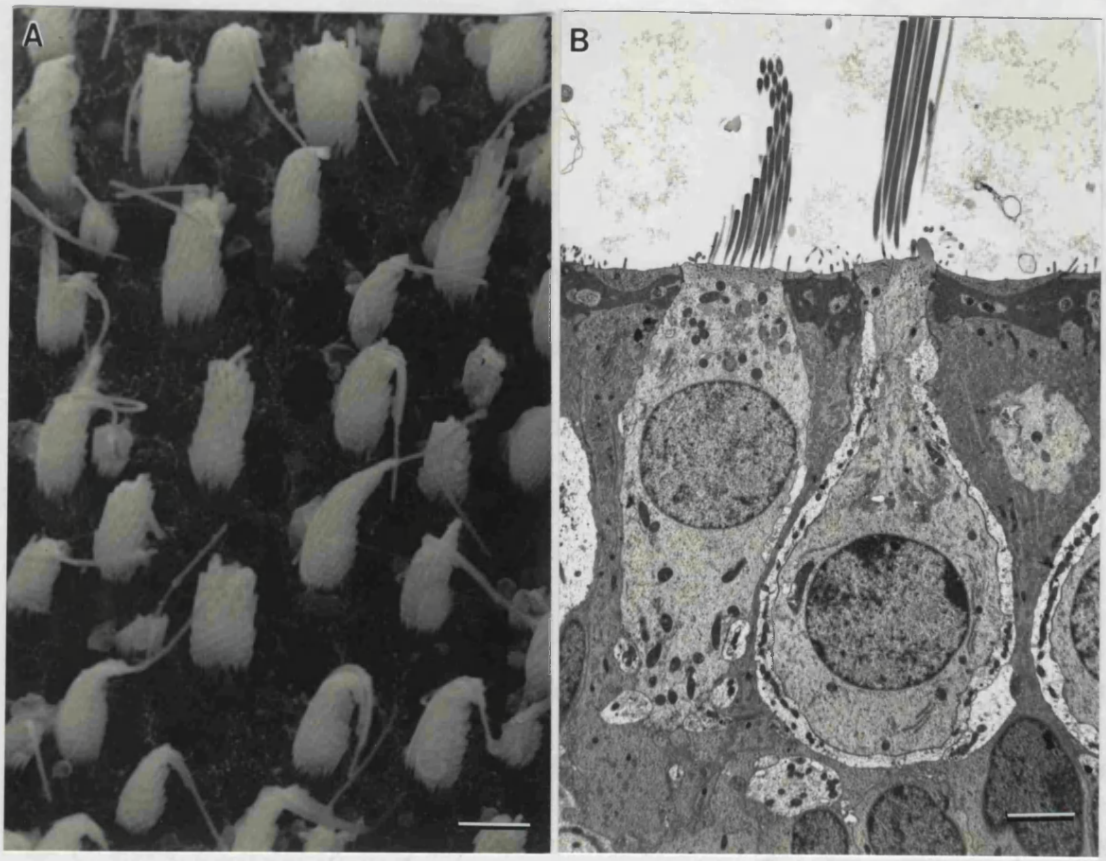

Figure 1.3 Mature Vestibular Hair Cells

A. Scanning electron microscopy, the mature vestibular hair bundle consists of a single kinocilium and a varying number of stereocilia in the vestibular sensory organs. The kinocilium is located in the periphery of the stereocilia bundle and the stereocilia are arranged in a regular pattern in rows, tall stereocilia near the kinocilium and short stereocilia away from the kinocilium. Bar = 4 μm.

B. Thin sections for transmission electron microscopy, the type I hair cell is pear-shaped and surrounded by a calyx nerve ending, the cylinder shaped type II hair cell is contacted by several small bouton-shaped nerve endings. Supporting cells are located in between hair cells and their intercellular junctions are present beneath the apical surfaces. Bar = 3 μm.

for cell morphology and internal organization (Flock et al., 1981; Flock et al., 1982; Sans et al., 1989; Slepecky and Chamberlain, 1982; Holley and Ashmore, 1990; Raphael et al., 1994a). The cytoskeleton is composed of the major filament systems including actin filaments, microtubules and many interconnecting structural elements. However, the absence of intermediate filaments within the auditory hair cells and their presence within non-sensory epithelial cells has been reported (Bauwens et al., 1991) although transient expression of neurofilament protein during hair cell development was found in the mouse cochlea (Hasko et al., 1990).

1.2.1 Vestibular Hair Cells

Two types of hair cells, called type I and type II hair cells presumably of different functional significance, are found in the vestibular sensory epithelia of the mammalian inner ear (Wersäll, 1954; 1956). They show differences in cell shapes and innervation patterns (Figure 1.3 B). Type I hair cells are flask shaped and surrounded, except at the apical surface, by the afferent nerve calyx. The typical type I hair cell has an enlarged bulbous basal portion containing the nucleus, a constricted neck and a flat head. Type I hair cells are found numerous in the striolar regions of the macular sensory epithelia and in the central part of the crista sensory epithelia (Wersäll, 1956; Lindeman, 1969c; Weisleder and Rubel, 1993). Type II hair cells are distributed throughout the vestibular sensory epithelia and they are shaped like cylinders and innervated by bouton type nerve endings. The nucleus is usually located in the middle part of the cell body and the cell length varies. The type II hair cells is generally considered to be phylogenetically older and morphologically less differentiated than the type I hair cell (Anniko, 1988; Sans and Chat, 1982; Kawamata and Igarashi, 1993; Weisleder et al., 1995). Type II hair cells are found in all vertebrates,

while type I hair cells occur only in higher vertebrates: reptiles, birds and mammals (Jørgensen and Andersen, 1973; Wersäll, 1956). The existence of separated subtypes of mammalian vestibular type I and type II hair cells has been reported by classification of the hair bundles (Lim, 1976; Bagger-Sjöbäck and Takumida, 1988; Rennie and Ashmore, 1991; Scarfone et al., 1991; Lapeyre et al., 1992).

The variation in the sensory hair cell population of the vestibular sensory neuroepithelia is great among individuals. According to Lindeman, there are about 8,400 sensory hair cells in the utricular macula, about 6,900 sensory hair cells in the saccular macula and about 6,000 hair cells in the cristae of the guinea pig (Lindeman, 1967). In humans, 20-40 years old, the mean population of the sensory hair cells is 33100 in the utricle and 18800 in the saccule (Rosenhall, 1972). The polarization of sensory hair bundles changes along the striolar region in the maculae of the utricle and the saccule (Figure 1.2). Since the striola is not a real dividing line, some hair bundle orientations overlap in this region. The stereocilia and the kinocilium of the hair cells in the maculae are considerably taller in the peripheral than the striolar region (Lindeman,1969c). In the utricular macula the tallest stereocilia and the kinocilium of hair cells are facing each other along the striola. In the saccular macula, the longest stereocilia and the kinocilium of hair cells face away from the striola towards the periphery, which is essentially opposite to that of the utricular macula. In each crista, the sensory cells are oriented in the same direction over the entire surface. In the lateral crista, hair bundle polarization is towards the utricle while in the superior and posterior cristae hair bundle polarization is away from the utricle.

1.2.2 Cochlear Hair Cells

There are two types of sensory hair cells in the auditory organs. However, these hair cells are arranged in a much more regular manner and are more highly specialised and differentiated than other hair cell types. The sensory hair cells of the organ of Corti of the mammalian inner ear are classified as inner hair cells (IHC) and outer hair cells (OHC). They are distinguished by their location within the organ of Corti, by their relation to the supporting cells, by their morphology and innervation, by the relationship of the hair bundles to the tectorial membrane, and by the structure of the cytoplasm. The hair cells lie on supporting cells whose projections enclose the sensory hair cells. The inner hair cells lie in a single row along the length of the basilar membrane and are surrounded completely by supporting cells. The inner hair cells have a rounded base and a short neck, similar in appearance to type I hair cells of the vestibular sensory epithelia. The outer hair cells are cylindrical in shape, similar to type II hair cells in the vestibular organs and lie in three to four rows along the length of the basilar membrane. In mammalian organ of Corti, supporting cells do not surround the outer hair cell body and only the upper part and basal surfaces of the outer hair cells are in contact with the supporting cells. Other lateral surfaces of the outer hair cells are bathed by the extracellular fluid in the tunnel of Corti. The round nuclei of the outer hair cells are usually located in the basal part of the cell body. The outer hair cells show some particular structural specialisations that are related to their unique functioning. The most striking cellular feature is an organised endoplasmic membrane forming lateral cisternae running parallel to the inner surface of the outer hair cell plasma membrane (Forge et al., 1993). In the mammalian organ of Corti, the kinocilium of the cochlear hair cells generally disappears after birth, only the basal body remains in the mature hair cell. The number of the stereocilia on the apical surface of the

hair cells varies along the length of the cochlea. The stereocilia are arranged in three to four rows protruding from the cuticular plate and they are oriented radially, towards the periphery of the cochlea. The longest stereocilia define the cell orientation. The stereocilia show a linear formation on the inner hair cells and a V or W configuration on the outer hair cells.

1.3 THE SUPPORTING CELLS

At the apical surface of sensory epithelia, the supporting cells and hair cells form a regular mosaic pattern, called the reticular lamina, in both the vestibular system and the cochlea. The role of supporting cells inside the sensory epithelia is to separate adjacent hair cells and also to separate the hair cells from the basement membrane. The supporting cells play important roles in repair of the inner ear epithelia after damage (Forge, 1985; Raphael and Altschuler, 1991a; 1991b; Meiteles and Raphael, 1994b).

The general morphology of supporting cells is similar in the vestibular and cochlear sensory epithelia. The number of supporting cells contacting a single hair cell in vestibular epithelia varies, but in most cases there are two to six supporting cells involved (Meiteles and Raphael, 1994b). The supporting cells extend from the thin basement membrane to the lumenal surface of the sensory epithelium. At the apex of the supporting cell, there are many microvilli. The nuclei of the supporting cells are located in the basal parts of the cells, and are aligned in a fairly straight row, parallel with the basement membrane. They may be round or oval in shape with one or two large nucleoli. Their basal portions form a continuous sheet, which the myelinated nerve fibres pierce at intervals. The supporting cell bodies vary in their shape (Engström et al., 1972). Above the nuclei the cells are narrow,

but at the apical surface they are wider and fill in the region around the constricted necks of the hair cells. The basal cytoplasm is restricted to a rather thin perinuclear layer. The cytoskeletal framework of the supporting cell is composed of actin, intermediate filaments and microtubules, which interact with various membrane bound organelles (Anniko and Arnold, 1990; Kuijpers et al., 1991b; Takumida and Anniko, 1994; Takumida et al., 1995).

1.3.1 Vestibular Supporting Cells

The supporting cells described above, are probably the only non-sensory cell type in the vestibular maculae and cristae. They may be of metabolic importance for the sensory cells and nerve fibres and provide nutritive role in the vestibular sensory neuroepithelia. Vestibular supporting cells contain numerous large vesicles and they are believed to be secretory in nature. During the early stage of development, supporting cells in the vestibular epithelia are generally considered to produce the otolithic membrane, extracellular matrices that cover the apical surface of the epithelia (Goodyear et al., 1995). Sometimes globular substance protrudes on the apical surface of supporting cells, which may be related to the production and formation of the otolithic membrane (Harada, 1988). One immunohistochemical study of cytokeratin polypeptide expressions suggested there is a more complex tissue organisation in the vestibular sensory epithelia than previously shown by routine histomorphology. A small population of vestibular supporting cells expresses a particular cytokeratin pattern not seen in the majority. The significance of this is unknown (Kuijpers et al., 1991a).

1.3.2 Cochlear Supporting Cells

The supporting cells in the mammalian organ of Corti show a greater degree of differentiation and organisation than that seen in the vestibular system. The cochlear

supporting cells are classified as inner and outer pillar cells, inner and outer phalangeal (Deiters') cells, border cells, Hensen's cells, Claudius' cells and Boettcher's cells (not found in humans). The nuclei of the supporting cells lie basally whereas those of the hair cells sit close to the lumenal surface of the epithelium. The mammalian cochlear hair cells are rigidly supported by the supporting cells. The heads of the inner and outer pillar cells are interlocked or interdigitated with the apical surfaces of the inner hair cells and the first row of outer hair cells. The pillar cell bases are widely separated and their bodies filled with organised microtubule bundles. The inner and outer phalangeal cells provide support more directly to both the inner and outer hair cells. Each phalangeal cell body is positioned between a sensory hair cell and the basilar membrane, and an extended process that lies between the upper parts of the hair cells to form the reticular lamina. Deiters' cells only contact the apical and basal surfaces of the outer hair cells. Actin is present in the pillar cells and Deiters' cells (Flock et al., 1982, Slepecky and Chamberlain, 1983). The remaining cells associated with the organ of Corti are located either more medially or more laterally to the sensory hair cells and, therefore, are less involved in direct support to the sensory hair cells. Some of these cells may be involved in the production of certain components of the tectorial membrane during development (Lim, 1986; Goodyear et al., 1995).

1.3.3 Intercellular Junctions

The supporting cells in both the vestibular organs and cochlea are joined to each other and to the sensory cells by intercellular junctions. Intercellular junctions play an important role in the maintenance of the highly specific inner ear environment (potassium rich endolymph) and hair cell function. The presence of junctional complexes in the inner ear sensory

epithelia has been documented in several animal species (Jahnke, 1975; Bagger-Sjöbäck and Anniko, 1984; Forge, 1986; Meiteles and Raphael, 1994a; Raphael et al., 1994a). The junction complexes associated with supporting cells including tight junctions, adherens type and gap junctions. Tight junctions, frequently referred to as the **zonula occludentes**, are located at the apical part of the lateral membranes of the supporting cells and the sensory hair cells of the organ of Corti and the vestibular sensory epithelia. Tight junctions act as permeability barriers limiting the passage of substances between adjacent cells, sealing the endolymphatic surface from the inferior extracellular spaces, thereby maintaining the ionic and resting potential differences between endolymph and perilymph. Adherens junctions reside below the tight junctions which is also important during response to trauma in the inner ear sensory epithelia. Gap junctions are highly specialized intercellular connections formed by two parallel, closely apposed lateral plasma membranes that direct cell to cell communication across this site. The general distribution of the gap junction between supporting cells was reported in the vestibular and auditory sensory epithelia of most vertebrates from fish to mammals. The supporting cells of all inner ear sensory epithelia are coupled by gap junctions of different sizes (Jahnke, 1975).

1.4 INNERVATION OF THE INNER EAR

The vestibulocochlear, the eighth cranial, nerve innervates the inner ear. It is divided into a vestibular division, which innervates the sensory receptors associated with the vestibular system, and a cochlear division, which innervates the sensory receptors associated with the auditory system. The vestibular nerve is further subdivided and the superior branch innervates the macula of the utricle, part of the macula of the saccule, and the cristae of the

superior and lateral semicircular canals. The inferior branch innervates the macula of the saccule and the crista of the posterior semicircular canal.

The primary vestibular neurons originate from bipolar cells in the vestibular division. Their cell bodies (ganglion of the vestibular nerve: Scarpa's ganglion) are located in the internal auditory meatus. The axons of the vestibular nerve fibres project to the brainstem and terminate in the vestibular nuclei. Some secondary neuronal fibres travel to the cerebellum, the spinal cord, and to the nuclei of the third, fourth, and six cranial nerves that innervate muscles of the eyes to interact with other systems. The dendrites pass through the basement membrane, entering the vestibular sensory epithelium where they make synaptic contacts with the vestibular hair cells. The structural organisation of the innervation patterns within the vestibular sensory epithelia is complex and three basic types of nerve/calyx patterns are found in the rat saccular macula (Ross et al, 1986). Both chalice and bouton types of afferent nerve endings have been found in all mammals examined to date. There are also efferent fibres conveying impulses from the brain which pass parallel to the ascending afferent nerve fibres of the vestibulocochlear nerve to the vestibular sensory organs. Efferent nerve fibres synapse either on the afferent calyx surrounding the type I hair cell or on the basal part of the type II hair cell (Anniko, 1988). It is generally accepted that neurotransmission within the vestibular sensory organ (both afferent and efferent nerves) is chemical in nature.

The major difference of type I and type II hair cells is their innervations (Wersäll, 1956). The type I hair cell is surrounded by a chalice formed from the terminal end of an afferent nerve fibre of the vestibular nerve. The nerve chalice encloses the body of the type I hair

cell completely except for the apex just below the tight junction (See Figure 1.3 B). Most afferent nerve chalices enclose one hair cell or occasionally enclose two or more, but the same nerve fibre may divide to serve as chalices for two type I hair cells or more. The synaptic region is formed by the hair cell membrane and that of the nerve chalice. On the hair cell side of the synaptic membrane, there are found a number of synaptic bars. Efferent nerve endings regularly synapse with the afferent chalice mostly at its base. The innervation pattern of type II hair cell is characterised by bouton endings. Both afferent and efferent nerve endings form bouton like synapses with type II hair cells generally in the lower one-third of the cell body. The efferent nerve endings are filled with round synaptic vesicles and inside the type II hair cell a sub-synaptic cistern is often present. At the contact between the nerve ending and the hair cell a synaptic bar is regularly found with a short rod surrounded by a single layer of vesicles that rests on the hair cell membrane.

There are nerve fibres from the cochlear nerve division innervating at the bottom of the auditory hair cells and the nerve fibres are divided into afferents and efferents. Afferents are dendrites from the bipolar spiral ganglion neurons; efferents refer to the axonal endings of neurons located in the brain stem that carry information to the cochlea. The shape of inner hair cells is similar in appearance compared to type I hair cells of the vestibular epithelia, but many nerve endings from afferent nerve fibers are synapsed basally with single inner hair cell. Efferent nerve fibers synapse either on afferent nerve endings of the inner hair cells or on the basal aspect of an outer hair cell. The innervations of the outer hair cells are predominately many bouton like efferent nerve endings and with some small afferent nerve endings in the basal part of the outer hair cells.

1.5 THE PHYLOGENESIS OF THE INNER EAR

In fishes, amphibians, reptiles, birds and mammals, the basic structural organisation of the inner ear sensory epithelia is composed of the receptor hair cells, supporting cells and nerve endings. The hair cells in the vertebrate inner ear are very similar to the neuromast hair cells found along the lateral line system in fishes and in aquatic larval amphibians. The elongated neuromast hair cells are located in patches and their hair bundles are embedded in a layer of acellular substance. The function of neuromast hair cell device is to respond to the water movements relative to the body. Similar to the lateral line organ, the inner ear originates from ectodermal thickenings on either side of the head and it is speculated as a specialised part of the lateral line system (Romer, 1958). The inner ear and the lateral line system are termed as the acousticolateralis system due to the similarity in development, structures and functions. A considerable amount of information on hair cell regeneration in the acousticolateralis organs in lower vertebrates have been compiled in the past years (Balak et al., 1990; Baird et al., 1996; Corwin, 1981; 1985; Popper and Hoxter, 1984; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Song et al., 1995; Wever, 1981). It is necessary to briefly discuss the differences among the vertebrate inner ears for understanding the ear evolution.

1.5.1 The Evolution of the Ear

The evolution of the vertebrate ear is generally considered to begin in the fish ear. There are two types of inner ear sensory organs, the maculae and cristae, in the fish ear to perceive head and body position and movement. From the structural and functional points of view, the maculae and cristae are typical lateral line organs, but deeply embedded inside the skull bone. Certain types of the macular sensory epithelium have been found to be used

for sound reception in some fishes (Wever, 1981). Both vestibular and auditory organs derive from the ectodermal otocyst and the auditory system is phylogenetically younger than the vestibular system. Most of the amphibians have two acoustic organs, the amphibian and basilar papillae. These papillae consist of hair cells and supporting cells resting on a solid base. There is a basilar membrane in the reptilian ear with hair cells borne on it to form the basilar papilla which can move in response to the action of sound. The development of avian and mammalian acoustic organs followed the pattern of the reptilian ear. The specialised auditory organs, the basilar papilla or the organ of Corti, are present in the reptiles, birds and mammals only. The inner ear of vertebrates from reptiles to birds and mammals is a complex of six to eight sensory receptors. Of these organs only one, the basilar papilla or the organ of Corti, is definitely known as the auditory organ. Other sensory receptors serve the vestibular functions. The vestibular structures are highly conserved across virtually all classes of vertebrates. The hair cells are closely surrounded by the supporting cells in the vestibular sensory organs from fishes to human beings and in the auditory organs from the amphibians to the birds. The mammalian auditory organ, the organ of Corti, is highly differentiated and formed in a spiral shape which is different from the long tube shaped auditory organs in the reptiles and birds. The outer hair cells of the mammalian cochlea are not closely surrounded by the supporting cells.

1.5.2 The Avian Inner Ear

The morphological structures of the avian inner ear compared with the mammalian inner ear are highlighted. The vestibular labyrinth of birds contains three cristae in three (lateral, superior, and posterior) semicircular canals and three (the utricle, the saccule, and the lagena) otolithic macular organs. Mammalian animals only have utricular and saccular

maculae, the macula lagena is absent. A mountain-like process, called the crista eminentia, is present in the avian cristae. There are no hair cells in the eminentia and the crista sensory epithelium is divided in two portions giving an impression of a cross. The crista eminentia is also found in some mammalian species, like gerbils and mice. The structural organisation of the avian vestibular sensory epithelia is almost identical to that of the mammals and very similar to that of lower vertebrates. There are type I hair cells and type II hair cells in the avian vestibular epithelia (Jørgensen and Christensen, 1989) and they extend to various depths from the lumenal surface of the epithelium toward the basal lamina, but do not contact it. The supporting cells, by contrast, extend from the basal lamina to the lumenal surface of the epithelium, surround and separate the sensory hair cells. Jørgensen and Andersen showed that the extrastriolar regions of the maculae in birds are comprised almost entirely of type II hair cells (Jørgensen and Andersen, 1973). Both type I and type II hair cells are located in the striolar regions of maculae in the chicken inner ear (Goodyear and Richardson, 1992).

The avian auditory sensory organ, the basilar papilla, is a long, slightly curved epithelium with a narrow proximal (basal) end and a wider distal (apical) end. The surface of the basilar papilla appears as a regular mosaic formed by supporting cells and hair cells. Three types of hair cells can be recognised in the pigeon basilar papilla (Takasaka and Smith, 1971). Short hair cells, which are located on the free basilar membrane and adjacent to the hyaline cells from inferior edge of the basilar papilla, have an almost exclusively efferent innervation. Tall hair cells, which are situated on the superior side of the papilla, have a predominantly afferent innervation. Intermediate hair cells are intermediate in their height versus width ratio and are frequently found between the tall and short hair cells. The

chicken has only two types of hair cells, tall and short (Jahnke et al., 1969; Tanaka and Smith, 1978). Hair cells have oval or hexagonal apical surfaces and hair bundles are oriented with the longest stereocilia and the kinocilium directed towards the inferior edge of the papilla. On the basis of their position and innervation patterns, the tall and short hair cells can be considered as similar to the inner and outer hair cells in the mammalian organ of Corti. The supporting cells in the avian basilar papilla have narrow elongated apical surfaces that form borders around each hair cell and between hair cells. In a cross section of the basilar papilla, the hair cells are closely surrounded by supporting cells, packed in one row, without space between the bodies of hair cells and supporting cells. The architecture of the avian basilar papilla more closely resembles vestibular sensory epithelia of mammals and the auditory sensory epithelia of the lower vertebrates.

1.6 FUNCTIONS OF THE INNER EAR

The sensory hair cells of the inner ear in vertebrates have the capacity to assimilate the mechanical energy from the surrounding medium and transfer it, through a receptor transducer mechanism, to the innervating nerve fibres, which send the signal to the central nervous system. The hair bundles at the apex of the sensory cells play an important role in the biological activities of hearing and balance. Displacement of stereocilia toward the kinocilium results in excitation (depolarization) of the hair cell, while bending the stereocilia away from the kinocilium results in inhibition (hyperpolarization) of the hair cell. These changes stimulate the hair cells, creating ionic movement in their apical regions and result in the discharge of neural impulses at their bases.

1.6.1 The Vestibular System

Sensory receptors of the mammalian vestibular system are located in the inner ear and they function independently from both the external and middle ears. By interacting with vision and musculo-skeletal proprioception, the vestibular system keeps the body balance and co-ordinates head and eye movements.

The Otolithic Organs

The function of the otolithic organs is to perceive gravity, linear acceleration and the centrifugal force. The positional sensation and smooth movement of the body are also dependent on the balance of the body muscular tonus, extremities and ocular muscles. Stimulation of the hair cells in the maculae of the utricle and saccule is different from that in the cristae ampullares of the semicircular canals. It is easy to understand that the utricle and the saccule are located on the different planes, the utricle responds to horizontal linear acceleration and the saccule to vertical linear acceleration. Maximum stimulation of the macula of the utricle occurs when the head is bent forward or backward. Maximum stimulation of the macula of the saccule occurs when the head is bent side to side. The sensory hair bundles of the utricle and saccule are embedded in the otolithic membrane and the weight of otoconia on the hair cells makes these structures behave like gravity receptors. Changes in head position result in changes of the otolithic membrane position. There are three main hypotheses on the excitatory mechanism of otolithic organs, which depend on shearing, traction and pressure. Because of the curvature and overlap of each striola, hair cells are oriented at different angles, making the macula multidirectionally sensitive.

The Semicircular Canals

The crista of each semicircular canal is the receptor of angular accelerations of the head movement, but it also responds to non-natural stimuli, such as cold or warm water applied into the external ear canal, or electrical stimulation to the vestibule. The anterior and posterior canals, which are also called vertical semicircular canals, respond to rotation in the vertical planes, while the horizontal canal responds to rotation in the horizontal plane. The pendulum model is the most useful model for describing the physiologic properties of the semicircular canals. The function of the cupula is to transmit the endolymphatic flow of the semicircular canal to the sensory hairs and to shift the sensory hairs in one direction in detection of the angular acceleration of the head, leading to the production of action potentials in the vestibular afferent fibres. Because of the configuration and three dimensions of the canals, movement of the endolymph fluid within each semicircular canal causes displacement of the cupula. Semicircular canals in the same plane, but at different sides of the head, work antagonistically. For example, a right direction rotation causes stimulation of hair cells on the crista of right horizontal semicircular canal and inhibition of hair cells in the crista of the left horizontal semicircular canal. Movement of the endolymph within the vestibular semicircular canals can be induced by creating a temperature gradient. A simple clinical procedure, the bithermal caloric procedure, makes use of this effect for evaluating the functional integrity of the vestibular system. Briefly, this procedure involves irrigating the external ear canal of each ear, in turn, with warm water and cool water to induce convection current within the endolymph. This current displaces the cupula and causes stimulation of sensory hair cells in the vestibular neuroepithelia. Thus, this caloric stimulation produces a vestibular-ocular reflex (VOR), producing measurable eye movements (Bárány, 1916).

1.6.2 The Cochlea

The external ear and middle ear collect and convey sound to the inner ear where cochlear sensory receptors detect sound waves and transform the energy into neural impulses that can be processed in the brain. The mammalian auditory organ has a dual sensory system (inner and outer hair cells) with distinctly different innervation patterns. Sound vibrations enter the cochlea at the oval window via the foot plate of the stapes. The inward and outward movements of the stapes result in displacement of the inner ear fluid and a change in pressure gradient across the basilar membrane. The movement of the basilar membrane induces a shearing motion between the reticular lamina of the organ of Corti and the tectorial membrane, that deflect the stereocilia of the hair cells leading to stimulation. The anatomic organization and gradation of the basilar membrane, the tectorial membrane and the organ of Corti complex along the cochlear duct determine the frequency-specific area that is maximally stimulated by a given stimulating frequency. The high frequencies are analysed towards the base whilst the lower frequencies are analysed progressively towards the apex of the cochlea. It is now well accepted that in human subjects and experimental animals the cochlear hair cells can also transduce electric energy to mechanical energy, which can be recorded in the external ear canal. Pure tone signals are able to evoke from the ear an acoustic response, known as the otoacoustic emission (Kemp, 1978). Evoked otoacoustic emissions using acoustic distortion products (ADP) is a sensitive indicator of hair cell integrity in mammals and is independent of the integrity of the eighth cranial nerve (Kemp, 1978; Brown et al., 1989; Hotz et al., 1994). This technique provides frequency specific information and it is possible to test high frequency activity for detecting early cochlear dysfunction, as many cochlear lesions start at the basal part of the cochlea.

CHAPTER 2: OTOTOXICITY

To study ototoxicity, it is essential to define clearly what is meant by ototoxicity. Ototoxicity for the vestibular system and the cochlea is described as "the tendency of certain therapeutic agents and other chemical substances to cause functional impairment and cellular degeneration of the inner ear and especially of the end organs and neurons of the cochlear and vestibular divisions of the eighth cranial nerve" (Hawkins, 1976). It is well known that many groups of drugs may cause damages to the sensory epithelia of the inner ear. They include: the aminoglycoside antibiotics (streptomycin, kanamycin, gentamicin, neomycin, etc.); the loop diuretics (furosemide, ethacrynic acid); the salicylates; quinine and derivatives; and certain chemotherapeutic agents (cisplatin, nitrogen mustard). A summary is given only on the subject of aminoglycoside ototoxicity in this chapter.

2.1 AMINOGLYCOSIDE OTOTOXICITY

Streptomycin was first isolated from the Actinomycete genus Streptomyces in 1944 (Waksman et al., 1944; Waksman, 1953). It was the first antibacterial agent to be effective against tuberculosis and soon introduced into clinical use. Many other drugs of the aminoglycoside family, neomycin (1949), kanamycin (1959), gentamicin (1963), tobramycin (1967), sisomysin (1970), amikacin (1972), netilmicin (1975), etc., were discovered and clinically used to treat life threatening infections. Tuberculosis incidence has increased world wide over the past 10 years and streptomycin still remains important for the treatment of tuberculous infections despite drug resistance. The main limit to the use of aminoglycoside antibiotics is that most of them damage the inner ear sensory

epithelia, the kidney and neural tissue. The remarkable nephrotoxicity and ototoxicity, on the cochlea or the vestibular system or both, of aminoglycosides have been well established by their clinical case reports and from experimental studies. Ototoxicity is perhaps the most important and serious of the various side effects, in that the inner ear damage has been thought to be irreversible in mammals and quite unpredictable. Some reports showed that streptomycin is predominantly vestibulotoxic; gentamicin and tobramycin are mainly vestibulotoxic in man but can affect both the auditory and vestibular systems; whereas neomycin and kanamycin produce cochlear toxic effects in most species (Keene and Hawke, 1981; Chiodo and Alberti, 1994).

2.1.1 Pharmacology of Aminoglycoside Antibiotics

Aminoglycoside antibiotics are a group of polycationic compounds poorly absorbed upon oral administration from the gastrointestinal tract. Aminoglycoside antibiotics are highly water soluble, and their positive charges prevent easy entry into cells via diffusion across the cell membrane. Excretion of aminoglycosides is predominantly via the kidney through glomerular filtration (Lerner and Matz, 1980). Aminoglycoside antibiotics enter the fluids of the inner ear by way of the blood stream either directly by intravenous injection, or following intramuscular or subcutaneous injections, but the exact mechanism by which the ototoxic drugs reach the inner ear fluids and damage the hair cells is unknown. Studies of rodent animals have suggested that following topical application of the aminoglycosides to the middle ear cavity, the drug may enter the inner ear by passing through the round window membrane or annular ligament to the perilymph in the scala vestibuli and through the Reissner's membrane into the endolymph space in the scala media (Stupp et al., 1973; Jahnke, 1988).

2.1.2 Gentamicin

Gentamicin was discovered in 1963 (Weinstein, et al., 1963a; 1963b) and it was unique in that it was the first aminoglycoside to be isolated from a source other than the *Streptomyces,* coming from *Micromonospora purpurea.* The formula weight of gentamicin is 463. To date gentamicin is one of the most commonly used, effective, broad spectrum aminoglycosides due to its efficient bactericidal nature, synergism when combined with other antibiotics, low rate of true resistance and relatively low cost. Gentamicin has been shown to affect both vestibular and cochlear systems but is less toxic than streptomycin in man (Lange, 1989). The ototoxicity from gentamicin has been reported to range from 3% to 25% (Moffat, 1987). Its toxic effects in humans were said to be predominantly upon the vestibular system within the range of human dosage (Meyers, 1970; Dayal et al., 1974; 1979). In guinea pigs, gentamicin appears to be toxic to the cochlea and the vestibular system (Kitasato et al., 1990), and the effects are dose dependent (Bamonte et al., 1980; Collins and Twine, 1985; McDowell, 1982; Twine, 1985).

2.2 HEARING LOSS AND VESTIBULAR DISTURBANCE

Cochlear toxicity can be measured by means of pure tone audiometry, auditory brainstem evoked responses and otoacoustic emissions in the clinic. Vestibular toxicity might be more difficult to measure. Reliable, reproducible and subjective assessments of vestibular function are much less readily available than that of cochlear function (Scott and Griffiths, 1994). Rotational stimulation with electronystagmographic recording or caloric stimulation might be used in clinics to evaluate vestibular dysfunction. Clinical signs of peripheral vestibular damage caused by systemic administration of aminoglycoside antibiotics usually affect both ears. Usually no nystagmus is observable and the caloric tests show a bilaterally

diminished or absent response. Rotating chair tests confirm significantly reduced or no evoked vestibular-ocular responses when both ears are affected (Hain and Zee, 1991). Local application with aminoglycoside antibiotics would cause dysfunction of the treated ear. The classic pattern of cochlear damage caused by aminoglycoside usually goes unnoticed until significant hearing loss occurs. The initial symptom of cochlear damage is often tinnitus, which is usually high-pitched and continuous. The mechanism of the production of the tinnitus is unclear.

Improvements in administration practices and in particular therapeutic drug monitoring have reduced ototoxic incidence in patients, but the problem of aminoglycoside ototoxicity still persists. Ototoxicity induced by aminoglycoside antibiotics in the developing world is exacerbated where aminoglycosides are freely prescribed or easily abused as remedies, without efficient monitoring. In some developing countries, aminoglycosides are used as the main antibiotic to combat infectious diseases because of economic constraints and other factors. Sheng et al. reported 175 cases of Chinese children suffering from hearing loss induced by gentamicin, 113 cases (65%) were given the drug as a treatment for common cold and fever (Sheng et al., 1985). Similarly, the increasing use of aminoglycoside antibiotics to treat pneumonia, diarrhoea and tuberculosis has also led to ototoxicity in significant numbers of patients with childhood deafness (Matz, 1990). According to the 1994 WHO working report, it is estimated that there are 1.7 millions children with hearing disability in China, and aminoglycosides are responsible for up to a third of profoundly deaf children in China in the last ten years. The incidences of aminoglycoside cochlear and vestibular toxic side effects were estimated at 7.5% each (Govaerts et al., 1990). Other clinical reports revealed the aminoglycoside cochleotoxic

incidence from 26% to 47% and vestibular toxicity from 5% to 15% (Fee, 1980; Winkel et al., 1978; Fausti et al., 1992). However, most of these prospective trials have been performed without control groups. The auditory or vestibular impairments may be directly caused by the infectious diseases, rather than the antibiotics used for treatment (Davey et al., 1983).

Severe acute vestibular dysfunction was reported on two patients with large tympanic membrane perforations after using Garasone ear drops containing 0.3% gentamicin and the author recommended caution in the use of ototoxic agents (Longridge, 1994). However, a previous report in the treatment of 300 patients with tympanic membrane perforation and otorrhea found no evidence of ototoxicity following the preoperative use of 0.3% gentamicin topically (Gydé, 1976). Clinically, ototoxicity from topical aminoglycoside antibiotic drops was reported to be extraordinarily low (Roland, 1994) although most of the aminoglycosides showed ototoxicity in animal models (Brummett et al., 1976; Rohn et al., 1993; Kuhweide, 1995).

2.3 EXPERIMENTAL AMINOGLYCOSIDE OTOTOXICITY

The ototoxic side effects of streptomycin were revealed in 1945 by Hinshaw and Feldman. They reported irreversible deafness and balance disturbance during the first clinical trial of the drug in tuberculous patients (Hinshaw and Feldman, 1945). Caussé first showed that streptomycin caused lesions in the sensory neuroepithelium of the inner ear rather than at a central site (Caussé, 1949). The destruction and disappearance of the hair cells of the inner ear demonstrated the nature of streptomycin action in early histopathological serial sections of celloidin embedded specimens from experimental animals (Caussé, 1949; Berg,

1951; Hawkins et al., 1952). Investigations of numerous aspects of the inner ear morphological damage induced by the aminoglycoside antibiotics and the pathophysiologic consequences have been performed in a variety of laboratory animals. Much of our understanding of aminoglycoside drug induced ototoxicity has been derived from the results of histopathological studies of the lesions in the animal ears (Hawkins and Johnson, 1981). A few cases of comparable human temporal bone pathology of ototoxic aminoglycosides were reported in the literature. Human temporal bone specimens are few because it is not possible to biopsy the inner ear during life and it is difficult to acquire post-mortem specimens free from artefact (Wright, 1986).

2.3.1 Damage Patterns of Inner Ear

Comparison of the experimental pathology of ototoxicity in animals with the histopathology in human temporal bones has demonstrated that the major changes occurring in the inner ear in response to aminoglycosides are the same. The histopathology of human temporal bones demonstrated that aminoglycosides induced hair cell death in the organ of Corti and vestibular organs is associated with permanent loss of hearing and balance functions (Moffat, 1987). Inner ear damage of cochleotoxicity is broadly similar for the various aminoglycoside antibiotics. Degenerative changes start in the outer hair cells of the basal turn and extended to the apex (Wright, 1969; Tange and Huizing, 1980; McDowell, 1982; Forge, 1985; Huizing and De Groot, 1987; McDowell et al., 1989). The basal turn is the high frequency sensing area of the cochlea and this damage pattern explains the clinically observed initial rise in threshold at high frequency. Threshold increases subsequently extend to lower frequencies including the speech frequencies if the progressive ototoxicity results in the loss of hair cells towards the apical turn. Damage to

the outer hair cells spreads from the inner most to the outer most row. Aminoglycoside toxic effects on the vestibular function were successfully monitored in guinea pigs by nystagmic responses to rotational stimulation (Aran et al., 1982; Meza et al., 1992). Gentamicin caused an early and severe reduction of the vestibular-ocular reflex (VOR) gain in guinea pigs and histological examination revealed that vestibular function deteriorated in parallel with the damage of the vestibular sensory epithelia (Pettorossi et al., 1986). The initial and most extensive hair cell damage occurred in the crest of the cristae and the striolar regions of the utricular and saccular maculae in the vestibular system (Lindeman, 1969a; Wersäll et al., 1973). Under scanning electron microscopy, the hair bundle of the sensory cells showed various degenerative changes: the stereocilia collapse, fuse to form giant stereocilia, even become lost and the apical surface becomes covered with debris (Wersäll, 1981; Takumida et al., 1989). With increasing ototoxicity, progression of hair cell loss extends towards the periphery of the vestibular receptors (Lindeman, 1969a; Twine, 1985). There is also differential sensitivity among the different vestibular sensory epithelia, the cristae showing proportionately greater hair cell loss than the utricular macula, and the utricular macula more than the saccular macula (Lindeman, 1969a). The reasons for this pattern of differential damage are not clear.

Immunocytochemical studies using antibodies raised against aminoglycosides that enable localization of the drugs in the inner ear tissues have shown that they are selectively distributed in hair cells (De Groot et al., 1990; Hiel et al., 1992). Generally, outer hair cells from the cochlea and type I hair cells from vestibular organs are more susceptible to ototoxic aminoglycosides than cochlear inner hair cells and type II vestibular hair cells (McDowell, 1982; Forge, 1985; Watanuki et al., 1972; Wersäll et al., 1973; Wersäll and

Hawkins, 1962). The supporting cells may stay intact for a considerable length of time. From animal experiments, aminoglycoside damage also occurred at the stria vascularis (Hawkins, 1973; Forge and Fradis, 1985; Forge et al., 1987), neurons and the spiral ganglion (Bicher et al., 1983; Chiodo and Alberti, 1994; Koitchev et al., 1982; Matz, 1993) and affected cochlear physiological responses (Brummett et al., 1978; Aran, 1981; Brown et al., 1989). Damages to the otoconia and otolithic structure (Cañizares et al., 1990; Campos et al., 1994), the vestibular non-sensory cells (Pender, 1985; Ge and Shea, 1993; Yoshihara et al., 1994; Oda et al., 1995) and vestibular ganglion (Harada et al., 1991; Black and Pesznecker, 1993) have also been reported. But, the effects on these structures are probably secondary to hair cell damage.

Kimura and colleagues reported that the vestibular lesions resulting from local application were greater than those produced by systemic injection (Kimura et al., 1991). Concentrated gentamicin solution was introduced into the guinea pig bulla and progressive degeneration of the hair cells was observed in the vestibular and cochlear sensory epithelia 24 hours after the first injection (Wersäll et al., 1969). Hair cell loss in the central part of the cristae and at the striolar region of maculae was found after a single application of 4% gentamicin into the middle ear through the tympanic membrane and, the pattern of hair cell loss extended towards the periphery of the cristae and maculae when animals survived longer (Harada, 1988). The aminoglycoside sisomysin was locally applied to guinea pig ears and damage to vestibular hair cells took place as early as the fifth day. After two years, the entire sensory epithelium disappeared, only kinociliary-like structures were observed at the apical surface of the vestibular epithelia (Dupont et al., 1993). In experimental studies, where the animals used are healthy, high doses and/or a considerable

length of treatment are necessary in order to create an ideal animal model of aminoglycoside ototoxicity. The extent of hair cell loss is generally dose related both on the vestibular and auditory sensory neuroepithelia (McDowell, 1982; Collins and Twine, 1985; Twine, 1985). There is, however, a considerable variation in the progression of damage and in the percentage of hair cell loss in the inner ear tissues using the same dose regime.

The procedures for the organotypic culture of inner ear tissues and for hair cell isolation have been developed (Brownell et al., 1984; Valat et al., 1989; Van De Water and Ruben, 1971) that makes it possible to study the effects of ototoxic aminoglycosides directly. Studies of acute aminoglycoside ototoxicity in isolated hair cells have been performed and the acute effects are distinctly different from the chronic effects of aminoglycosides (Dulon et al., 1989; Ernst et al., 1994). Cultured explants from the neonatal mouse cochlea have been shown to be a useful means to examine in vitro the ototoxic effects of aminoglycosides (Anniko et al., 1982; Richardson and Russell, 1991; Kotecha and Richardson, 1994). The latter studies demonstrated that in neonatal mouse cochlear cultures, hair cells in the apical coil are less sensitive to neomycin than those in the basal coil and outer hair cells appear to be more severely damaged by neomycin than inner hair cells (Richardson and Russell, 1991). The differential sensitivity of hair cells along the cochlea in vitro mimics the ototoxic response of cochlear hair cells in vivo, which suggests that the differential sensitivity is inherent within the tissues and is not related to the drug distribution.

2.3.2 Modes of Hair Cell Loss

Hair cell loss from the inner ear sensory epithelia occurs in most vertebrate animals when they are exposed to various damaging factors. As hair cells are damaged and lost, the adjacent supporting cells expand to replace the degenerating hair cells and close the space once occupied by the lost hair cells to keep the lumenal surfaces of the epithelia intact (Forge, 1985; Hawkins and Johnson, 1981; Wersäll et al., 1973). The supporting cells form "phalangeal scars" to repair the sensory neuroepithelium of the cochlea (Raphael and Altschuler, 1991a; 1991b). Freeze fracture examinations showed no significant alterations of the tight junctions associated with the hair cell apex and thin sections also showed no breakdown of permeability barriers in the reticular lamina (McDowell et al., 1989). Damaged and lost hair cells were replaced by expansion of adjacent supporting cells to form the scar in the vestibular neuroepithelia (Meiteles and Raphael, 1994b; Wersäll et al., 1973), similar to the repair processes in the organ of Corti. Microfilaments, cytokeratins, adherens junctions, and tight junctions of the supporting cells rearrange their distribution in damaged areas and maintain the reticular lamina intact. The lumenal surfaces of the inner ear sensory epithelia are bathed in the potassium rich endolymph. The repair process of supporting cells may prevent the mixing of the endolymph and perilymph after hair cell loss which would have an effect on the inner ear functions if it happened.

In the adult mammalian organ of Corti, the damaged hair cells degenerate within the epithelium. Following administration of aminoglycosides to guinea pigs, outer hair cell loss in the organ of Corti appears to follow an ordered sequence, the hair cell body degenerated and was taken up by the phagocytic activity of the neighbouring cells inside the sensory epithelium, without destruction of the reticular lamina (Forge, 1985;

McDowell et al., 1989; Raphael and Altschuler,1991a). The process of hair cell loss does not cause an inflammatory reaction in the damaged area. In acoustic trauma experiments, the degenerating hair cell bodies and debris from degenerated hair cells can be found scattered in the space beneath the reticular lamina (Bohne and Rabbitt, 1983; Fredelius et al., 1990). Another mode of hair cell loss, extrusion of some degenerated and killed hair cells between the expanded supporting cells from the epithelia into the lumenal surface has been reported in the vestibular and auditory sensory epithelia of lower vertebrates. Damaged hair cells were extruded from the lumenal surface of the avian basilar papilla after noise trauma (Cotanche, 1987) and after aminoglycoside intoxication (Marean et al., 1993). Hair cell extrusion appeared to occur in the vestibular sensory epithelia of fish, amphibian, and birds as a consequence of ototoxic drug treatment (Yan et al., 1991; Baird et al., 1993; Weisleder and Rubel, 1993). In addition, degenerated hair cells were also found within the avian basilar papilla after noise exposure (Raphael, 1993). However, compared with the mammalian inner ear tissues, the extrusion mode may be a predominant means to remove degenerated hair cells in the compact tissues of lower vertebrates in which hair cells are closely surrounded by adjacent supporting cells.

In TEM sections, a typical sign of hair cell damage was an increase in liposomes, proliferation of the endoplasmic reticulum and formation of Hensen's body (De Groot et al., 1991). Some of the hair cells undergoing degeneration following aminoglycoside treatment resemble the phenomenon of apoptosis (Forge, 1985). Apoptosis refers to the changes a cell undergoes during programmed cell death, which is a gene-directed process (Alison and Sarraf, 1992; Raff, 1992). Programmed cell death or apoptosis is the most common form of cell death, which is associated with a multistage process of DNA

fragmentation in many cell types and can be examined morphologically and biochemically (Majno and Joris, 1995). Apoptosis occurs individually or in small groups of cells surrounded by normal tissue and apoptotic cells are easily recognisable by their characteristic morphological features. These apoptotic features include shrinkage and condensation of the cytoplasm; margination of nuclear chromatin; and fragmentation of the cell and of the nucleus but preservation of other intact organelles with little swelling of mitochondria. These features distinguish apoptosis from necrosis, in which cells dying as a result of acute injury swell and lyse, spilling their contents into the extracellular space, eliciting an inflammatory response (Kerr et al., 1972). Perhaps the most fundamental feature of programmed cell death is that the dead cells or fragments are rapidly phagocytosed by neighbouring cells or macrophages, before there is any leakage of cytoplasmic contents and without an inflammatory response.

2.4 MECHANISMS OF AMINOGLYCOSIDE OTOTOXICITY

It appears that aminoglycoside ototoxicity can take two forms, acute and chronic, with different mechanisms. Acute reversible loss of cochlear microphonics in animal experiments may occur with local and high doses of aminoglycosides, as a result of competitive antagonism between the drug and calcium (Takada and Schacht, 1982). The acute effects may result from displacement of calcium ions by aminoglycosides from the plasma membrane and the blockage of tranduction channels and currents of the hair cells, which can be blocked by raising calcium concentration (Dulon et al., 1989; Richardson and Russell, 1991). Most clinically observed aminoglycoside ototoxicity is, however, of the chronic type which is generally accepted to be largely irreversible in mammals and it is based on the metabolism of the drug to cause hair cell death. Early studies of

aminoglycoside pharmacokinetics gave rise to the hypothesis of the accumulation and high concentration of the aminoglycosides in the inner ear fluids (Voldrich, 1965; Vrabec et al., 1965). However, due to the technical difficulties in obtaining the endolymph, most studies have been conducted on the perilymph. The perilymph concentrations of different aminoglycosides were tested lower than the peak serum levels and the tissue levels achieved by a drug did not correlate with its ototoxic effect (Tran Ba Huy et al., 1981; 1986; Harpur and Gonda, 1982). A recent paper reported that aminoglycosides were taken up equally by the guinea pig vestibular and cochlear hair cells although there may be differences in vestibulotoxic and cochleotoxic potentials (Aran et al., 1995). The selective toxicity of aminoglycosides to the inner ear cannot be explained by selective tissue penetration of aminoglycosides.

The most consistent and favourable hypothesis proposed to date to account for the specific mechanism of ototoxicity envisages a specific binding interaction between the aminoglycoside and phosphatidylinositol 4'5' bisphoaphate ($PhIP_2$) of hair cell membrane (Schacht, 1986; Schacht and Weiner, 1986; Henley and Schacht, 1988). The aminoglycoside drugs have been found to interact with polyphosphoinositides and the metabolism of $PhIP_2$ in the inner ear is inhibited by long term drug treatment. The specific binding sites for aminoglycosides are located on the inner leaflet of the hair cell plasma membrane and the drug has to cross the membrane to reach it. The first step in ototoxicity is suggested to be an electrostatic interaction of the aminoglycoside with a negatively charged component of the outer plasma membrane. The aminoglycoside is then transported into the cell by an energy dependent process that can be inhibited by metabolic blockers. The next and most crucial step is the binding of the drug to the plasma

membrane phosphatidylinositol-4,5-biphosphate (Williams et al., 1987). Once inside the cell, the aminoglycoside may interfere with further necessary intracellular reactions, thus leading to progression of the ototoxic mechanism. These studies strongly challenge the concept of the accumulation theory and suggest that ototoxicity manifested by aminoglycosides is likely to be an intrinsic property of the drugs, and a result of drug induced specific effects on metabolic disruption of hair cells, rather than a result of their concentrations in the inner ear fluids.

How to answer why some of the drugs cause more damage to the cochlea than to the vestibular system and vice versa? Why some hair cells are more susceptible than other hair cells in the cochlea and in the vestibular system? An oxidative metabolism was demonstrated to be involved in the formation of a cytotoxic metabolite from aminoglycosides (Huang and Schacht, 1990) and the existence of a drug derived cytotoxic metabolite may exert the deleterious intracellular effects to hair cells. Further investigations of the enzymatic activity and the nature of the metabolite localized the enzyme(s) of gentamicin metabolism in subcellular fraction from guinea pig liver and provided evidence that the toxin is a gentamicin derivative (Crann et al., 1992). The hypothesis is that the aminoglycosides are metabolized or "activated" to a cytotoxic metabolite and this complex produces free radicals that can damage the target cells of the inner ear and proximal renal tubules (Schacht, 1993; Priuska and Schacht, 1995). Free radical scavengers and iron chelators demonstrated their protective effects to gentamicin ototoxicity in guinea pigs in vivo (Song and Schacht, 1996). The balance between synthesis of intracellular toxin and its detoxification may determine the degree of toxicity in different types of cells and for different aminoglycoside antibiotic molecules. Selective

tissue distribution of the metabolizing enzymes may explain the selective aminoglycoside toxicity toward the kidney and the inner ear as well as why some aminoglycosides preferentially damage the cochlea and others the vestibular system, and why particular cell groups within these tissues are susceptible (Barclay and Begg, 1994).

CHAPTER 3: REPAIR AND REGENERATION OF HAIR CELLS

Sensory hair cells are produced throughout life in the ears and the lateral line organs of fish and amphibians (Corwin, 1981; Corwin, 1985; Jørgensen, 1981; Popper and Hoxter, 1984). The cold-blooded fish and amphibians also have the ability to restore a damaged population of hair cells (Lombarte et al., 1993; Baird et al., 1993). The continuous post-embryonic production of sensory hair cells has been found in all avian vestibular sensory organs but not in the basilar papilla (Jørgensen and Mathiesen, 1988; Roberson et al., 1992). The avian auditory epithelium and mammalian inner ear sensory organs were considered to cease production of new hair cells shortly after birth (Ruben, 1967; Bredberg, 1968; Katayama and Corwin, 1989). Over the last few years, it has been demonstrated that production of new sensory hair cells following trauma induced hair cell loss occurs in the auditory and vestibular epithelia of the avian inner ear (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Weisleder and Rubel, 1992). Some review papers have summarised the work on hair cell regeneration (Corwin, 1992; Cotanche and Lee, 1994; Cotanche et al., 1994; Duckert and Rubel, 1993a; Tsue et al., 1994b; Forge, 1996).

3.1 HAIR CELL REGENERATION IN THE AVIAN INNER EAR

The evidence that led to this discovery was from the avian auditory epithelium, where hair cells are normally produced only during a few days of embryonic life (Katayama and Corwin, 1989) and the number of hair cells reaches a plateau before birth (Tilney et al., 1986). In 1987, two independent discoveries were made, indicating the hair cells in the avian basilar papilla were capable of proliferative repair after hair cell loss induced by acoustic overstimulation (Cotanche, 1987) or ototoxic aminoglycoside administration

(Cruz et al., 1987). Cotanche produced hair cell loss in chicks by exposure of 10 day old chicks to an 1500 Hz pure tone at 120 dB for 48 hours. After 48 hours of recovery, scanning electron microscopy identified the presence of immature hair cell stereocilia bundles in the area of noise induced hair cell loss. Over a period of ten days, the new stereocilia bundles underwent a progressive maturation, which was similar to embryonic hair cell development (Cotanche, 1987). Cruz and colleagues investigated the temporal pattern of hair cell loss in the basilar papilla after a 10 day course of gentamicin administration in hatchling chicks. Unexpectedly, a progressive partial restoration of the normal hair cell counts over several weeks was observed by light microscopy, suggesting regeneration of the sensory cells in the auditory epithelium (Cruz et al., 1987).

Two parallel studies provided further evidence that the repopulating of the damaged avian basilar papilla results from the production of a new generation of hair cells. Tritiated thymidine (^{3}H-thymidine), a radioactive analogue of the nucleic acid thymidine, has been used as the cell proliferation marker which is readily incorporated into the replicating DNA of proliferating cells during "S-phase" of the cell cycle. After mitosis, the nucleus of each daughter cell containing the radioactive marker may be detected using autoradiographic techniques. Tritiated thymidine was administered during the recovery period following noise exposure in neonatal chicks (Corwin and Cotanche, 1988) and mature quails (Ryals and Rubel, 1988). After ten days of survival, clearly labelled nuclei of hair cells and supporting cells were found in the noise damaged region of the basilar papilla. No labelled hair cells and supporting cells were found outside the damaged region or in birds not exposed to noise. These results suggest that post-trauma replacement of hair cells by cell division depends on the precise location of damage in the avian basilar papilla. Hair cell

regeneration in the avian basilar papilla occurs only after extrusion of degenerated hair cells and does not occur where damaged hair cells remain (Cotanche, 1987). Therefore, this regeneration process is not simply a post-embryonic extension of the production of hair cells, like the lateral line organs of fish and amphibians. After 10 days of gentamicin treatment, hair cells and supporting cells incorporated with tritiated thymidine were also seen in hair cell loss regions of the basilar papillae from experimental animals (Lippe et al., 1991). Even a single high dose of gentamicin injection could cause hair cell loss in the basilar papilla and immature hair cells were present three days later in regions where native hair cells had been killed (Janas et al., 1995).

Immunohistochemical labelling of proliferative cells has also been used to detect 5-bromodeoxyuridine (BrdU, a thymidine analogue) incorporated into DNA in S-phase cells. Monoclonal antibodies specifically generated against BrdU molecules have been produced (Gratzner, 1982). In recent years, immunohistochemical labelling of BrdU has been used to examine hair cell regeneration in the inner ear (Hashino and Salvi, 1993; Raphael, 1992; Raphael et al., 1994b; Roberson et al., 1992; Stone and Cotanche, 1994; Weisleder and Rubel, 1992). Although the avian cochlea may regenerate hair cells in response to damage induced by acoustic trauma or ototoxic drugs, ongoing proliferation of hair cells is not the characteristic of this organ. The ongoing postnatal regeneration of vestibular epithelial cells suggests that the vestibular epithelia may retain the potential for lesion repair. Hair cell regeneration had been observed in the avian vestibular epithelia after streptomycin toxicity (Weisleder and Rubel, 1992; 1993). One day after the 7 days drug injection, clear evidence of vestibular toxicity was present in treated chickens and hair cell loss occurred progressively over 20 days. Immunocytochemical BrdU labelling and tritiated thymidine

autoradiography revealed a larger number of labelled mitotic nuclei in the treated group than in the untreated group. At 60 days, the tissue had essentially recovered the normal hair cell number but labelled type II and type I hair cells as well as supporting cells still could be identified. These studies demonstrate that the avian vestibular system is able to regenerate hair cells, both on an ongoing basis and through up-regulation of proliferative regeneration after hair cell loss.

Long term studies demonstrated that regeneration of hair cells is involved in the recovery of avian inner ear sensory epithelia, with hair cell counts returning to normal and hair cell re-innervation. In adult quail, both tall and short hair cell numbers were significantly reduced 10 days following acoustic insult. But tall hair cell number was near normal within 60-90 days after trauma, and short hair cell recovery had a slower time course (Ryals et al., 1989). Serial thin sections for transmission electron microscopy analysis showed that afferent neural terminals attached on remaining cells appeared normal, but were not attached to the immature regenerated hair cells at early times after noise trauma. By 10 days following acoustic trauma, however, both afferent and efferent synaptic contacts were found on newly regenerated hair cells (Ryals and Dooling, 1996). Three to six months later afferent terminals had regained a more normal appearance but were less numerous on tall hair cells. Some return of efferent like terminals was seen often contacting two tall hair cells (Ryals et al., 1992).

After termination of ototoxic gentamicin treatment, the new hair cells in the chick basilar papilla were easily distinguished from supporting cells but were immature within the first 28 days. Hair cell numbers were equivalent to untreated control animals by six weeks, but

full maturation of the short hair cells and their synaptic contacts were not complete until 20 weeks. At this time point, the cells have assumed the closely packed mosaic pattern typical of the control ears (Duckert and Rubel, 1990; 1993b). During the maturational process, hair cell apical surfaces expanded, numbers and complexity of cellular organelles increased, cell shape elongated. In addition, stereociliary bundles progressively grew in length and changed in arrangement and orientation.

Given that avian hair cells are able to regenerate following acoustic or ototoxic drug exposure, are regenerated hair cells capable of restoring their function? In theory, complete restoration of function to normal sensitivity is dependent on several conditions. The regenerated hair cells must be physiologically mature, they must be innervated by appropriate axons, and the other elements of the peripheral and the central nervous system must be functional. Both behavioural and electrophysiologic studies suggested that the avian hair cell recovery also resulted in a recovery of the auditory and vestibular functions (Girod et al., 1989; McFadden and Saunders, 1989; Tucci and Rubel, 1990; Saunders et al., 1992; Bohne and Harding, 1992; Duckert and Rubel, 1993b; Niemiec et al., 1994; Jones and Nelson, 1992; Carey et al., 1996). Ototoxic aminoglycosides can cause near complete hair cell loss in the proximal part of the basilar papilla without affecting the tectorial membrane. After 16 to 20 weeks post-treatment, there was a significant recovery of the threshold shifts in all but the high-frequency regions in chicks (Tucci and Rubel, 1990). This pattern of functional recovery parallels the structural recovery of the hair cells in the basilar papilla (Girod et al., 1991; Duckert and Rubel, 1993b). Following kanamycin poisoning of the European starling, behavioural tests of auditory function were also used to demonstrate hearing recovery during regeneration (Marean et al., 1993). Destruction of

the regenerated hair cells by re-treating the animals with kanamycin reinstated the high frequency hearing loss, suggesting that the regenerated hair cells are responsible for the functional recovery (Hashino et al., 1992; Marean et al., 1995). Like the basilar papilla, regenerated hair cells in the avian vestibular sensory epithelia appear to be functional and capable of relaying information to higher vestibular centres. Virtually complete physiological recovery of vestibular function occurred in chicks over a period of 70 days following hair cell destruction by aminoglycoside streptomycin (Jones and Nelson, 1992). Compound action potentials of the vestibular nerve partially recovered within two weeks of the last treatment day. This period of recovery corresponded temporally with the appearance of new, but immature hair cells. Full recovery of normal activation latencies and amplitudes required new sensory hair cell maturation, perhaps in association with the appearance of type I hair cells, and re-innervation. Vestibular ocular reflex recovery in chickens was reported during the course of hair cell regeneration. The hair cell loss and recovery in the vestibular cristae after streptomycin treatment is associated with changes in the gain of the vestibular ocular reflex (Carey et al., 1996).

3.2 IN VITRO STUDIES OF HAIR CELL REGENERATION

Morphological studies in organotypic cultures provide the investigator with some clear advantages relative to the in vivo condition. In vitro studies offer a convenient means for examination of tissues under controlled, easily manipulated conditions. Development of organotypic cultures can be studied to the exclusion of other external inductive influences. For introducing the organotypic cultures and subsequent studying the development of the avian inner ear, techniques and methods were developed and published (Friedmann, 1959; Corwin and Cotanche, 1989; Stone and Cotanche, 1991). These authors reported on

specific aspects of sensory cell development in culture, including stereocilia development and orientation. Cultures of avian vestibular sensory epithelia have been used to examine regeneration phenomenon in vitro because these tissues appear to be continually producing hair cells (Warchol and Corwin, 1993) and proliferation can be increased after aminoglycoside induced hair cell loss in the avian vestibular epithelia (Tsue et al., 1994a). Compared with explanted normal utricles, cultured utricles pre-damaged by streptomycin in vivo also demonstrated an increase in proliferation and differentiation of progeny into hair cells (Oesterle et al., 1993). Hair cell markers, calmodulin and β tubulin antibodies, have been used together with BrdU labelling to observe supporting cell proliferation in the sensory epithelium of the chick basilar papilla after hair cells were killed by streptomycin in vitro (Stone et al., 1996). Some supporting cells generated in culture after hair cell loss begin to differentiate into hair cell specific phenotypes in vitro.

3.3 PRECURSOR CELLS

In lower vertebrates, supporting cells in the sensory epithelia proliferate throughout the life of animal and can give rise to new hair cells (Corwin, 1981; 1985). Immunocytochemical study has shown that hair cell precursors and supporting cells are closely related. An antibody which labels supporting cells also labels the proliferating cells that give rise to new hair cells and supporting cells in fish (Presson, 1994). In vivo studies of hair cell regeneration in amphibian lateral line organs demonstrated that hair cell loss stimulates mitosis of supporting cells that surround the hair cells and eventual differentiation of new hair cells (Balak et al., 1990; Jones and Corwin, 1993; 1996).

Evidence for the role of supporting cell mitosis in repopulating the avian auditory sensory epithelium showed that supporting cells in the hair cell loss area incorporated the proliferation markers, tritiated thymidine, adjacent to the labelled hair cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Following multiple injections of thymidine to label proliferating cells of the chick basilar papilla after 18 hours noise exposure, labelled supporting cells can be identified by 72 hours (Girod et al., 1989). Supporting cells in the basilar papilla incorporated the DNA specific marker BrdU within the region of the noise induced lesion, suggesting that supporting cells proliferate after acoustic trauma induced hair cell loss (Hashino and Salvi, 1993). The number of labelled cells increased and many of the cells were present in pairs at 36 hours after noise exposure (Raphael, 1992). The first cells in the basilar papilla labelled by BrdU were nuclei of supporting cells directly below the areas of hair cell loss. Increasing numbers of labelled nuclei were found with time after noise exposure and they could go on to give rise to new hair cells (Stone and Cotanche, 1994). Hair cells of cultured chick basilar papillae were killed individually by a laser microbeam, the first cells to show DNA synthesis in response to hair cell death were supporting cells within the sensory epithelium, suggesting that supporting cells are the precursors of regenerated hair cells (Warchol and Corwin, 1996).

Supporting cells also appear to play a progenitor role in the avian vestibular system. A significant pairwise association between labelled supporting cells and hair cells in the normal utricular maculae and crista ampullae was reported (Roberson et al., 1992). High numbers of labelled supporting cells were also observed after explantation of the normal saccules and utricles into serum free culture media, containing mitotic tracers (Warchol and Corwin, 1993) and some of the labelled supporting cells began to differentiate as hair

cells after seven days in culture. Using a short pulse fix autoradiographic protocol to detect avian vestibular cells in S-phase from control and streptomycin treated birds, nuclei of supporting cells in the basal layer and located lumenally were labelled by tritiated thymidine. After three hours survival, there was a consistent increase in the percentage of labelled nuclei in the lumenal nuclear layer. This can be explained only by migration of basely located proliferating supporting cells into the lumenal cell layer during the preparation for mitosis (Tsue et al., 1994c). In the streptomycin damaged avian vestibular system, supporting cells are the predominant tritiated thymidine labelled cell type initially after damage. The mitotic supporting cells migrate towards the lumen of the epithelium where they differentiate into type II hair cells and some of the new type II hair cells further differentiate into type I hair cells (Weisleder et al., 1995). During the progressive recovery from aminoglycoside induced hair cell loss in the avian vestibular system, proliferation labelling appears in supporting cell nuclei before they appear in hair cell nuclei (Weisleder and Rubel, 1992; 1995). The results confirm that hair cells originate from supporting cells.

The subject of hair cell regeneration is one of substantial scientific and clinical interest. It was thought that hair cells, if damaged during life, could not be regenerated in the mammalian inner ear. Results obtained from the avian ear may not be directly applicable to auditory organs in the mammals because they are highly specialised organs. The mammalian vestibular organs have similar morphological structures as the inner ear of birds and other lower vertebrates. Hair cell regeneration in the avian inner ear has shed light on triggering hair cell regeneration in the mammalian inner ear, but there are many problems to be solved. The aim of this thesis was to investigate the potential for hair cell regeneration in mammalian vestibular organs.

CHAPTER 4: MATERIALS AND METHODS

This project included two parts of studies: 1) in vivo experiments; and 2) in vitro experiments. The progression of hair cell loss due to effects of gentamicin on the inner ear sensory neuroepithelia and consequent structural repair and morphological changes have been examined by fluorescence microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and differential interference contrast microscopy (DIC) for in situ end labelling (ISEL) apoptosis and immunohistochemical labelling of BrdU.

4.1 IN VIVO EXPERIMENTS

Healthy pigmented guinea pigs and albino animals without middle ear infection were used. The initial study was performed using 25 male albino guinea pigs (Dunkin-Hartley strain), which were purchased from a commercial supplier. All subsequent experiments were carried out using pigmented guinea pigs, of either sex. The pigmented animals (Mill Hill strain 13) were bred from the stock at the animal house located in the Institute of Laryngology and Otology under the supervision of the Department of Biological Service in University College London. Pigmented animals' age and body weight varied due to the limitation of the animals breeding rate. Animals were housed at a maximum of five per cage with free access to food and water. For identification purpose, albino animals were marked with methyl violet and brilliant green dyes on the surface of the pinna. Pigmented guinea pigs were identified by drawing pictures of the fur colour and patterns.

Two protocols were used to induce hair cell loss in the inner ear vestibular sensory epithelia of guinea pigs: 1) systemic injections with gentamicin for 10 days; and 2) topical application of a single dose of gentamicin to the right middle ear cavity.

4.1.1 Systemic Treatment with Gentamicin

A total of 101 guinea pigs were used in the experiments of systemic gentamicin treatment, 25 of them served as controls including 20 normal pigmented guinea pigs without any injection and those of five albino animals which received an equivalent volume of 0.9% sterilised normal saline (Antigen Pharmaceutical Ltd) by systemic injection.

Gentamicin Dose Regime for Systemic Injection

The clinical gentamicin injection B.P. solution contains gentamicin sulphate equivalent to 40mg / ml gentamicin base in 2 ml ampoules or vials. In total, 20 albino and 56 pigmented guinea pigs were randomly selected and given injections with gentamicin sulphate solution (Cidomycin Injection, Roussel Laboratories Ltd; Gentamicin Injection B.P. Faulding Pharmaceutical Plc.). Gentamicin was given subcutaneously on the animal's back in a single, daily adjusted dose of 100 mg / kg / day for albino animals and 125 mg / kg / day for pigmented animals for 10 consecutive days. These drug dose regimes have been shown to reliably produce almost complete loss of outer hair cells in the basal turn of the cochlea in the respective strains (Forge, 1985; Brown et al., 1989; McDowell et al., 1989). The animals' ages and body weights were recorded at the beginning of the gentamicin injection. In order to adjust the injection dosage, animals were weighed everyday prior to the gentamicin injection. Sterile needles of 25 gauge (0.5x16 mm) and sterilised disposable syringes were used at the volume of 1 ml , 2 ml and 2.5 ml as necessary to keep the drug injection as accurate as possible.

Experimental Groups

Five separate experiments were performed: a preliminary study using albino guinea pigs, and four subsequent studies using pigmented guinea pigs (Tables 4.1- 4.5).

Table 4.1 Preliminary Experiment on Albino Guinea Pigs

SURVIVAL TIME	CONTROL ANIMAL	TREATED ANIMALS	EXAMINATIONS
1 hour	1	4	SEM; TEM; Phalloidin
2 days	1	4	SEM; TEM; Phalloidin
4 days	1	4	SEM; TEM; Phalloidin
1 week	1	4	SEM; TEM; phalloidin
3 weeks	1	4	SEM; TEM; Phalloidin

Twenty five albino guinea pigs were divided into five subgroups. The animals were 3-4 weeks old, and body weight ranged from 251 grams to 289 grams at the beginning of the gentamicin injection. All inner ear tissues from one ear of each animal were processed for TEM. The utricle, saccule, one crista and a piece of organ of Corti from the other ear were processed for fluorescence microscopy using phalloidin-FITC labelling. The other cristae and organ of Corti were processed for SEM.

Table 4.2 First Group of Pigmented Guinea Pigs

SURVIVAL TIMES	CONTROL ANIMAL	TREATED ANIMALS	EXAMINATIONS
2 days	1	2	6 ears for SEM
3 days	1	2	6 ears for TEM
4 days	1	2	6 ears for Phalloidin
28 days	1	2	6 ears for SEM
29 days	1	2	6 ears for TEM
30 days	1	2	6 ears for Phalloidin

Eighteen animals were selected for use at 4-10 weeks of age and body weight ranged from 248 grams to 570 grams. According to survival time after the last gentamicin injection, specimens were pooled to process and examined in an "early" group (2, 3 and 4 days) and a "late" group (28, 29 and 30 days). Four ears from treated animals and two ears from control animals (total 9 animals 18 ears for each early or late group) were prepared for SEM, TEM and fluorescence microscopy separately.

Table 4.3 Second Group of Pigmented Guinea Pigs

SURVIVAL TIMES	CONTROL ANIMALS	TREATED ANIMALS	EXAMINATIONS
4 weeks	1	4	SEM, TEM
8 weeks	1	3	SEM, TEM
12 weeks	2	3	SEM, TEM

Fourteen animals, weighing from 212 grams to 335 grams, were used at 3-4 weeks of age. This experiment was designed to repeat the previous late group result and to investigate whether hair cells recovered further after long survival time. Specimens from one ear were processed for SEM and those from the opposite ear for TEM from same animal.

Table 4.4 Third Group of Pigmented Guinea Pigs

SURVIVAL TIMES	CONTROL ANIMALS	TREATED ANIMALS	EXAMINATIONS
2 weeks	1	4	SEM, BrdU
4 weeks	1	4	SEM, BrdU
8 weeks	1	3	SEM, BrdU
12 weeks	2	4	SEM, BrdU

Twenty animals were selected for use at 4-11 weeks of age and body weight ranged from 284 grams to 723 grams. The animal number given in the Table 4.4 did not include six gentamicin treated guinea pigs died unexpectedly (see page 93). For immunohistochemical BrdU labelling, different techniques were used, see Table 4.6 .

Table 4.5 Fourth Group of Pigmented Guinea Pigs

SURVIVAL TIMES	CONTROL ANIMALS	TREATED ANIMALS	EXAMINATIONS
1 week	nil	5	SEM, TEM
4 weeks	nil	5	SEM, TEM
12 weeks	2	5	SEM, TEM
33 weeks	3	4	SEM, TEM

Twenty four animals were selected for use at 5-10 weeks of age and body weight ranged from 258 grams to 586 grams. The animal number in this group excluded two gentamicin treated guinea pigs which died of unknown reasons and there was no control animal in one week and four week subgroups. Previous control animals were used.

4.1.2 Topical Application with Gentamicin

The local application of aminoglycosides represents an almost direct application of the drug to the inner ear in vivo and the purpose was to dose one ear of each animal once with the opposite ear used as a control. Sixty guinea pigs received topical application with gentamicin or normal saline in the right ear and the left ear was used as the control. Among them, 29 animals were used for SEM study and the other 31 animals for BrdU immunolabelling. Animals were 2-7 months old and body weight varied from 375 grams to 885 grams at the beginning of the experiment.

Animal Anaesthesia

The guinea pigs received diazepam (10 mg in 2 ml ampoules, Phoenix Pharmaceuticals Ltd) injection at the dosage of 2.5 mg / kg body weight, equivalent to 0.5 ml / kg intraperitoneally. Then, animals were given an intramuscular injection with hypnorm (10 mg in 10 ml vial, Janssen Pharmaceutical Ltd) at the dosage of 1 mg / kg, equivalent to 1 ml / kg body weight. This anaesthetic regime maintained the guinea pig in a stable anaesthetic situation with satisfactory analgesia and good muscle relaxation for 60 minutes, at least. No supplementary anaesthesia was necessary during the surgery. Attention was paid to the body temperature of the animal during the whole operation, which was achieved by placing a heating pad under the animal's body.

Surgery Procedures

Once satisfactory anaesthesia was obtained, the skin hair over the head behind the right ear was cut and shaved. The skin was then cleaned by "alcowipe", sterile alcohol surface wipe (Seton Prebbles Ltd). The animal was placed on the heating pad and the animal's head was

held in position by hand. Under aseptic conditions, a 1.5 cm long incision was made on the skin behind the right pinna. After separation of the connective tissues and the muscles under the skin, the smooth white surface of the bulla was exposed post-auricularly. A hole of 1.5 mm in diameter was drilled through the bone in the superior compartment of the exposed bulla, using a dental drill with an 1 mm diameter burr. Under an operating microscope, at 10x magnification, through the hole, the lateral and superior semicircular canals which are located in the epitympanic cell could be seen. A 25 gauge needle was used to inject 0.15-0.2 ml (6-8 mg gentamicin sulphate) gentamicin sulphate solution (40 mg /ml Cidomycin Injection; Roussel Laboratories Ltd) into the epitympanic cell of the middle ear cavity. Using this procedure, the tympanic membrane was kept intact, to ensure that the drug entered and remained within the middle ear cavity. Often after gentamicin solution was applied topically, a nystagmus was observed towards the unoperated ear side which provided a means to ensure the gentamicin entered into the middle ear. Presumably, this was a response induced by a caloric stimulation, by the "cold" room temperature gentamicin solution. The hole was sealed by a piece of connective tissue and the incision was sutured by one or two stitches. The whole procedure was usually completed within 15-20 minutes for each animal. After surgery, the animal was allowed to recover in a hay box or "vet-bed", then they were returned to the stock cages. To relieve post-surgery pain, buprenorphine hydrochloride (Temgesic injection, Reckitt and Colman Pharmaceuticals Ltd) was injected subcutaneously at dose of 0.05 mg / kg body weight on the surgery day once or twice.

Saline Control Treatment

For SEM study, a single dose of 0.2 ml of 4% gentamicin was applied to the right middle ear cavity of 24 guinea pigs. The right ears of five other animals and the left ear of one guinea pig with gentamicin on the right ear were given 0.2 ml 0.9% normal saline (Antigen Pharmaceutical Ltd) as method controls to test whether the operation itself could damage the inner ear tissues. The surgery procedures were the same as described above.

4.1.3 In Vivo BrdU Administration

Two procedures were used for delivery of 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical Ltd) to guinea pigs in vivo: 1) intraperitoneal systemic injections; and 2) implantation of an osmotic pump.

Intraperitoneal Systemic Injection with BrdU

Crystalline BrdU was dissolved in water for injection (Antigen Pharmaceutical Ltd) or normal saline (Antigen Pharmaceutical Ltd) at 50°C in a water bath. The concentration of BrdU solution varied from 50 to 75 mg / ml. The dosages were adjusted from 50 to 100 mg / kg body weight for multiple injections or one injection for flash labelling.

At various survival times after 10 days systemic gentamicin treatment, the third group of pigmented guinea pigs were given BrdU by intraperitoneal injections every six hours over a 24 hour period and the fourth (last) injection was made one hour before sacrifice (Table 4.6). The tissues from one ear were processed for SEM to examine hair cell bundles; the other ear was used for BrdU immunolabelling in whole mount or wax section preparations.

Table 4.6 BrdU Administration after Systemic Gentamicin Injection

SURVIVAL TIME	GROUP SIZE	BRDU TREATMENT	SPECIMEN PREPARATIONS
2 weeks	4 treated + 1 control	50 mg/kg q.i.d.	whole mount
4 weeks	4 treated + 1 control	50 mg/kg q.i.d.	whole mount
8 weeks	3 treated + 1 control	50 mg/kg q.i.d.	whole mount
12 weeks	4 treated + 2 controls	50 mg/kg q.i.d.	wax sections/ whole mount

Table 4.7 summarises the details about BrdU injections to 24 guinea pigs which received topical gentamicin application at different schedules and at different dosages.

Table 4.7 BrdU Administration after Topical Gentamicin Application

ANIMAL NUMBERS	SURVIVAL TIMES	BRDU TREATMENT	SPECIMEN PREPARATION
3	10 days[1]	75 mg / kg	wax sections
3	2 weeks[2]	75 mg / kg	wax sections
3	2 weeks[3]	50 mg / kg	wax sections
3	5 weeks[4]	50 mg / kg	wax sections
8	5 weeks[5]	100 mg / kg	whole mount
4	6 weeks[6]	100 mg / kg	whole mount

1. Three days after topical gentamicin treatment, guinea pigs received BrdU intraperitoneal multiple injections every eight hours for seven days. Animals were sacrificed immediately after the last BrdU injection.
2. Animals received same BrdU treatment as above, but sacrificed four days later after the last BrdU injection.

3. Animals received BrdU intraperitoneal injections twice a day on day 3, day 7, and day 13 after topical gentamicin application.
4. Same BrdU treatment as above plus two more injections on the day of sacrifice.
5. BrdU was intraperitoneally injected once to two animals on day 4, two animals on day 7 and another four animals on day 13 after topical gentamicin application. Animals survived up to five weeks.
6. Two animals received BrdU intraperitoneal injection once on day 4 and other two animals on day 7 after topical application. Animals survived up to six weeks.

Continuous Delivery of BrdU by Implanted Osmotic Pump

ALZET osmotic pumps were implanted on the back of the guinea pig subcutaneously under aseptic condition immediately after topical application with gentamicin. ALZET osmotic pumps 2ML2 (Alza Corporation), were filled with 2 to 2.3 ml of BrdU dissolved in normal saline at a concentration of 25 mg / ml before the implantation. Seven guinea pigs after topical gentamicin application received a single implant of a BrdU osmotic pump, five of them survived for two weeks and the other two survived for three weeks. The pumping rate of this model of Alzet osmotic pump has been tested at 5 μl / hour and the duration of drug delivery lasts for two weeks. The BrdU solution inside the pump would be delivered into the local subcutaneous space and absorbed by local capillaries resulting in continuous systemic administration. The pumps were checked at the time the animals were sacrificed and the small intestine and liver tissues of the animals were collected and processed for BrdU immunolabelling as an internal control. The inner ear tissues from each animal, the utricles from the treated ear and from left control ear were processed for BrdU immunolabelling using paraffin wax sections.

4.2 IN VITRO STUDIES

To examine more directly the processes of hair cell loss and epithelial repair, organotypic cultures of the explants of the vestibular sensory epithelia from mature mammalian animals were established and developed. The vestibular utricular and saccular maculae from mature guinea pigs and gerbils were dissected out and maintained in organotypic cultures, using procedures essentially the same as those developed by Dr Guy Richardson for neonatal mouse organ of Corti (Richardson and Russell, 1991).

4.2.1 Preparations

All glassware and instruments were dry heat sterilised one day before use in a GS150 sterilizer at 180 ° C for two hours. An autoclave 280EH machine or "Little Sister" were used to autoclave all other materials, e.g. rubber, distilled water and vaseline, for 30-40 minutes at 120 ° C at 10 pounds pressure. On the same day before the dissection, the stage of the laminar flow hood and the dissection microscope were wiped with 80% ethanol. In case of accidental contamination during dissection, the instruments were dry heat sterilised in a portable hot bead sterilizer (Steri 250, InterFocus Ltd).

Hank's balanced salt solution (HBSS from Sigma Chemical Ltd), was modified with sodium bicarbonate, without phenol red. Immediately before use, one millilitre of 1 M HEPES buffer (Sigma Chemical Ltd) was added to 100 ml HBSS to obtain buffered HBSS at pH 7.3. This solution was used to rinse specimens after 80% ethanol sterilisation and used as the dissection medium.

Minimum essential medium (MEM, from Gibco, Life Technologies) with Earle's salts was used as the basic culture medium throughout the incubation period. MEM was supplemented with heat inactived horse serum (Gibco, Life Technologies). Before mixing MEM with heat inactived horse serum, one millilitre of 200 mM L-glutamine (Sigma Chemical Ltd) and one millilitre of 1 M HEPES were added to 100 ml MEM. Then, 10 ml of heat inactived horse serum was added to 90 ml of the modified and buffered MEM to provide a culture medium containing 10% heat inactived horse serum.

Rat tail collagen, which was kindly provided by Dr Richardson, was used to coat glass coverslips to ensure attachment of explanted tissues. To prepare collagen coated coverslips, one and half drops of collagen solution were placed on a 22 mm diameter round glass coverslip and spread with the tip of the glass Pasteur pipette. The coated coverslips were exposed to 25% ammonia solution (NH_3) for 15 minutes in the fume cupboard to gel the collagen solution and then rinsed in sterile distilled water to remove ammonia and finally stored in a Columbia staining jar containing 8 ml modified HBSS and 3-4 drops of heat inactived horse serum at room temperature until use.

4.2.2 Tissue Culture Establishment

Pigmented guinea pigs of either gender and of ages from five weeks up to 13 months were used to collect inner ear vestibular sensory epithelia. Mature gerbils, eight month old, were also used to collect inner ear vestibular tissues for organotypic cultures to compare the response to gentamicin treatment in different species. The animals were killed by anaesthetic overdose and decapitated. Both bullae were removed from the head under aseptic conditions and the intact bullae were rapidly immersed in cold 80% ethanol

solution to sterilise for 10 minutes. Dissections were performed using a stereo microscope inside a laminar flow cabinet. The specimens were washed twice in cold buffered Hank's balanced salt solution, and the utricle and saccule from each ear were dissected out on top of a cold plate to keep specimens cool and reduce autolysis. The dissected tissues were transferred into a small petri dish with fresh HBSS for fine dissection. The membranous sacs were opened and the otoconia layer was removed with a pair of fine tweezers.

4.2.3 Tissue Culture Maintenance

The vestibular tissues were initially maintained in Maximow slide assemblies. These offer the investigator a means to continuously observe and photographically record active events in the cultured tissues. To set up the Maximow slide assembly, the collagen coated coverslip was centred face up on one 44 mm square coverslip on a piece of black paper in a large petri dish. With a Pasteur pipette, the prepared tissues were transferred and explanted on to the collagen surface of the round coverslip with the apical surface of the epithelium uppermost. The explanted tissues were fed with a single drop (50 µl) of MEM culture medium, then enclosed in the Maximow depression slide which was sealed with a paraffin vaseline mixture. The cultures were maintained in the lying drop position and incubated at 37 ° C incubator (Heraeus Instruments) for 24 or 48 hours. During the whole period in vitro, the cultures were removed from the incubator to observe the development of the explants, to check for microbial contamination and to photograph under a Nikon inverted light microscope (Diaphot 200) at various times.

4.2.4 Tissue Culture Treatments

After settlement for 24 or 48 hours in vitro, the Maximow slide assembly was split open, and the round coverslip with the explanted cultures was transferred into a 35 mm petri

dish. From this point, some cultured explants were incubated in the agent of interest. In parallel with in vivo studies, gentamicin sulphate powder (Sigma Chemical Ltd) dissolved in 9:1 MEM culture medium at final concentration 1 mM or 2 mM was directly applied to cultures at 37 ° C to induce hair cell damage in vitro. Twenty four guinea pigs were used to set up vestibular tissue cultures for electron microscopy. To examine gentamicin toxicity, 23 cultures were treated with 1 mM gentamicin for 4, 6 or 24 hours, another 9 cultures were incubated in 2 mM gentamicin for 24 hours and 16 cultures were used as controls. At the end of the drug treatment, the cultures were rinsed in drug free culture medium three times, then they were maintained in 250-300 μl of normal MEM and horse serum culture medium for further periods of up to 14 days (total 16 days in vitro). Control cultures were fed in normal MEM and horse serum culture medium without gentamicin immediately on transfer to the petri dishes and cultured in the same condition parallel with the treated specimens. The culture medium was changed every two days and the incubator temperature was 37 ° C with constant 5% CO_2 supply.

The vestibular utricular cultures from another seven guinea pigs were treated with 1 mM gentamicin for 24 hours, then the cultured utricles were rinsed and continuously grown in gentamicin free medium but containing 3 μg /ml BrdU (Warchol et al., 1993) up to 16 days. Finally, these vestibular utricles incubated with BrdU were processed for BrdU fluorescence labelling.

To test different mammalian species in response to aminoglycoside gentamicin in the culturing system, vestibular tissues from nine mature gerbils were used and maintained in vitro as the procedures for guinea pig cultures. After 24 hours settlement, 12 utricles were

incubated with 1mM gentamicin for six hours, then rinsed and cultured in drug free medium. The other six utricles were used as controls. By four days in vitro, six treated and three control cultures were processed for TEM and other cultures survived up to 13 days in culture medium containing 3 μg /ml BrdU in vitro. Finally, these BrdU incubated gerbil cultures were processed for routine and BrdU immunoelectron microscopy.

The vestibular utricles from 32 mature guinea pigs were used to set up cultures for the apoptotic nucleus labelling. After 44 hours settlement in vitro, control cultures were incubated in normal MEM medium with 10% horse serum without gentamicin. The treated cultures were added to the above culture medium containing 2 mM gentamicin sulphate for 6 hours or 24 hours incubation at 37°C, then the gentamicin medium was washed off. Some utricular maculae were processed for apoptotic nucleus labelling by light microscopy immediately after gentamicin treatment. The other cultures were allowed to grow in gentamicin free medium for five more days.

4.3 TISSUE PREPARATION AND MICROSCOPIC EXAMINATION

4.3.1 Tissue Harvesting in Vivo

Animals were killed in a carbon dioxide (CO_2) chamber or anaesthetized with 6% pentobarbitone sodium (Rhone Merieux Ltd) following required procedures laid down by the British Home Office. The animals were decapitated and both bullae were removed. The bullae were widely opened to expose the vestibular parts and the cochlea. Holes were made in the apex of the cochlea and the stapes was removed from the oval window, or the specimens were cut in two parts, then perfused directly with fixative by gentle injection

before the whole bulla was immersed in the fixative for further processing. The time from anaesthesia overdose, decapitation, perfusion and fixation was less than five minutes for each animal on average. Each time group at least three ears from gentamicin-treated guinea pigs were prepared.

4.3.2 Tissue Harvesting in Vitro

When the required incubation period was completed for in vitro studies, the cultured explants remained in situ on the coverslip were rinsed in cold buffered HBSS to wash out the residual culture medium, then processed for various microscopic examinations.

4.3.2 Fluorescence Light Microscopy

Fluorescence light microscopy was applied for whole mount examination of the in vivo and in vitro specimens (Raphael and Altschuler, 1991a; 1991b; Meiteles and Raphael, 1994b; Goodyear and Richardson, 1992). The specimens from in vivo experiments for phalloidin staining of actin were fixed in 4% paraformaldehyde containing 0.5% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.3. Following two hours fixation with gentle rotation at room temperature, the bullae were washed in 0.01M phosphate buffered saline (PBS) and then the vestibular and cochlear tissues were dissected out under the dissecting microscope. The utricles and saccules were further dissected to remove the otoconia and reveal the apical surfaces. The cupula of the cristae and the tectorial membrane of the organ of Corti were also removed carefully, if possible.

Phalloidin-FITC (Sigma Chemical Ltd) was prepared as a stock solution of 0.1 mg / ml in methanol. Before use, it was diluted 1:100-500 in 0.01M phosphate buffer saline (PBS) or in Hank's Balanced Salt Solution (HBSS) with 5 mM HEPES at pH 7.3. The final staining

concentration varied between 0.2-1μg / ml. The cristae, the utricular and saccular maculae, and the organ of Corti were immersed in 0.3% triton X-100 (Sigma Chemical Ltd) for 15 minutes before they were incubated in phalloidin-FITC staining solution. The phalloidin-FITC solution contained 3% bovine serum albumin (Sigma Chemical Ltd) to reduce background staining. The incubation time was two hours at room temperature. After rinsing in PBS three times, phalloidin-FITC labelled specimens were mounted on glass slides using Citifluor (Agar Scientific Ltd) or in glycerol with 0.1% paraphenyldiamine (Sigma Chemical Ltd). The preparations were examined and photographed with a Zeiss universal microscope or Nikon Optiphot-2 microscope equipped for epifluorescence. Fluorescence images were obtained using FITC-filters and photographs were taken on Kodak Tmax 400 or Ilford HP5 400 films.

4.3.3 Electron Microscopy

The specimens for electron microscopy were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer with 3 mM calcium chloride ($CaCl_2$), pH 7.4, total osmolarity 550 mOsm. Fixation was continued for 1.5-2 hours at room temperature with constant slow rotation. After washing in the 0.1M cacodylate buffer, specimens were post-fixed in 1% 0.1M cacodylate buffered osmium tetroxide (OsO_4) for another 1.5 hours at room temperature. The cristae, the utricular and saccular maculae, and the organ of Corti were dissected out in buffer or 70% ethanol under the stereo dissecting microscope. Some specimens were kept in buffer or in 70% ethanol at 4°C overnight for group processing.

The cultured vestibular explants were also processed for electron microscopy using the routine procedures. For transmission electron microscopy, the cultured explants were fixed

in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer with 3 mM calcium chloride ($CaCl_2$), which was supplemented with 0.3% tannic acid (Goodyear and Richardson, 1992) for thin sectioning.

Specimen Preparation for Scanning Electron Microscopy

Samples for scanning electron microscopy were processed with double thiocarbohydrazide (TCH) and osmium procedure (Hunter-Duvar, 1978; Davis and Forge, 1987; Forge et al., 1992). Dissected specimens were placed in a processing basket and incubated in a freshly prepared and filtered saturated solution of TCH for 20 minutes at room temperature. Following six washes in distilled water, the samples were incubated in 1% aqueous OsO_4 for one hour. The whole procedure was then repeated. After dehydration through a graded series of ethanol, critical point drying was carried out in Polaron critical point dryer using liquid CO_2. The specimens were mounted on the surface of a stub and sputter coated with a thin layer of gold before examination using JEOL JSM 35C SEM. Observation of the specimens was usually carried out at low magnification first and increased to high magnification.

Data Analysis of SEM Photographs

The number of hair bundles was counted from SEM pictures taken along the striolar region of the treated and control utricular and saccular maculae after systemic and topical treatment with gentamicin. Separate areas along the striolar regions where hair bundles were oriented in different directions to confirm the accurate selection were photographed under SEM (the number of fields photographed from each specimen varied). Counting was carried out and repeated either from original negatives using a light box or from contact prints of photographs taken at a nominal magnification of 2000x only, providing a field

of 3200 μm^2 real area. Hair bundle numbers in the individual field of the striolar regions from the different post-treatment survival groups and from control groups were compared using one-way analysis of variance (ANOVA). ANOVA is a widely used statistical technique to test statistical significance of many different trials which examines the variability of the observations within each group as well as the variability between the group means. Duncan's test for ANOVA was used to compare multiple group data with each other to determine which group means are significantly different from others and the difference between each two groups is considered as significance at p level 0.05. The ANOVA results from each experiment are reported in Chapter 5 in the simplified tables with the number of samples, the mean ± s.d. and the median only.

Thin Sections for Transmission Electron Microscopy

For transmission electron microscopy, the inner ear tissues were incubated en bloc in 1% uranyl acetate in 70% ethanol for one hour at room temperature. Then, the dehydrated specimens were infiltrated in the plastic resin (Agar Scientific Ltd) in three changes each time over two hours (2 parts propylene oxide : 1 part resin; 1 part propylene oxide : 2 parts resin; 100% resin). Finally, specimens were embedded in fresh resin using a flat mould and the plastic resin was polymerised at 60°C for 24 hours. Semi-thin sections were cut at 0.5-1 μm thickness on a Reichert-Jung ultramicrotome and stained with toluidine blue for light microscopy. Ultra-thin sections were cut at 90-110 nm thickness and efforts were made to cut sections as closely as possible perpendicular to the lumenal surface of each epithelium. For most of the embedded tissue blocks, sections of the entire width of the epithelium were taken at three different levels across the tissue. Serial thin sections were collected at each level and mounted on 200 mesh copper grids. The grids were stained in 4% uranyl acetate and Reynold's lead citrate in an enclosed petri dish containing 1M sodium hydroxide (NaOH) to prevent lead precipitate on the tissue sections, then examined by JEOL 1200 EXII TEM at an accelerating voltage of 80 kV.

4.4 BRDU IMMUNOLABELLING

Immunolabelling the DNA synthesis marker BrdU with antibody has been used in this study to detect inner ear cell proliferation.

4.4.1 Light Microscopy of BrdU Immunolabelling

Differential interference contrast microscopy and fluorescence microscopy were use to examine whole mount specimen preparations processed for BrdU immunolabelling. Conventional light microscopy was used to examine wax sections.

Specimen Fixations and Preparations

The animals were sacrificed and bullae were obtained using the procedures described in text 4.3.1 after completion of appropriate BrdU treatment. After the removal of the bullae, an abdominal incision was made and a piece of the duodenum and a slice of the liver were removed and fixed as positive control tissues for BrdU immunolabelling. The bullae and control tissues were fixed in Carnoy's fixative (freshly made 100% ethanol 6 parts; chloroform 3 parts; glacial acetic acid 1 part) or in freshly made 4% paraformaldehyde in 0.1M phosphate buffer for two hours at room temperature. Carnoy's fixed bullae were dissected out in 70% ethanol after rehydration. Paraformaldehyde fixed bullae were rinsed and dissected out in 0.1M phosphate buffer.

Fixed specimens from 29 animals were prepared for whole mount surface preparations and specimens from 22 guinea pigs were processed using paraffin-wax embedding for cutting sections. For whole mount surface preparation, inner ear tissues were kept in aqueous solution after fixation and dissection. For wax embedding, dissected tissues were

dehydrated in an ethanol series, for 15 minutes each and then immersed in chloroform overnight. Specimens were infiltrated with melting paraffin wax at 60°C using standard procedures. Each specimen was finally embedded in a tissue cassette. Wax blocks of the specimens were cut at 4-5 μm thickness using an Autocut 2040 (Reichert-Jung) and serial sections were collected on "Poly-Prep" slides coated with a thin layer of poly-L-lysine (Sigma Chemical Ltd), then flattened and dried on a hot plate. After fixation, the small intestine and liver tissues were cut into small pieces or cubes and processed for paraffin wax sections at 5 μm thickness as positive control tissues.

Antibodies

The monoclonal mouse anti-bromodeoxyuridine, Dako-BrdU, (Dako Ltd) was used throughout the experiment to identify BrdU in single stranded DNA produced by partial denaturation of double stranded DNA. Different secondary antibodies and ABC kits were tested to label the primary antibody. An avidin / Biotin detection system which involves the introduction of an unlabelled primary antibody followed by a biotinylated secondary antibody and then the avidin / biotinylated enzyme complex, was the most commonly employed method in this study. Both biotinylated rabbit anti-mouse immunoglobulins and StreptABComplex / HRP (Dako Ltd) and biotinylated horse anti-mouse immunoglobulins and Vectastain elite ABC-peroxidase kits (Vector Laboratories Ltd) were used for the identification of antigen in the processed tissues.

DNA Denaturation

Wax sections had to be immersed in CNP-30 twice, two minutes each to remove the wax and were rehydrated. Dewaxed sections or whole mount specimens were incubated for

over 10 minutes with 3% hydrogen peroxide in distilled water to remove endogenous peroxidase activity that may be present in the tissues. Enzymatic digestion was applied to destroy the cross linking of nuclear protein to DNA created by paraformaldehyde fixation. The specimens were incubated in 0.1% trypsin (Sigma Chemical Ltd) in 0.1% calcium chloride ($CaCl_2$) dissolved in distilled water for 15-20 minutes at room temperature. The trypsin solution was warmed to 37°C before use. To disassociate histone and partially denature double stranded DNA, specimens were treated with 2-4 M hydrochloric acid (HCl) for 30-60 minutes at room temperature or some section slides were immersed in heated 1M HCl at 60°C for 5-10 minutes. Between the different steps, specimens were rinsed in distilled water three times for five minutes each time.

BrdU Detection

For all subsequent processing steps, 0.5% triton X-100 was added to 0.01M phosphate buffered saline (pH 7.4) used for washing and dilution. Specimens were incubated in this buffer for 10 minutes to permeabilise the cell membranes before they were treated with a blocking solution containing 5% normal rabbit or horse serum in PBS for 20 minutes in a moisture chamber to block non-specific staining. The primary monoclonal mouse anti-BrdU was diluted 1:20 in the above PBS blocking solution and applied to fully cover specimens which were then incubated overnight at 4°C or one hour at room temperature in a moist chamber. Negative method control slides or specimens were treated without the primary antibody using the blocking solution only or using normal mouse serum (Dako Ltd) diluted 1:20 in the blocking solution. After incubation with the primary anti-BrdU antibody solution, specimens were rinsed in PBS twice. Biotinylated rabbit anti-mouse immunoglobulins plus StreptABComplex / HRP kit and Vectastain elite ABC-peroxidase

kit containing biotinylated horse anti-mouse immunoglobulins were used in most of the experiments. Specimens were incubated for 60 minutes at room temperature with biotinylated rabbit or horse anti-mouse immunoglobulins diluted 1: 200-100 in PBS. ABC reagent was finally used to incubate specimens for 30-45 minutes at room temperature. For preparations of StreptABComplex / HRP or Vectastain elite ABC reagent the manufacturer's instructions were followed.

Chromogenic Substrate for Peroxidase

3,3'-Diaminobenzidine (DAB) is a precipitating substrate for localization of peroxidase activity in immunohistology. DAB substrate solution is used to develop peroxidase which can produce an insoluble brown end product and can be observed visually. To prepare the staining DAB chromogen solution, one 10mg DAB tablet (Dako Ltd) was dissolved in 10 ml of 0.01M PBS, pH 7.4, to make a stock solution stored at -20°C. Before use, the stock solution was thawed and diluted in distilled water and mixed well. The final concentration of the DAB staining solution was 0.5 mg / ml containing 0.03% hydrogen peroxide (H_2O_2). When Vector DAB substrate kit (Vector Laboratories Ltd) was used, 2 drops of buffer stock solution, 4 drops of DAB stock solution and 2 drops of hydrogen peroxide solution were mixed well in 5 ml distilled water and prepared just before use. Each specimen was stained with DAB solution for 3-6 minutes to develop peroxidase and the brown colour was checked under a microscope. The whole mount specimens were rinsed thoroughly with distilled water after DAB development and mounted using an aqueous "Uvinert" mountant (BDH Ltd.). For wax sections, cell nuclei were counterstained in 1% Ehrlich's haemotoxylin for one minute. Glass slides were washed in running tap water, dehydrated, immersed in CNP-30 and then mounted with a coverslip using permanent

mounting medium. The specimen preparations were examined and photographed with a Zeiss Universal or Nikon Optiphot-2 microscope. Dark brown stained cell nuclei were identified as BrdU positive labelling. BrdU labelled cell nuclei were counted in utricular series wax sections from 4 animals which received BrdU osmotic pump implantation.

4.4.2 Fluorescence Microscopy of BrdU Immunolabelling

BrdU treated guinea pig utricular cultures were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for one hour and after rinsing in PBS, the specimens were immersed in 2M hydrochloric acid (HCl) for 30 minutes at room temperature to partially denature double stranded DNA. The primary monoclonal mouse anti-bromodeoxyuridine (Dako Ltd) was diluted 1:20 and applied to whole mount specimens for overnight incubation. FITC-conjugated rabbit anti-mouse immunoglobulins (Dako Ltd) were used as secondary antibody at dilution 1:20 to label the primary antibody at room temperature for one hour. The specimens were mounted for whole mount preparation using Citifluor (Agar Scientific Ltd) and examined under fluorescence microscopy.

4.4.3 Electron Microscopy of BrdU Immunolabelling in Cultured Utricles

Immunogold technique was used to examine BrdU immunolabelling in ultra-thin sections of BrdU treated cultured gerbil utricles by electron microscopy. Cultured gerbil utricles were fixed in 4% paraformaldehyde / 0.05% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 30 minutes or fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for one hour, and post-fixed in 1% osmium tetroxide (OsO_4) in 0.1M phosphate buffer for 30 minutes. After fixation, specimens were washed in the same buffer three times, followed by two washes in distilled water. After rapid dehydration, specimens were infiltrated and embedded in resin in a

flat mould as routine procedures. Ultra-thin sections were cut at 100nm thickness and collected on clean nickel grids washed in acetone.

For electron microscopy of indirect immunogold single labelling, all incubations were carried out in multiwell microtest plate and all solutions were microfiltered by a syringe top 0.45 mm pore size filter (Millipore Ltd). The grids mounted with sections were etched in 10% hydrogen peroxide solution and osmicated tissues were treated with saturated aqueous sodium metaperiodate solution ($NaIO_4$), both for five minutes at room temperature. This step was believed to permeabilise the resin thereby aiding antibody penetration.

The sections on grids were denatured in 1% periodic acid (H_5IO_6) at 50°C in a water bath for five minutes. The grids were thoroughly rinsed after each step. The grids were incubated for 30 minutes at room temperature in normal goat serum (British BioCell). Normal goat serum was diluted 1:20 in PBS-BSA-NaN_3 antiserum diluent [0.01M phosphate buffer, pH 7.4, with 0.15M sodium chloride (NaCl), containing 0.5% bovine serum albumin (Sigma Chemical Ltd, fraction V, globulin free) and 0.01% sodium azide (NaN_3)]. The monoclonal mouse anti-BrdU antibody (Dako Ltd) was diluted 1:20 in the above diluent, the grids were placed in the primary antibody solution for one hour incubation at room temperature in a humid chamber. Normal mouse serum and diluent were used to replace the primary antibody to act as essential method controls.

After primary antibody incubation, the nickel grids were washed thoroughly in 0.05M Tris HCl buffer I, (pH 7.4) and in 0.05M Tris HCl buffer II, containing 0.1% bovine serum albumin (pH 7.4) with agitation. Before the secondary antibody, the grids were incubated in Tris HCl buffer

III, containing 1% bovine serum albumin, pH 8.2. Secondary antibody, goat anti-mouse IgG, conjugated to 15 nm gold particles (British BioCell) was gently centrifuged for five minutes at 700G to remove particle aggregates before use, then diluted 1:20 in Tris HCl buffer III. The grids were incubated in drops of gold particle labelled secondary antibody solution for one hour at room temperature in a humidity chamber. After immunogold labelling, the grids were rinsed very thoroughly in large quantities of 0.05M Tris HCl buffer II, then in Tris HCl buffer I, followed by double distilled water. Finally, the grids were lightly counterstained for conventional electron microscopy using aqueous uranyl acetate and Reynold's lead citrate before examination using JEOL 1200 EXII TEM at an accelerating voltage of 80 kV.

4.5 APOPTOTIC NUCLEI LABELLING

After gentamicin treatment, the cultured guinea pig utricles were processed in whole mount preparations to examine apoptotic nuclei labelling. Control and treated utricles were fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer for one hour at room temperature. After rinsing in PBS, they were removed from the collagen layer and transferred to a multiple well plate. Phalloidin-FITC and propidium iodide were used to double label actin and nuclei, respectively, and examined by fluorescence microscopy. Differential interference contrast microscopy was used to examine in situ end labelling (ISEL) apoptotic nuclei.

4.5.1 Double Labelling of Cultured Utricles

Before incubation with the double labelling solution, the fixed utricles were treated with 0.3% triton X-100 in PBS for 15 minutes at room temperature to permeabilise the cell membrane. The double labelling solution contained 100 µg / ml DNase-free ribonuclease A (Sigma Chemical Ltd), propidium iodide (Sigma Chemical Ltd) 1µg / ml, and phalloidin-

FITC 1μg / ml in PBS (Jacobson et al., 1993). Specimens were incubated for 45 minutes in a 37°C incubator and then rinsed in PBS twice, finally equilibration buffer was applied to the specimens for 10 minutes. All specimens were mounted in "Slowfade Light" antifade glycerol mountant (Molecular Probes Inc). Observation and photographing of the fluorescence images were carried out using a Nikon Optiphot-2 microscope (Table 4.8).

4.5.2 In Situ End Labelling (ISEL)

The ISEL method (ApopTag in situ apoptosis detection kit, Oncor Inc) has been applied to determine apoptotic hair cells in the cultured utricles. The ApopTag kit utilizes terminal deoxyribonucleotidyl transferase (TdT) to add digoxigenin-nucleotide to the free 3'-OH ends of double or single stranded DNA generated during apoptosis. The incorporated to digoxigenin-nucleotides could be labelled by an anti-digoxigenin antibody conjugated peroxidase enzyme, which generates an intense signal from DAB substrates. Fixed utricular specimens were permeabilised in 90% methanol containing 0.3% hydrogen peroxide to quench the endogenous peroxidase for 20 minutes. Specimens were rinsed in PBS and then blocked in 5% horse serum in PBS containing 0.3% Triton X-100 for 30 minutes and washed again in PBS. These procedures were all performed at room temperature. For subsequent procedures, the manufacturer's instructions were followed. Specimens were applied with equilibration buffer directly and incubated up to 5-10 minutes. Two drops of reaction buffer and one drop of TdT enzyme (terminal deoxynucleotidyl transferase) were mixed well by vortexing before use and the working strength TdT enzyme applied to the specimens. Incubation was carried out at 37°C for one hour. Distilled water was used in the reaction buffer as negative staining control and incubation time and conditions were the same. When the required incubation was

completed, specimens were rinsed in prewarmed 37°C working strength stop buffer for over 30 minutes at 37°C. Stop buffer was changed once and the specimens were agitated every 10 minutes. After thoroughly washing in three changes of PBS for five minutes each, the specimens were incubated with anti-digoxigenin-peroxidase in a humidified chamber for 60 minutes on a rocking plate at room temperature. Development of the peroxidase reaction with DAB solution was the same as the procedures described for BrdU immunolabelling. Finally, the whole mount specimens were mounted by "Uvinert" mountant. Positively labelled apoptotic nuclei, stained brown, were examined and photographed with Nikon Optiphot-2 Microscopes (Table 4.8).

Table 4.8 Treatments and Preparations for Apoptotic Labelling

CULTURE GROUPS	PROPIDIUM IODIDE	IN SITU END LABELLING
Immediately after gentamicin treatment		
controls	4 utricles	5 utricles
2 mM gentamicin 6 hrs	4 utricles	7 utricles
2 mM gentamicin 24 hrs	4 utricles	8 utricles
5 days after gentamicin treatment		
controls	4 utricles	4 utricles
2 mM gentamicin 6 hrs	5 utricles	4 utricles
2 mM gentamicin 24 hrs	6 utricles	5 utricles

4.5.3 Data Analysis

Three areas in the central striolar region and three areas in the periphery were used to perform counting on each cultured utricle. Each area was equivalent to the real size of 75μm by 55μm. The central area was obtained along the striolar region of the cultured utricles and the peripheral area was along the edge of the sensory epithelium. In each area, the level of hair cell nuclei was focused. The number of hair cell nuclei was counted using a 100x oil, immersion lens in a Nikon Optiphot-2 microscope using fluorescence microscopy for propidium iodide labelling and differential interference contrast microscopy for in situ end labelling. The total number of hair cell nuclei and the positive labelled apoptotic hair cell nuclei from central and peripheral areas were counted and the data were analysed by ANOVA using Duncan's multiple range test (P value at 0.05).

CHAPTER 5: RESULTS

The results are presented as follows: 1) in vivo experiments; 2) in vitro experiments. The magnification of micrographs presented in this chapter is determined by the scale bar labelled in the bottom right corner of the first photograph in each plate.

5.1 IN VIVO EXPERIMENTS

A total of 161 guinea pigs were used in the in vivo experiments. Fluctuation of body weight did occur in animals from different groups and generally, treated guinea pigs did not gain weights as much as those of control animals during and after the period of 10 days gentamicin injection. In the 3rd experiment, three treated female guinea pigs were found dead on the 9th injection day and the post-mortem examination revealed their stomachs were distended and split open, apparently due to excessive carrot intake. Another three treated female animals died on day 2, day 4, day 6 after completion of the 10 days gentamicin injection and their stomachs and intestines were distended with bile juice accumulated inside these organs. Another two treated female guinea pigs died unexpectedly in the 4th experiment after completion of 10 days gentamicin injection. Up to 33 weeks post-treatment, all other treated animals progressively gained body weight by the time of sacrifice. Neither control nor gentamicin treated male guinea pigs were found dead during and after systemic gentamicin treatment. No guinea pigs were found dead after topical application with gentamicin.

5.1.1 Fluorescence Microscopy of Specimens from Systemic Treatment Experiments

Comparative observations of inner ear specimens from control and gentamicin treated animals were performed to determine phalloidin-FITC labelled actin differences in the

inner ear tissues after hair cell loss. Normal morphological structures were observed in animals which received systemic injection of 0.9% sterilised normal saline and from control animals without any injection. The injection of 0.9% sterilised normal saline showed no damage to the inner ear tissues compared with the animals without any injection. After staining actin in the inner ear epithelia with phalloidin-FITC, the whole mount preparations of the control vestibular tissues were covered with an even lawn of hair cells (Figure 5.1 A). At high magnification of the utricular maculae, the samples showed fluorescent stained F-actin in the hair bundles and the mosaic pattern of the cuticular plate of hair cells and the apical part of supporting cells was delineated by labelling of a ring of actin associated with the intercellular junctions (Figures 5.1 B, C). The stained actin ring around the supporting cell was thick and the apical surface of the supporting cell was polygonal in shape differing from the circular cuticular plate of hair cells. With fluorescence microscopy, supporting cells were clearly differentiated from hair cells as they lack hair bundles at their surface and are free of actin staining in the central zones. In the normal organ of Corti, phalloidin staining of actin was intense in the cuticular plate of three rows of outer hair cells and single row of inner hair cells. The stereocilia were arranged regularly and the cuticular plate and adherens junctions which connect adjacent sensory and supporting cells to form the mosaic of the reticular lamina could be seen clearly (Figure 5.1 D).

Two or three days after systemic gentamicin treatment, hair cell loss and scar formation could be identified in the striolar region of the utricular macula and in the central part of the cristae with fluorescence microscopy. An area staining weakly with phalloidin was apparent in the striolar region of the utricles, but the remaining peripherally located hair cells were stained intensely and the hair bundle density looked normal (Figure 5.2 A).

Focus on the epithelial surface of the striolar region showed that hair cell loss had occurred and an actin band stained with phalloidin was revealed in the centre of the lost hair cell to show the scars formed by adjacent supporting cell expansion in the sites of lost hair cells (Figure 5.2 B). Some apparently normal hair cells and some more intensely stained hair bundles were also present. In the utricular macula and in the cristae, the weakly stained region became progressively wider and extended peripherally when animals survived up to three or four weeks. The crista tissue was technically difficult to make a flat preparation for whole mount surface examination, but the fluorescence image was good enough to do comparison. Fused, enlarged, and intensely stained hair bundles were mostly seen in the crista epithelium and the density of hair bundles decreased as hair cells were lost and replaced by supporting cells (Figure 5.2 C). However, the saccular macula showed no hair cell damage with this 10 days of gentamicin treatment, from one day up to four weeks survival examined by fluorescence microscope (Figure 5.3 A). The organ of Corti showed a substantial reduction in the intensity of actin labelling in the stereocilia and the cuticular plate after gentamicin treatment. Lost hair cells were replaced by the expanded supporting cells and actin rings met to form the "scar patterns" at the sites of the lost hair cells (Figure 5.3 B).

5.1.2 SEM of Specimens from Systemic Treatment Experiments

In guinea pigs, the three cristae from three semicircular canals are not flat and are best described as a "saddle". The hair cells with long hair bundles fully cover the crista sensory epithelium (Figure 5.4 A). In cristae from gentamicin treated animals, the hair bundles had fused and became greatly enlarged. Most of the hair bundles disappeared in the central part of cristae and extended progressively towards the periphery in animals which survived

longer time post-treatment (Figures 5.4 B, C). At higher magnification, hair bundles in the control sensory epithelium of the crista are oriented in the same direction (Figure 5.5 A). In the treated cristae, degenerated hair cells and lost hair cells were replaced by expansion of the adjacent supporting cells (Figures 5.5 B, C). Although the mosaic pattern of hair cells and supporting cells disappeared, there was no evidence of lesions through the epithelial surface. Expanded supporting cells sealed the sites of hair cells and the enlarged polygonal surfaces of supporting cells formed a new meshwork pattern. Damaged hair cells displayed swollen apical ends on the epithelial surface (Figure 5.6 A). When the bizarre shaped hair bundle disappeared, there was a shallow depression of the size and shape of the hair cell apex, sealed by expansions of supporting cells from the bottom (Figure 5.6 B). The apical parts of the involved supporting cells expanded continuously and joined together to form different shaped scars at the sites of lost hair cells (Figure 5.6 C). As new intercellular junctions were formed, the hair cell outlines disappeared, the positions of lost hair cells were completely obliterated by the expansion of supporting cells (Figure 5.6 D).

In addition to the supporting cell expansion to replace the lost hair cells in the epithelium, some hair cells with immature like hair bundles were observed by SEM in a crista of an albino guinea pig which had survived three weeks after 10 days of systemic gentamicin treatment. Immature like hair bundles appeared with the normal and degenerating hair bundles in the apical surface of the crista in the region where hair cell loss was apparent at earlier time (Figures 5.6 E, F). The small bundles of stereocilia like projections were found together with a relatively taller and thicker cilium and, subsequently, similar immature hair bundles were also found in other cristae (Figure 5.6 G). Interestingly, immature hair

bundles only appeared at the meeting point of several surrounding supporting cells, not close to the existing mature hair bundles. The saccular maculae from gentamicin treated animals examined by SEM were normal in appearance without hair cell loss.

Subsequent SEM examinations were performed on all inner ear sensory epithelia, but the results presented here are focused on the vestibular organs, mainly the utricles. The hair cells are evenly distributed on the surface of the control utricles and saccules. The utricle is in a shell shape and the saccule looks like a hook (Figures 5.7 A, B). In high power views of the utricles and saccules, there does not appear to be any regularity in the arrangement of hair cells and supporting cells. The varying number of stereocilia are organised in rows, tall stereocilia near the kinocilium and short stereocilia away from the kinocilium. The orientation of the tallest stereocilia and the kinocilium of the hair bundles face to the striola in the utricular macula and in the saccular macula the longest stereocilia and the kinocilium face away from the striola towards the periphery (Figures 5.7 C, D).

In utricles, loss of hair cells was apparent during the first week following the end of systemic gentamicin treatment. By four days post-treatment, hair cell loss was found in the striolar region of affected utricular maculae (Figure 5.8 A). In high power view of the utricles which had suffered hair cell loss, it was seen that supporting cells expanded to replace the lost hair cells and formed an uninterrupted surface (Figure 5.8 B). Some degenerating hair bundles fused to form giant stereocilia and debris was present in some places. At the early time after systemic gentamicin treatment, the saccular macula from the treated guinea pig appeared normal by SEM (Figure 5.8 C). Four weeks after systemic gentamicin treatment, more hair bundles disappeared in the utricular striolar region and in

the central part of cristae (Figure 5.9 A, Figure 5.10 A) and the area of hair cell loss became wider towards the periphery. The extent of hair cell loss appeared to be greater in the cristae than in the utricles, but no quantitative study was performed. At high magnification, hair cells displaying at their apical surfaces immature hair bundles at different developing stages were found along the striolar region of the utricles where hair cells had been lost originally (Figure 5.9 B). At the same time, short, immature hair bundles were also present on the epithelial surface of the cristae (Figure 5.10 B). Again, the saccular macula was apparently not affected by the drug at four weeks after the 10 days of systemic gentamicin treatment (Figure 5.10 C) and hair cells along the striolar region displayed the normal organised pattern.

The immature hair bundles were easily distinguished from the microvilli on the apical surfaces of supporting cells. Immature hair bundles had a distinctive morphology resembling developing stereocilia, which consisted of stereocilia like projections and a kinocilium like projection arising on a small apical surface of the hair cell (Figure 5.11). The thin and short stereocilia were similar in length and angled towards the centre. The kinocilium was taller and thicker and was initially located in the centre of the cell surface surrounded by thin and short stereocilia (Figures 5.11 A-C). Immature hair bundles showed different stages consistent with their development. As the immature hair bundles developed, the stereocilia become taller. Regularly arranged lateral cross-links on the tips of adjacent stereocilia were present (Figures 5.11 D, E) and the centrally located kinocilium was positioned eccentrically towards the edge of the stereocilia bundle (Figures 5.11 D-F). Hair cells with these immature characteristics of hair bundles were rare in the control vestibular organs (Figure 5.12 A, Table 5.3) and in the areas where hair cells were

not affected by the drug. Most immature hair bundles were present in the areas of hair cell loss after gentamicin treatment along with surviving hair cells and expanded supporting cells. Hair cell loss and immature hair bundle development occurred between one week and four weeks post-treatment. Two weeks after 10 days of systemic gentamicin treatment, hair cell loss had occurred in the striolar region of the utricle and most mature looking hair bundles had disappeared in the area. However, hair bundles with immature forms equivalent to those illustrated in Figure 5.11 were predominant in the striolar area (Figure 5.12 B). Four weeks post-treatment, immature hair bundles showing differentially lengthened stereocilia, but were still shorter than controls, and with clear orientations of hair bundles (Figure 5.12 C). The orientation of the immature hair bundles was similar to that of mature bundles on surviving hair cells in their vicinity. The immature hair bundles that emerged in the damaged hair cell area, together with the surviving mature hair bundles, increased the hair cell density in the striolar region by four weeks after gentamicin treatment (Table 5.3).

In further experiments animals were allowed to survive more than four weeks after systemic gentamicin treatment. Many, but not all, short immature hair bundles present in the striolar region developed continuously towards maturity. In most of the specimens, at eight weeks or 12 weeks post-treatment, the area that suffered hair cell loss did not extend further to periphery than at four weeks (Figures 5.13 A, B). Hair bundles with the most immature form were fewer in the striolar region by 12 weeks post-treatment (Table 5.3), and most hair bundles showed the characteristic of mature hair bundles with stereocilia arranged in a staircase of ascending height (Figure 5.14 A). However, compared with age matched control animals, most of the longest stereocilia on these hair cells in the 12 week

group appeared shorter than their counterparts in similar areas of controls and the density was lower than normal (Figure 5.14 B, Tables 5.1, 5.2). The saccules examined at eight to 12 weeks after systemic treatment revealed reasonably normal morphology. The only saccule which suffered hair cell loss along the striolar region was found in an animal which survived eight weeks after systemic gentamicin treatment. Under SEM, there was a narrow, long band with low density of hair cells in the hook shaped saccular epithelia (Figure 5.13 C), and the number of hair cells in the striolar region of the utricular macula from the same ear was also low and the existing hair bundles were short (Figure 5.14 C). In this same animal, damages to both the cristae and the cochlea were extensive and more severe hair cell loss was found in cristae and the organ of Corti.

After eight months (33 weeks) post-treatment survival, an area with shorter hair bundles could be identified in the striolar region of the treated utricles and the hair cells appeared normal in the periphery (Figures 5.15 A, B, Table 5.4). Although most of the stereocilia were quite well organised and not many typical immature hair bundles were present in the striolar region of the treated utricles, the normal pattern of hair cell distribution was not re-established at eight months (33 weeks) after gentamicin treatment (Figure 5.15 C). In a particular case, many round balloon shaped extrusions were scattered on the apical surfaces along the striolar region of the utricles from an animal after eight month survival, suggesting hair cell degeneration was in progress (Figure 5.16 A). The hair bundles along the striolar region of the saccular macula showed typical mature morphology with normal distribution even at 33 weeks after systemic gentamicin treatment (Figure 5.16 B).

5.1.3 SEM of Specimens from Topical Gentamicin Application

Above results from systemic gentamicin treatment showed that hair cell loss occurred mainly in the striolar region of the utricle and in the central part of the crista epithelium in the guinea pig and the saccular macula was generally not affected by the protocol of systemic gentamicin treatment. After topical application with single dose of gentamicin, SEM results revealed that hair cell loss occurred in the saccular macula as well as in the utricular macula, cristae and the organ of Corti.

Guinea pigs had usually recovered within 2-4 hours after general anaesthesia. At 48-72 hours after the unilateral application with gentamicin into the right ear, the guinea pigs developed involuntary movement of the eyeball (nystagmus) with the fast component towards the untreated ear. However, nystagmus could last a couple of days and it was not found in animals which received normal saline perfusion, indicating ototoxic damage by gentamicin to the vestibular system in the treated ear. Some animals suffered head tilting towards the treated side. In the first week after surgery, most of the guinea pigs lost body weight which was subsequently regained. The wound sealed well without signs of infections.

Degeneration and loss of hair cells happened as early as three days after single topical application with gentamicin and occurred in the saccules as well as the utricles and cristae. There was a narrow band with hair cell loss along the striolar region in the saccular macula (Figure 5.17 A), and in the utricular macula (Figure 5.17 B). The utricular macula suffered hair cell loss in the striolar area similar to the pattern after systemic gentamicin treatment. Only the hair cells in the striolar region were damaged while hair cells located peripherally

survived. The sites of hair cell loss were replaced by the supporting cells to keep the apical surface intact in the damaged saccules and utricles (Figures 5.18 A, B). Progression of hair cell loss and repair after topical application was generally similar to the course of systemic gentamicin treatment. The area of hair cell loss became wider at one week post-treatment but hair cells located in the periphery appeared normal (Figure 5.17 C). A high magnification of the affected area, normal hair bundles were few and some swollen, balloon shaped extrusions were attached to the striolar epithelium. The supporting cells had expanded to seal the epithelium when the degenerated hair cells disappeared (Figure 5.18 C). At 4-5 weeks post-treatment, the hair cell lost area became extended in the striolar regions of the saccular and utricular maculae (Figures 5.19 A, B). However, hair bundles with characteristics of development appeared in the areas of hair cell loss in the saccular maculae as well as in the utricular maculae (Figures 5.19 C, D). The immature hair bundles were different from microvilli at the apical surfaces of the supporting cells and their morphology was identical to the immature hair bundles found from systemic gentamicin treated animals. In most cases, the utricular and saccular maculae and the cristae from the same ear showed a similar pattern of hair cell loss, but hair cell loss and repair did not progress at the same rate. The utricular maculae appeared more reparative than other vestibular sensory epithelia. At 12 weeks, many hair bundles appeared to be further developed, but the cell density was still lower than that in controls and immature hair bundles were apparent (Figures 5.20 A, B). In saline treated ears, normal morphology of hair cells was displayed in the vestibular sensory epithelium and in the organ of Corti (Figures 5.20 C, D).

5.1.4 Data Analysis of Hair Cell Recovery

SEM results from both systemic and topical treatments with gentamicin revealed that hair cell loss occurred in the striolar region of the otolithic organs and in the central part of cristae. Immature hair bundles appeared in the areas where hair cells had been lost and developed towards maturity as the survival time increased post-treatment. The statistical data of hair bundles counted in the utricular and saccular maculae showed a general trend of hair cell recovery after gentamicin induced hair cell loss.

Data of Systemic Gentamicin Treatment

Although hair cell loss appeared more severe and immature hair bundles were first found in the central part of the cristae, due to technical difficulties data analysis was performed only on the utricular maculae from different experiments after systemic gentamicin treatment. The data (hair cell numbers per field) are summarised in Tables 5.1-5.4 and in Figures 5.21-5.23.

Table 5.1 Results from 1st and 2nd Experiments

	No Fields/No Utricles	MEAN±S.D.	MEDIAN
Controls	12/7	54.3±3.89	55
1 week	5/2	19.0±2.92	18
4 weeks	20/6	31.6±3.95	32
8 weeks	10/3	35.5±3.41	36
12 weeks	12/3	39.8±2.82	39

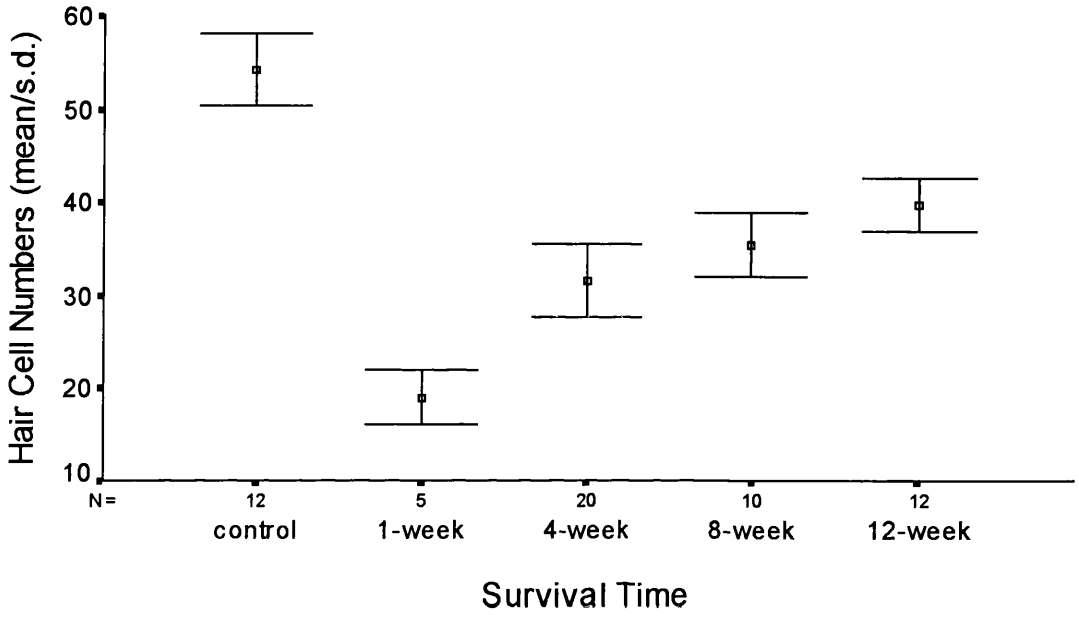

Figure 5.21 Hair Cell Numbers of 1st and 2nd Experiments

The 1st and 2nd experiments were carried out separately, but results were pooled in order to compare the hair cell number decrease and increase. The results demonstrated that the hair cell numbers of treated groups increased significantly following initial hair cell loss after systemic gentamicin treatment (P value at 0.05). When gentamicin treated groups were compared with each other, the mean number of hair cells was significantly different (P value at 0.05), at the sequence of 1week<4weeks<8weeks<12weeeks.

Table 5.2 Results from 3rd Experiment

	No Fields/No Utricles	MEAN±S.D.	MEDIAN
Controls	21/5	55.2±3.52	55
2 weeks	17/4	22.2±6.99	19
4 weeks	15/4	33.9±5.13	33
8 weeks	11/3	28.5±8.13	33
12 weeks	17/4	35.5±2.07	36

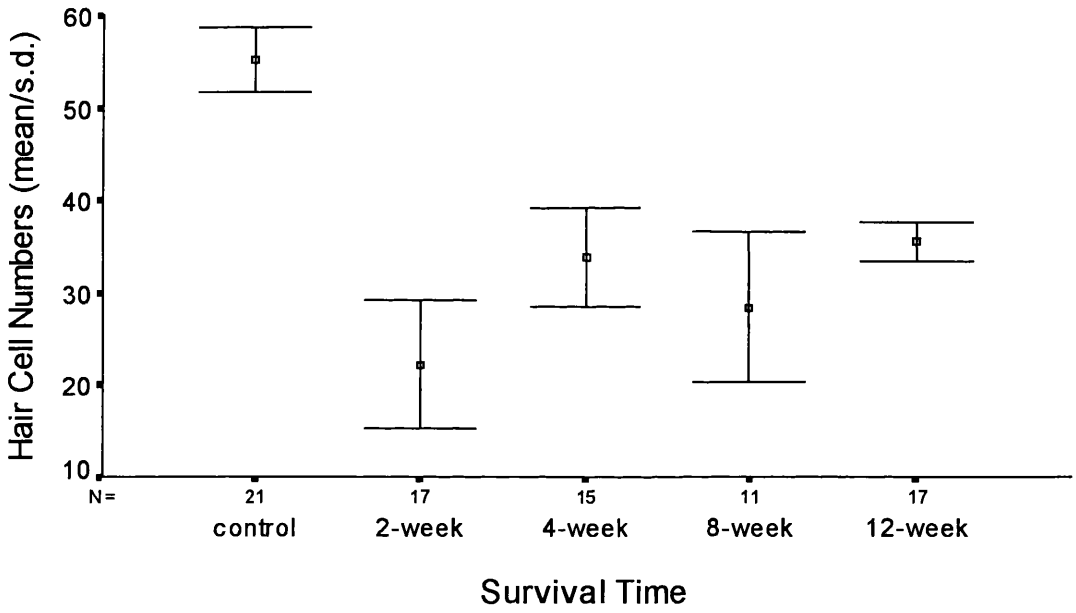

Figure 5.22 Hair Cell Numbers of 3rd Experiment

The mean number of hair cells per field in the striolar regions of gentamicin affected utricles decreased at early time, followed by an increase. Analysis of variance demonstrated that the mean number of hair cells by four weeks and 12 weeks post-treatment significantly increased (P value at 0.05).

From above 140 SEM photographs, hair bundles were divided into three categories by the shape of hair bundles and counted separately: 1) the immature hair bundles equivalent to those illustrated in Figures 5.11; 2) the short hair bundles equivalent to those illustrated in Figure 5.11 F including the immature bundles; and 3) mature looking hair bundles. The data are given in Table 5.3.

Table 5.3 Hair Bundle Shapes in Utricular Maculae (mean±s.d.)

Groups (Fields)	Immature	Short	Mature	Total
Controls (33)	0.4±0.7	6.3±2.26	48.6±3.28	54.9±3.63
1-2 weeks (22)	6.4±4.29	12.5±4.7	9.1±6.74	20.2±7.19
4 weeks (35)	4.1±1.54	16.4±2.59	16.2±5.3	32.6±4.63
8 weeks (21)	3.2±1.45	17.6±3.16	14.2±6.02	31.8±7.16
12 weeks (29)	1.3±1.01	18.2±2.55	19.1±4.72	37.3±3.2

The immature form of hair bundles was less than one per 100 hair bundles in the striolar region of control utricles. It was over 30 per 100 hair bundles in the striolar region of treated utricles by two weeks after gentamicin treatment. The short and immature hair bundles reached half of the total number from the treated utricles. By 12 weeks, the number of the immature hair bundles decreased and the mature hair bundles increased. The mean number of total hair bundles at 12 weeks nearly doubled the mean number of hair cells at 1-2 weeks post-treatment. The results suggested that hair cell loss and cell number reduction occurred in the striolar region of the utricles after systemic gentamicin treatment. Following the hair cell loss, immature hair bundles emerged in the areas where most hair cells had been lost. As animals survived longer, the immature hair bundles continued to develop towards maturity which increased the hair cell density.

Table 5.4 Results from 4th Experiment

	No Fields/No Utricles	MEAN±S.D.	MEDIAN
Controls	17/5	45.2±4.78	45
1 week	21/5	23.0±3.57	22
4 weeks	18/5	26.8±4.15	28
12 weeks	18/5	33.1±4.63	32
33 weeks	16/4	29.1±6.21	30

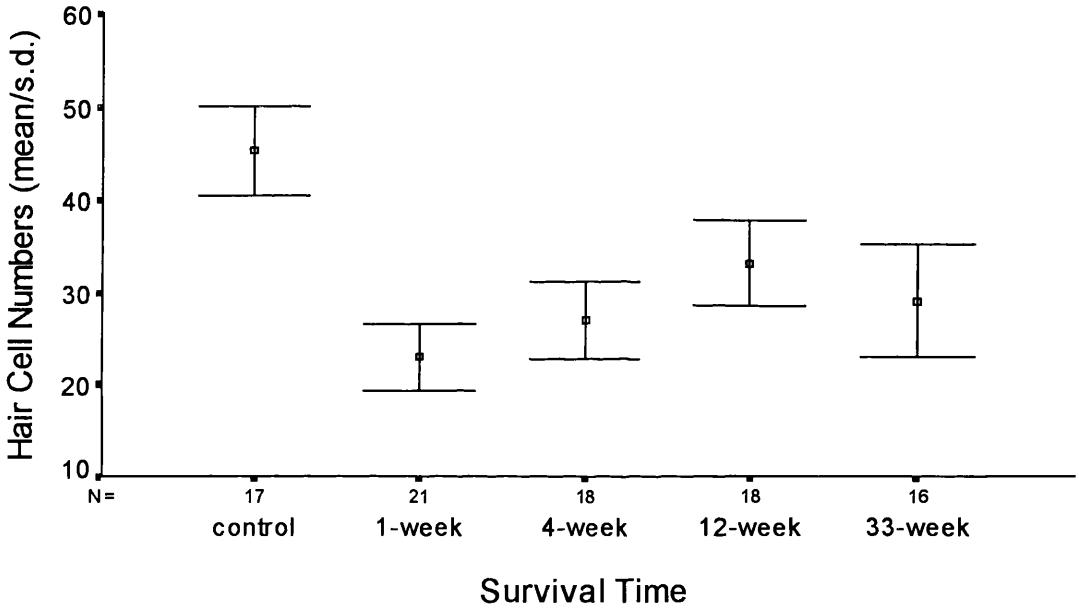

Figure 5.23 Hair Cell Numbers of 4th Experiment

The hair cell numbers counted from three control animals (6 to 10 months old at the time of sacrifice) in this long term experiment were low. The general trend of hair cell loss followed by recovery was the same as in the previous experiments. There was a statistically significant increase in hair cell numbers between one week and four weeks, then between four weeks and 12 weeks in gentamicin treated groups (P value at 0.05). There was no further increase of the hair cell number between 12 and 33 weeks.

Looking at the combined data from different experiments of systemic gentamicin treatment as a whole, a total 230 SEM photographs from 65 utricles were analysed and the results demonstrated that the recovery was the same as the individual experiment. The data are given in Table 5.5 and the mean hair cell numbers are graphically presented in Figure 5.24.

Table 5.5 Combined Results from Systemic Gentamicin Treatment

	No Fields/No Utricles	MEAN±S.D.	MEDIAN
Controls	50/17	51.6±6.11	53
1 week	26/7	22.2±3.75	22
2 weeks	17/4	22.2±6.99	19
4 weeks	53/15	30.6±5.21	30
8 week	21/6	31.8±7.16	34
12 weeks	47/12	35.7±4.29	36
33 weeks	16/4	29.1±6.21	30

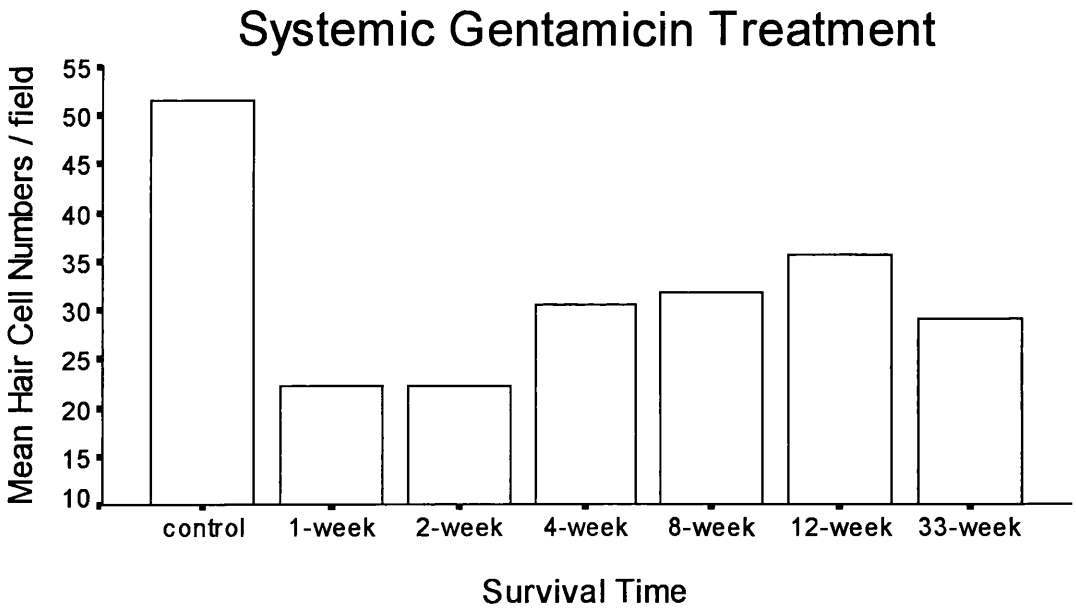

Figure 5.24 Hair Cell Numbers of Systemic Gentamicin Treatment

The mean number of hair cells of gentamicin treated groups, no matter the survival time, was significantly lower than the mean number of control hair cells, suggesting the systemic gentamicin treatment did affect the utricles and cause hair cell loss. Statistical significance was also found between mean hair cell numbers of different survival groups post-treatment. The mean number of hair bundles at 1-2 weeks decreased in the striolar region, down to 40% of the control number. As survival time increased to four weeks, the hair cell number significantly recovered after the initial loss. By 12 weeks, the hair bundle numbers increased further (the highest in these experiments) with significant difference compared with the data of one week and four week groups, but it was still significantly lower than the control mean, indicating that recovery was incomplete. The mean number of hair bundles did not increase further in the longest survival group up to 33 weeks post-treatment. Table 5.6 shows the difference and statistical significance (P value at 0.05) of the mean number of hair cells tested between each two groups from the combined result.

Table 5.6 Duncan's Multiple Range Test

COMPARATION	DIFFERENCE	SIGNIFICANCE
Controls vs 1 week	29.4	*
Controls vs 2 weeks	29.4	*
Controls vs 4 weeks	21.0	*
Controls vs 8 weeks	19.8	*
Controls vs 12 weeks	15.9	*
Controls vs 33 weeks	22.5	*
1 week vs 4 weeks	-8.4	*
1 week vs 8 weeks	-9.6	*
1 week vs 12 weeks	-13.5	*
1 week vs 33 weeks	-6.9	*
2 weeks vs 4 weeks	-8.4	*
2 weeks vs 8 weeks	-9.6	*
2 weeks vs 12 weeks	-13.5	*
2 weeks vs 33 weeks	-6.9	*
4 weeks vs 12 weeks	-5.1	*
8 weeks vs 12 weeks	-3.9	*
12 weeks vs 33 weeks	6.6	*

Data of Topical Gentamicin Treatment

After topical application with gentamicin, hair cell loss and recovery occurred in both saccular and utricular maculae. A total of 115 fields from the striolar regions of 41 saccules and 137 fields from the striolar regions of 44 utricles were assessed in control and topical gentamicin treated ears at different survival times (Figure 5.25, Table 5.7).

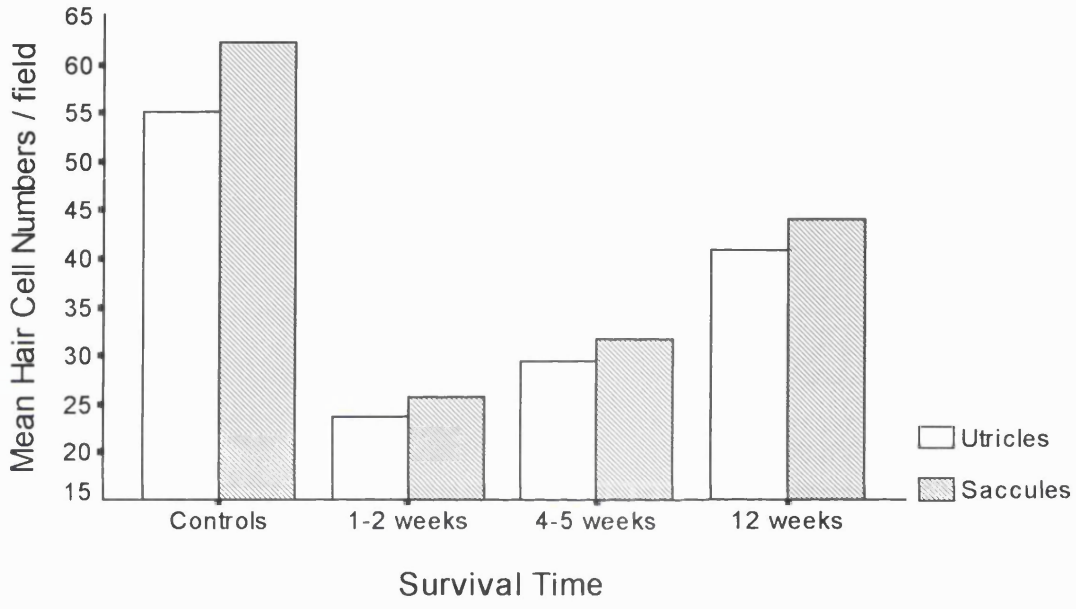

Figure 5.25 Hair Cell Numbers of Topical Gentamicin Treatment

Table 5.7 Data of Topical Gentamicin Application

	Utricular Maculae		Saccular Maculae	
	Fields/Utricles	**Mean±S.D.**	**Fields/Saccules**	**Mean±S.D.**
Controls*	71/23	55.1±7.23	47/20	62.2±5.67
1-2 weeks	26/9	23.7±5.6	29/9	25.6±7.71
4-5 weeks	24/7	29.3±7.43	23/7	31.6±9.82
12 weeks	16/5	40.9±10.05	16/5	44.1±8.15

(*) Controls include saline treated ears and untreated left ears.

Normal saline treated ears showed normal morphology of hair cells in the utricles and saccules from SEM and the mean number of hair bundles were tested without statistical significance between saline treated ears and untreated left ears. Analysis of variance demonstrated that statistical significance was found between mean numbers of hair cells of two compared groups from both saccular and utricular maculae after topical application with gentamicin (Table 5.8).

Table 5.8 Significance Results of Topical Gentamicin Treatment

	Saccular Maculae	Utricular Maculae
Controls vs 1-2 weeks	*	*
Controls vs 4-5 weeks	*	*
Controls vs 12 weeks	*	*
1-2 weeks vs 4-5 weeks	*	*
1-2 weeks vs 12 weeks	*	*
4-5 weeks vs 12 weeks	*	*

(*) Indicates statistical significance at P value at 0.05.

The mean number of hair cells in the striolar regions of the utricular and saccular maculae showed the same pattern of hair cell loss and partial recovery as seen after the systemic gentamicin treatment. The mean number of hair cells of control utricles and control saccules were significantly higher than those means of topical gentamicin treated utricles and saccules no matter what the survival time, suggesting topical gentamicin treatment induced hair cell loss in both the utricles and the saccules. Statistical significance was also found when the mean hair cell numbers of treated utricles and treated saccules at different survival times were compared with each other. As survival time increased, the mean

number of hair cells increased after an initial hair cell loss suggesting hair cell recovery. Table 5.9 summarises the data for differential analysis of the immature hair bundle types in the saccular and utricular maculae after topical application with gentamicin. The immature hair bundles seen by SEM equivalent to those of Figure 5.11 contributed to the increasing numbers of hair bundles in the treated utricles and saccules. However, the mean number of hair cells in the longest survival time up to 12 weeks was still significantly (P value at 0.05) lower than the mean number of control groups, indicating the recovery was incomplete in both utricles and saccules at 12 weeks post-treatment.

Table 5.9 Immature Hair Bundles in Striolar Regions

	Utricular Maculae		Saccular Maculae	
	Controls	4-5 Weeks	Controls	4-5 Weeks
Fields / Specimens	71/23	24/7	47/20	23/7
Total Immature Hair Bundles in Specimens	14	98	8	62
Mean Immature Hair Bundles/Field	0.2	4.08	0.17	2.95
Immature Bundles / 100 Counted Bundles	0.36	12.88	0.27	9.34

5.1.5 Scanning Electron Microscopy of the Organ of Corti

The cochlear sensory epithelium suffered hair cell loss to variable degrees from either systemic or topical treatments with gentamicin at different survival times. The cochleae and vestibular organs of all treated animals had been affected by gentamicin treatment. Every treated animal showed hair cell loss in the organ of Corti and in the specific areas in the vestibular sensory epithelia examined by SEM. The hair cell loss in the cochlea served as an important internal control indicating the drug had entered the ear and damaged the inner ear. Although immature hair cells and partial recovery of hair cell numbers occurred in the vestibular organs after longer survival post-treatment, no evidence of immature hair bundles was found in the cochlear sensory epithelium at any time point examined by SEM. In the damaged cochlea, hair cell loss started from the basal turn and extended towards the apical turns. The outer hair cells appeared more sensitive to the drug and were lost first by one week post-treatment. The inner hair cells were less affected by the drug and some remained intact by 12 weeks post-treatment. Supporting cells expanded to form scars in the sites of hair cell loss and to repair the lesions in the reticular lamina keeping the epithelial surface intact (Figures 5.26 A, B). In severe hair cell loss, supporting cells dedifferentiated and the sensory epithelium of the organ of Corti was formed by epithelial cells migrating in from either side of the organ of Corti (Figure 5.26 C). After topical application with gentamicin, hair cell damage was also seen in the cochlea, and as early as one week post-treatment the architecture of the basal turn of the organ of Corti was distorted (Figure 5.27 A). In some cases, second and third turns of cochlea were often found with extensive hair cell loss and both inner hair cells and outer hair cells had disappeared completely (Figure 5.27 B).

5.1.6 Transmission Electron Microscopy

Only the vestibular tissues from the systemic gentamicin treated guinea pigs were examined by thin sections for transmission electron microscopy. In control vestibular tissues, two types of hair cells were present in the sensory epithelium and hair cells and supporting cells were closely packed. The type I hair cell is pear-shaped and surrounded by a nerve calyx, while the cylinder shaped type II hair cell is contacted by several small bouton-shaped nerve endings (see Figure 1.3 B). The nuclei of most hair cells are spherical and located above the nuclei of the supporting cells and some nuclei of the type II hair cells are located more lumenally. The supporting cell nuclei are usually located at the base of the cell just above the basement membrane and the cell bodies extend the entire width of the sensory epithelium to the lumenal surface. The cytoplasm of the supporting cell usually appears more electron dense than the cytoplasm of hair cells. At the apex of supporting cells there is a distinct, thick bundle of microfilaments attached to the inner surface of the cell membrane at the level of the adherens junction, equivalent to phalloidin labelling. This bundle extends some distance across the apical cytoplasm, parallel to the surface and is quite distinct from the cuticular plates of hair cells.

In thin sections of gentamicin treated vestibular tissues, the normal organisation of hair cells and supporting cells disappeared and progressive degeneration of hair cells was observed in both hair cell types. Within one week post-treatment, hair cell loss appeared in the striolar region of the utricles and in the central part of the cristae. Supporting cells expanded to occupy the lesion sites in the hair cell loss region and their apical surfaces became wider and some nuclei migrated towards the lumenal surface of the sensory

epithelium. The remaining hair cells showed cytoplasmic shrinkage but mitochondria and other intracellular structures appeared intact (Figure 5.28 A, B). In most instances, the hair cell body was degenerated, showing cytoplasmic shrinkage and nuclei darkly staining. But the apical cuticular plate and hair bundle appeared relatively normal without stereociliary fusion. The normal looking cuticular plate was often retained in place and was attached to the adjacent supporting cells by undisrupted tight junctions (Figures 5.29 A, B). When stereocilia became fused or lost, the degenerated apical remnant of the damaged hair cell was being pushed out towards the lumenal surface, and the heads of the adjacent supporting cells expanded to come together and occupy the site of the lost cuticular plate (Figures 5.29 C,D).

The degenerated hair cells were enclosed inside the sensory epithelium by neighbouring supporting cells. The nucleus could break up and the hair cell body was fragmented. The degenerated hair cells showed many of the features described for apoptosis: condensed cytoplasm with little or no swelling of mitochondria or other organelles, compact chromatin in misshapen nucleus, and fragmented nucleus. These morphological features were found during the degeneration of both types of vestibular hair cells. The nuclear chromatin appeared condensed and marginated, packed into smooth masses against the nuclear membrane and they may move down to the level of the supporting cell nuclei, that was often seen as type I hair cells (Figure 5.30 A). Degenerated type II hair cells were present with darkly stained cell cytoplasm and nucleus suggesting apoptotic condensation (Figure 5.30 B). There was no inflammatory response associated with degeneration and loss of hair cells in the inner ear vestibular sensory epithelia.

In addition to hair cells degenerated inside the epithelium, thin sections also revealed extrusion of degenerating hair cells similar to those observed by SEM and phalloidin-FITC fluorescence labelling. At the apical surface of some hair cells, there was a fused and expanded protuberance containing cytoplasm and intracellular organelles (Figure 5.31 A). The protrusion developed through the cuticular plate with stereocilia. When the apical part of the hair cell was pushed out, the membrane around the stereocilia was expanded and the stereocilia and cytoplasm were protruded. In the partially ejected cells, the tight junctions between the hair cell and the adjacent supporting cells appeared intact (Figures 5.31 B, C). Larger and rounded protrusions contained the cell nucleus, suggesting the whole hair cell was extruded. The density of the cytoplasm and the nucleus in the protrusions appeared relative normal in morphology. The spaces left by the ejected hair cells were gradually closed by the expansions of adjacent supporting cells (Figures 5.31 D. E). Where the whole hair cell was ejected, the supporting cell expansions sealed the space completely (Figure 5.31 F). Tight junctions were present beneath the extruded hair cells and adjacent supporting cells formed new connections after hair cell loss.

Over a prolonged recovery period, there was an increase in the number of identifiable hair cells within the regions equivalent to those in which at early time post-treatment there was a loss of hair cells. Four weeks after systemic gentamicin treatment, the epithelium equivalent to the striolar region became thinner but intact. The existing hair cells appeared to be short with small apical cuticular plates and no innervation (Figure 5.32 A). More hair cells were found in the striolar region of the utricles from animals which had survived 12 weeks after systemic gentamicin treatment and most hair cells appeared like the type II hair cells, cylindrical in shape without the afferent calyx (Figure 5.32 B). Some hair cells were

innervated at the lower part of the cell body and developed cuticular plates were present at the cell apex.

Mature hair cells displayed the organised stereociliary bundles in staircase arrangement supported on a well developed cuticular plate (Figure 5.33 A). In comparison with mature hair bundles, the existing hair cells in the striolar region of gentamicin treated utricles at four weeks were immature having small apical cuticular plates, thin stereocilia and no innervation synapsing. Short stereocilia with relatively low density of microfilaments joined the cell with no construction and there was no rootlet in the poorly developed cuticular plate (Figure 5.33 B). The hair bundles did contain a single kinocilium, but the stereocilia were thin. Hair cells with relatively thick stereocilia and defined cuticular plates appeared to form bouton shaped nerve endings at the lower part of the body (Figure 5.33 C). Up to 33 weeks after gentamicin treatment, hair cells were densely packed in the striolar region of the utricular sensory epithelium. They all resembled type II hair cells with small bouton shaped nerve endings at the basal end and short hair bundles at the apex, the nerve calyx normally surrounding the type I hair cells were not found at this stage (Figure 5.34). The result suggested that type I hair cells had been lost after gentamicin treatment, and been replaced in the striolar region by cells with the characteristics of type II hair cells.

Hair cells in the utricular thin sections from the systemic gentamicin treated animals were counted by Dr Andrew Forge and the data are given in Tables 5.10, 5.11. The two sets of data showed the same trend of hair cell recovery after initial loss and supported the SEM results (Tables 5.1, 5.4). The mean total number of hair cells in the utricles (in a length of 80 μm across the striolar region) from gentamicin treated guinea pigs was significantly

lower than the control mean (P value at 0.05). As animal survival time increased post-treatment, the hair cell number significantly increased from one week to four weeks, then further advanced to 12 weeks (P value at 0.05). At the same time, type I hair cells significantly decreased in number accompanied by type II hair cells significant increase. By 33 weeks post-treatment, the total hair cell number increased slightly, but did not show significance compared with the 12 weeks result.

Table 5.10 Hair Cell Numbers in Thin Sections (1st and 2nd groups)

GROUPS	SECTIONS/ UTRICLES	TOTAL HAIR CELL	TYPE I HAIR CELL	TYPE II HAIR CELL
Controls	42/8	10.31	6.88	3.89
4 weeks	21/4	6.19	1.10	5.82
12 weeks	16/5	8.06	2.69	5.71

Table 5.11 Hair Cell Numbers in Thin Sections (4th group)

GROUPS	SECTIONS/ UTRICLES	TOTAL HAIR CELL	TYPE I HAIR CELL	TYPE II HAIR CELL
Controls	34/5	12.47	7.79	4.68
1 week	37/5	5.24	1.03	4.22
4 weeks	35/5	7.36	1.88	5.48
12 weeks	45/5	8.47	1.93	6.45
33 weeks	37/3	9.01	2.02	7.08

5.1.7 BrdU Immunohistochemistry

From statistical analysis of the hair cell numbers in the vestibular organs, hair cell loss was followed by a partial recovery when treated animals survived longer post-treatment. BrdU was used to detect whether the developing hair cells arose through proliferation in the inner ear. No animal was found dead in experiments by intraperitoneal systemic BrdU injections or by implantation of an osmotic pump.

BrdU Labelling in Whole Mount Preparations

The whole mount preparations of the inner ear tissues showed some BrdU positive labelling in the vestibular sensory epithelia parallel with the SEM results. Many immature like hair bundles were revealed by SEM in the utricles two weeks after systemic gentamicin treatment, and the utricle from the opposite ear processed for BrdU immunolabelling showed positive labelling in the sensory epithelium (Figure 5.35 A). It was not very convincing that all BrdU labelled nuclei were located inside the sensory epithelia although some labelled nuclei were in pairs and most of the labelling appeared below the sensory epithelium, in the cell nuclei of the connective tissue (Figure 5.35 B, C). Very small numbers of BrdU labelled nuclei were found in the treated utricles and saccules from animals which received topical gentamicin application (Figure 5.35 D, E). The utricles from control animals also showed BrdU positive labelled nuclei beneath the sensory epithelium in the connective tissue but the organ of Corti did not show BrdU labelling. In order to judge the location of the positive labelled nuclei, wax embedding and sectioning of the tiny vestibular tissues were made. Many utricular wax sections from gentamicin treated animals receiving BrdU injections were mainly used to test different techniques and no complete and consistent results were obtained.

BrdU Labelling in Wax Sections

The small intestine and liver processed as control tissues showed BrdU positive reactivity in animals which received BrdU treatment. In haemotoxylin counterstained sections, normal nuclei were stained blue and BrdU positively labelled nuclei were stained brown. In sections of the small intestine, the intestinal mucosa was present in the finger like projections into the intestinal lumen. BrdU labelled nuclei, brown in colour, were present along the epithelial layer of the villi as well as in the underlying lymphocytes and connective tissue (Figure 5.36 A). In sections of liver tissue, BrdU positive labelling was found in the nuclei of the hepatocytes and the stroma of connective tissue. Nuclei from liver parenchyma cells were labelled in closely adjacent pairs and labelled stroma nuclei were irregular in shape (Figure 5.36 B). These results demonstrated that BrdU had been distributed around the animal body after intraperitoneal injections or osmotic pump implantation. In BrdU negative control liver sections, the cell nuclei were stained blue by haemotoxylin without BrdU labelling (Figure 5.36 C), suggesting that BrdU antibody was a proper marker to detect cell proliferative activity.

BrdU positive labelling in the inner ear vestibular organs was found in guinea pigs which survived two weeks after receiving topical gentamicin application to the middle ear and implantation of BrdU osmotic pump. Wax sections of gentamicin affected utricular maculae showed hair cell loss across the striolar region that supported the SEM results reported in 5.1.3. In some cases the affected area was extended towards the periphery and the sensory epithelium was thinner (Figure 5.37 A). Brown positively labelled nuclei were present in the utricular maculae and the labelled nuclei were located at the level of supporting cell nuclei (Figures 5.37 B) or more lumenally, at the level normally occupied

by hair cells (Figures 5.37 C, D). The sections of left control utricular maculae showed regularly arranged hair cells and no BrdU positive labelling was found inside the sensory epithelia (Figure 5.38 A, Table 5.12). BrdU labelling was also found in cell nuclei in the connective tissue underlying the sensory epithelia in both the treated and untreated utricles (Figure 5.38 B, Table 5.12). BrdU labelled nuclei were counted on each utricle from 5 μm thickness serial wax sections and the total labelled nuclei varied from zero to 12 in the sensory epithelium of treated utricles. However, labelled nuclei in the connective tissue were much greater in all of the treated utricles in comparison with the number of labelled nuclei in untreated utricles (Table 5.12). The results of BrdU positive labelling in the utricles provided evidence that the proliferation occurred the mammalian vestibular sensory epithelia.

Table 5.12 BrdU Labelled Nuclei in Utricles

Animal Number	Treated Utricles			Untreated Utricles		
	Wax Sections	Sensory Epithelium	Connective Tissue	Wax Sections	Sensory Epithelium	Connective Tissue
408	146	0	215	158	0	48
409	132	12	500	113	0	26
413	110	7	199	116	0	19
414	111	1	82	118	0	51

5.2 IN VITRO EXPERIMENT RESULTS

To examine hair cell degeneration and recovery phenomena more directly, the use of vestibular sensory epithelia from mature guinea pigs and gerbils maintained in organotypic cultures was evaluated. In the in vitro experiments, gentamicin was added directly to the cultured utricles.

5.2.1 Morphology of Guinea Pig Vestibular Tissues in Vitro

The mature vestibular utricles and saccules were dissected out and explanted on to coverslips coated with rat tail collagen with the sensory epithelium uppermost. Explants became attached to the surface in MEM culture medium after one or two days settlement in vitro and tissue outgrowth was usually seen within 48 hours after explantation (Figure 5.39 A, B). The outgrowth served as a good indicator for culture survival and it was formed by the fibroblasts and epithelial cells from the cultured tissue through migration and mitotic divisions. After longer time in vitro, the cultured utricle was surrounded by a continues layer of outgrowth tissue (Figure 5.39 C).

The culture medium used throughout the in vitro experiments was the MEM culture medium supplemented with 10% heat inactived horse serum. SEM of the control cultured utricles showed that hair cells evenly covered the surface of the epithelia and stereocilia bundles were present with increasing height (Figure 5.40 A). The striolar region of the cultured explants could be identified, where hair bundle orientation was opposite. Although many hair bundles appeared normal, some of the stereocilia were fused or lost rigidity and bent to the surface. Some bleb-like structures were present at the surface of the epithelium. Thin sections of cultured explants showed both type I and type II hair cells

remained in the epithelium but morphology of hair cells in vitro was different (Figure 5.40 B). Most of the hair cells lost their innervation almost completely by four days in vitro, the earliest time examined. The stereocilia bundle showed preservation of closely packed microfilaments and extensive cross links between adjacent stereocilia (Figure 5.40 C).

Several different culture media were tested in attempts to determine the optimal conditions for preserving hair cells in the mature mammalian vestibular organotypic cultures. Vestibular utricles and saccules were cultured for seven days with 1) MEM medium only; 2) 10% heat inactive horse serum in MEM medium with N1 supplement (Sigma Chemical Ltd); 3) 5% foetal calf serum (Life Technologies Ltd) in MEM medium with and without N1 supplement. The optimal condition for culturing the mature mammalian vestibular maculae was judged by SEM morphology of the hair cells. After testing, no culture condition was found better than the MEM medium supplemented with 10% heat inactived horse serum.

5.2.2 Effects of Gentamicin Treatment to Cultured Vestibular Maculae

After settlement of 24-48 hours in vitro, cultured guinea pig vestibular utricles were treated with 1mM gentamicin for four hours. Hair cell loss was mainly found in the striolar region at seven days post-treatment. The hair cell loss extended towards peripheral area when cultured explants survived 14 days after gentamicin treatment, the longest time examined in this in vitro study. When cultured utricles were treated with 1mM gentamicin for 24 hours, hair cell loss was extensive at longer times in vitro (Figure 5.41 A). The hair bundles were fused and dome shaped extrusion enclosed the hair bundle on the apex of the affected hair cell. Some dead hair cells in cultured utricles appeared to be expelled from

the sensory epithelium. In central area of the cultured utricles, most of the hair cells were lost and replaced by supporting cells. The epithelial surface remained intact with debris of degenerated hair cells on the surface and a few cells showing the features similar to the immature hair cells found in vivo after gentamicin treatment (Figure 5.41 B). SEM revealed that cells with a small apical surface developed a bundle of tall and thin microvilli of approximately equal height, different from the short microvilli on the apical surfaces of the adjacent supporting cells. In the peripheral area, the surviving hair bundles were present with staircase arrangement (Figure 5.41 C). The saccular maculae were also affected by gentamicin treatment in vitro, but the extent of hair cell loss did not appear as great as in the equivalent utricle and detailed results are mainly given on cultured utricles.

Thin sections of gentamicin treated cultured utricles revealed hair cell loss found by SEM. Expelled hair cells attached at the apical surface of the epithelium showed the presence of the nucleus, mitochondria, the cuticular plate and stereociliary microfilaments (Figure 5.42 A). Occasionally, the cuticular plate and the stereociliary bundle were found to be enclosed inside the sensory epithelium (Figure 5.42 B). Degeneration of hair cells inside the sensory epithelium and less frequently, extrusion of hair cells to the lumenal surface of the epithelium, were similar to the hair cell loss revealed in the in vivo studies. Progressive loss of hair cells was found in the striolar region of the utricle and extended towards to the periphery. In the area of hair cell loss, supporting cells were present in the epithelium and their nuclei became elongated, enlarged and some of them appeared to migrate more lumenally (Figure 5.42 C). Supporting cells expanded their apical surface to repair the spaces in the epithelium that the hair cells once occupied. The expanded supporting cells formed new intercellular junctions between themselves to effect repair of the lesion caused

by the loss of hair cells. Hair cells individually degenerated inside the sensory epithelium displaying morphological features of apoptosis: compaction of nuclear chromatin into uniformly dense masses and margination, with cytoplasm shrinkage and condensation, membrane blebbing, but the mitochondria intact (Figure 5.43 A, B). Some apoptotic hair cells remained in situ and some were found at the level of supporting cell nuclei which appeared normal (Figure 5.43 C, D). Apoptotic bodies, containing electron-dense inclusions, were often seen in the area of hair cell loss and they became ingested by surrounding supporting cells

5.2.3 Hair Cell Loss in Gerbil Vestibular Utricles in Vitro

To confirm the value of the culture system, similar studies were performed with gerbil vestibular tissues. The cultured gerbil utricles, similar to the guinea pig cultured utricles, were evenly covered by hair cells on the whole epithelium examined by SEM (Figure 5.44 A). From thin sections, hair cells appeared normal in control gerbil utricles. Hair bundles were well preserved and erect on the cuticular plate and the hair cell types were easy to distinguish by differences in cell body shape. Type I hair cells were rounded with a basally located nucleus. The type II hair cells were elongated with a big round nucleus. Most hair cells lost nerve connection to the cell body by three days in vitro, the earliest time examined (Figure 5.44 B). The supporting cells appeared electron-dense and their nuclei were located beneath the hair cells. By 12 days after gentamicin treatment, hair cell loss was more extensive in the central area of the gerbil vestibular utricles and the area of hair cell loss became thinner compared with the peripheral area where intact hair cells remained (Figure 5.44 C). In the area of hair cell loss, only supporting cells were present and their cell bodies were cylindrical in shape and their nuclei formed an even layer above the

basement membrane. Lost hair cells were replaced by supporting cells which expanded their apical surface to form new junctions to effect repair of the lesion. The epithelium remained intact with debris of degenerated hair cells on the surface and remnants of degenerated hair cells inside the epithelium. The pattern of hair cell loss that occurred within the cultured gerbil utricles after gentamicin treatment was similar to the guinea pig results of both in vivo and in vitro studies. Degenerated hair cells also displayed morphological features characteristic of apoptosis. The nucleus showed compaction of chromatin into uniformly dense marginated masses (Figure 5.45 A). The cell cytoplasm was darkly stained suggesting cell shrinkage. Apoptotic cells appeared to stay in situ before they fragmented and some of them moved towards the basal part of the epithelium (Figure 5.45 B). Fragmented apoptotic bodies, containing electron-dense inclusions, were seen in the area of hair cell loss and became ingested by surrounding supporting cells (Figures 5.45 A, B). The cytoplasm of supporting cells contained fragmented electron-dense nuclei, indicating that the supporting cells digested the degenerated hair cells. Supporting cell nuclei were apparently normal without apoptotic changes after gentamicin treatment.

5.2.4 BrdU Immunolabelling of Cultured Utricles

Fluorescence microscopy of whole mount preparations revealed that the cell nuclei of the outgrowth tissue and connective tissue from control and gentamicin treated guinea pig utricles were incorporated BrdU and were positively labelled by antibody after 16 days in vitro (Figure 5.46 A). Using single label immunogold for electron microscopy, mitotic nuclei of gerbil utricular cultures were labelled by BrdU antibody (Figure 5.46 B). Most hair cells had been lost in the sensory epithelium 13 days after gentamicin treatment. A high density of gold particles was only found in the nuclei of the utricular outgrowth tissue and in the connective tissue on the sections examined. It indicated that cultures survived and some cells continued to proliferate in vitro. Several thin sections from each gerbil utricle were used for BrdU immunogold electron microscopy, there was no convincing BrdU positive labelling was found in the cell nuclei inside the sensory epithelium.

5.2.5 Apoptotic Nuclei Labelling

The morphological evidence from TEM sections suggested that gentamicin induced hair cell loss in the vestibular sensory epithelia of the mammalian inner ear through apoptosis both in vivo and in vitro. To substantiate this observation, the fluorescent dye for nucleic acids, propidium iodide, and in situ end labelling by ApopTag apoptosis detection kit were applied in cultured utricles to detect apoptotic nuclei.

Fluorescence Microscopy of Cultured Utricles

Cultured specimens were stained by propidium iodide to label the nuclei and phalloidin-FITC to label actin in hair bundles to identify regions of hair cell loss. The utricles in control cultures up to eight days in vitro showed the hair cell nuclei were round in shape (Figure 5.47 A) and formed a quite even layer below the cuticular plates of hair cells and above the layer of small nuclei of supporting cells (Figure 5.47 B). The peripheral hair cell nuclei were present in normal morphology (Figure 5.47 C). There were a few hair cell nuclei condensed or fragmented stained by propidium iodide in control tissues. After treatment with 2 mM gentamicin for 6 hours or 24 hours, the number of condensed or fragmented nuclei increased. The nuclei of degenerated hair cells appeared condensed and became smaller than normal hair cell nuclei with bright propidium iodide staining (Figure 5.48 A), although the hair bundles labelled by phalloidin existed on the cuticular plates in the sensory epithelium (Figure 5.48 B). Brightly stained fragmented and condensed hair cell nuclei were found more in the central area of the cultured utricles after 24 hours gentamicin treatment (Figure 5.49 A). The apical surface of hair cells absent of hair bundles and presence of many scars were noted (Figure 5.49 B). Different patterns of scars were seen depending on the number of the supporting cells involved. When the cultured

utricles were allowed to survive for further 5 days after gentamicin treatment, phalloidin-FITC labelling actin in the sensory epithelial surface revealed that most hair bundles disappeared and supporting cells formed scars and new intercellular junctions appeared (Figure 5.50 A). However, some immature like small and short hair bundles were apparent in the apical surface of the epithelium together with tall hair bundles. More hair cell nuclei became fragmented and some disappeared in the central area of the treated utricles (Figure 5.50 B). The number of hair cell nuclei decreased in these specimens after a total of eight days in vitro (Table 5.13). The supporting cells were not affected by gentamicin treatment and their nuclei were densely packed in an even layer beneath the level of hair cell nuclei (Figure 5.50 C). Many peripheral hair cell nuclei still remained and the density of hair cell nuclei decreased slightly after longer survival time post-treatment (Table 5.13). The result indicated that degeneration and loss of hair cells were predominant in the central area of the cultured utricles, but the hair cells in the periphery were less affected.

In Situ End Labelling of Cultured Utricles

In parallel with the propidium iodide staining, whole mount cultures of utricular maculae were processed for in situ end labelling apoptosis by ApopTag kit. Under differential interference contrast microscopy, hair cell nuclei in control utricles appeared in an even layer and the nucleus size was similar (Figure 5.51 A). Apoptotic nuclei could be detected by in situ end labelling method in control utricles but the number was small. More positively labelled hair cell nuclei were found in the gentamicin treated utricles in the central areas corresponding to the regions of hair cell loss in parallel propidium iodide and phalloidin double labelled preparations. Labelled nuclei by ApopTag kit were brown in colour showing nuclear fragmentation and chromatin margination (Figure 5.51 B). In

method control preparation, there was no labelling found in the gentamicin treated specimens (Figure 5.51 C). Apoptotic hair cell nuclei appeared predominately at the level of hair cell nuclei (Figure 5.52 A) and positive labelling was also found more lumenally (Figure 5.52 B). Hair bundles can be seen on the cuticular plates of the surviving hair cells. Some apoptotic nuclei moved lower towards the level of supporting cell nuclei inside the epithelium and enclosed by supporting cells (Figure 5.53 A). In the periphery regions, fewer apoptotic hair cell nuclei were labelled, and the number appeared greater than that in the central regions (Figure 5.53 B; Table 5.14). These findings supported the results obtained from electron microscopy.

Data Analysis

The normal and abnormal hair cell nuclei including fragmented and condensed nuclei labelled by fluorescent propidium iodide or by ISEL method were quantified in control and gentamicin treated cultured utricles. A minimum of four maculae were examined for each experimental paradigm. Three rectangular areas in the central striolar region and three areas in the periphery, a total of 360 fields on 60 cultured utricles, were counted at 100X oil lens under Nikon Optiphot-2 microscope using fluorescence microscopic attachment or differential interference contrast lens as appropriate (Tables 5.13, 5.14).

Table 5.13 Propidium Iodide Labelling in Utricles (mean±s.d.)

Treatment (Fields)	Centre Total	Periphery Total	Centre Apop.	Periphery Apop.
Controls (12)	38.7±3.85	40.5±2.5	2.2±1.27	1.3±1.34
Gen. 6 hs (12)	28.1±4.74	37.2±3.43	5.7±3.96	3.9±2.58
Gen. 24 hs (12)	28.2±3.61	34.8±2.79	11.8±5.58	5.3±3.86
5d Controls (12)	36.1±2.94	38.7±2.27	0.6±1.0	1.0±1.0
5d Gen. 6 hs (15)	23.0±6.04	37.3±2.74	2.1±1.77	0.7±1.28
5d Gen. 24 hs (18)	19.0±5.12	31.2±4.56	1.4±1.2	0.9±1.0

Table 5.14 ISEL Apoptosis Labelling in Utricles (mean±s.d.)

Treatment (Fields)	Centre Total	Periphery Total	Centre Apop.	Periphery Apop.
Controls (15)	39.1±2.66	41.7±2.66	2.1±2.1	2.0±1.46
Gen. 6 hs (21)	27.4±4.87	36.4±4.18	2.2±1.54	0.9±0.86
Gen. 24 hs (24)	27.2±3.64	35.5±2.9	4.3±4.31	1.6±1.66
5d Controls (12)	38.8±2.45	41.7±2.35	1.9±2.35	0.9±0.99
5d Gen. 6 hs (12)	24.8±5.12	35.5±3.6	5.4±2.19	3.2±1.99
5d Gen. 24 hs (15)	22.6±4.53	32.7±4.13	5.1±3.75	3.7±1.72

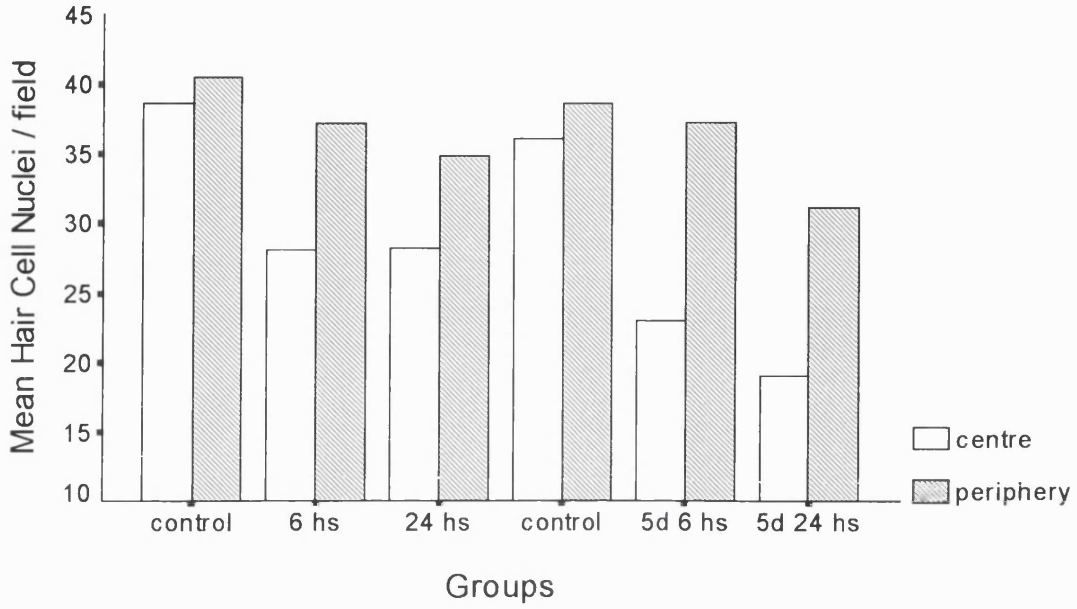

Figure 5.54 Hair Cell Nuclei by Propidium Iodide Labelling

The plot shows the mean total number of hair cell nuclei per field stained by propidium iodide in central and peripheral areas of the cultured utricles. There was no significant difference in the mean number of hair cell nuclei in control cultures at different locations and at different time points in vitro. The control mean number was significantly higher than the mean number of central hair cell nuclei in gentamicin treated cultures (P value at 0.05), suggesting in vitro gentamicin treatment damaged hair cells in central/striolar region. The mean number of peripheral hair cell nuclei in gentamicin treated utricles was significant higher (P value at 0.05) than its mean number of central hair cell nuclei, showing peripheral hair cells were less affected by gentamicin treatment.

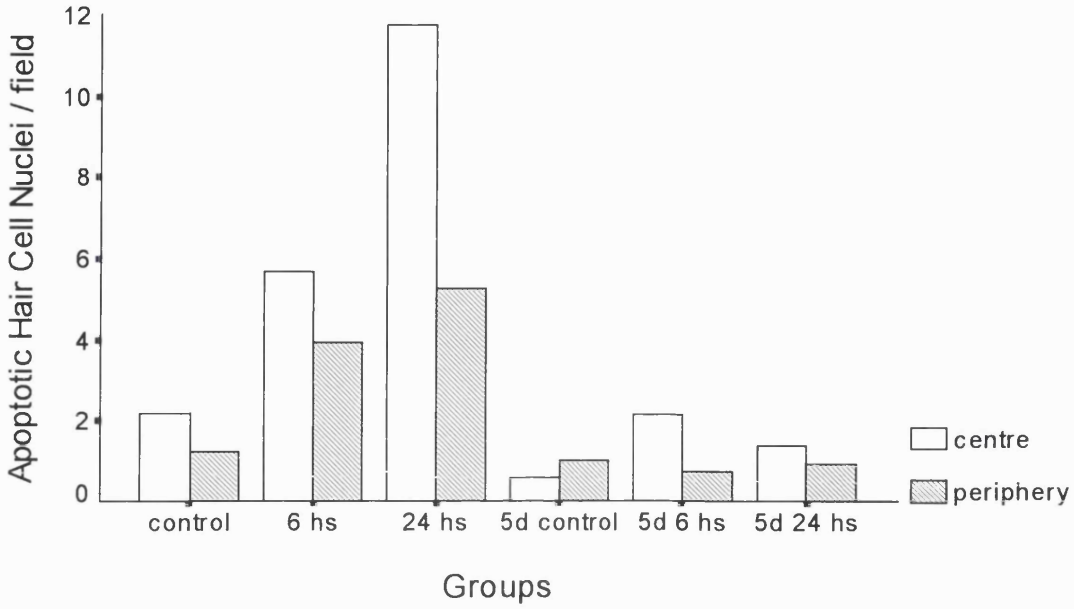

Figure 5.55 Apoptotic Hair Cell Nuclei by Propidium Iodide Labelling

The fragmented and condensed hair cell nuclei brightly stained by propidium iodide were counted separately. The plot shows the mean number of hair cell nuclei per field with apoptotic morphology after propidium iodide staining in central and peripheral areas of the cultured utricles. The number of central apoptotic hair cell nuclei was significantly high after gentamicin incubation for 6 hours and 24 hours. The cultured vestibular utricles after 24 hours gentamicin treatment immediately processed for propidium iodide labelling showed more brightly stained fragmented and condensed hair cell nuclei compared with any of the other groups.

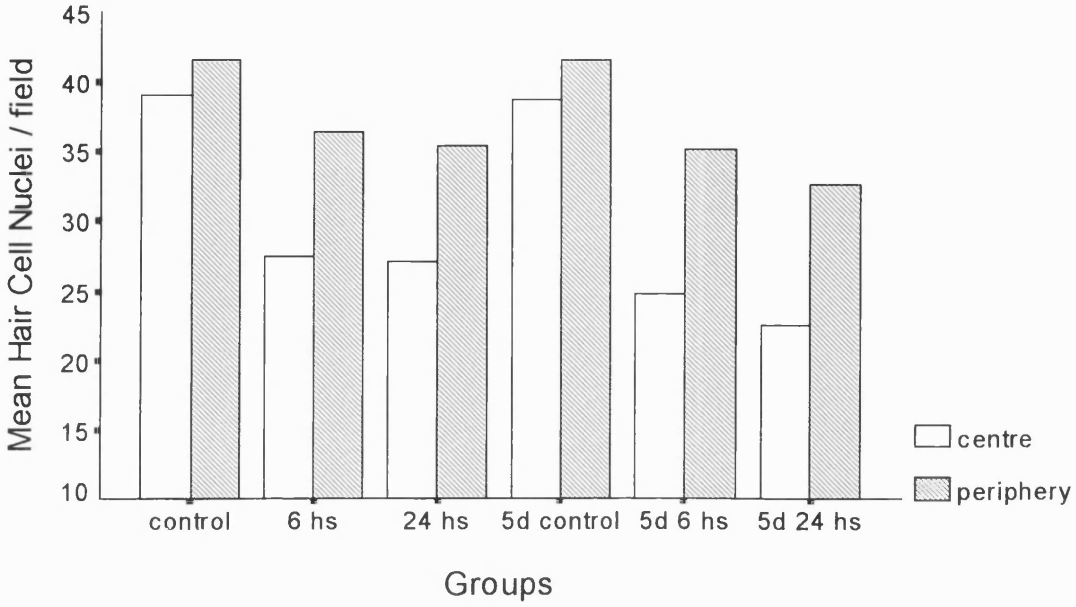

Figure 5.56 Hair Cell Nuclei by Differential Interference Microscopy

This graph shows the mean total number of counted hair cell nuclei (including brown positively stained apoptotic hair cell nuclei) per field in central and peripheral regions of the cultured utricles. In control cultures at different time points in vitro, the mean number of central hair cell nuclei was slightly lower than the mean number of peripheral hair cell nuclei, but there was no significant difference. The control mean number was significantly higher than the mean number of gentamicin treated cultures (P value at 0.05), suggesting in vitro gentamicin treatment damaged hair cells in the cultured utricles. In gentamicin treated utricles, the mean number of peripheral hair cell nuclei was significant higher than the mean number of central hair cell nuclei (P value at 0.05), showing more centrally located hair cells were lost than peripheral hair cells. However, significant difference was also found between the mean numbers of control cultures and periphery hair cell nuclei in gentamicin treated utricles.

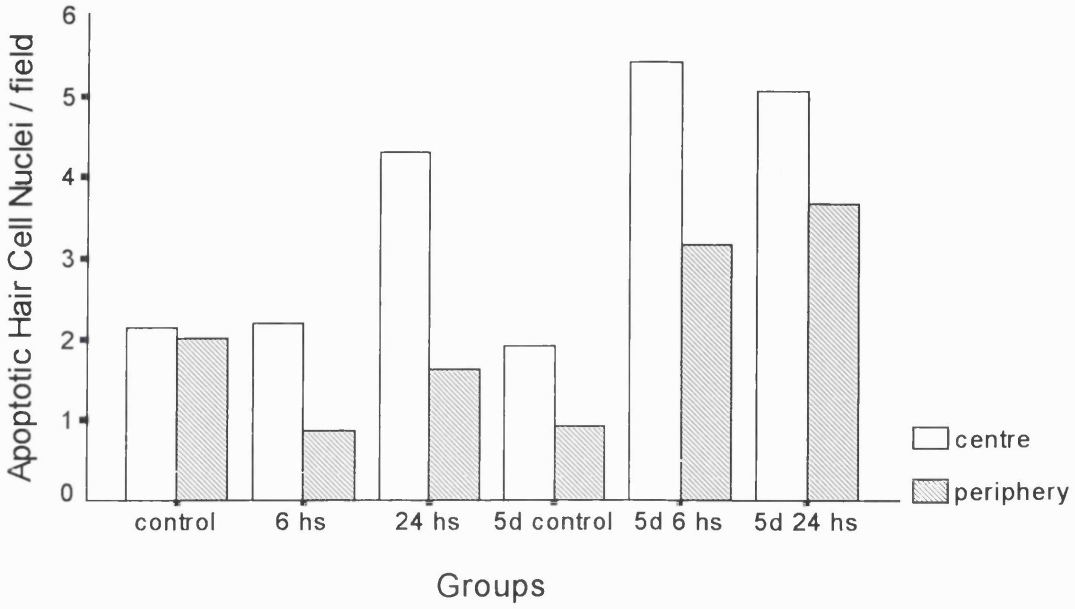

Figure 5.57 Apoptotic Hair Cell Nuclei by ISEL

This graph shows the mean number of brown stained apoptotic hair cell nuclei per field in central and peripheral regions of the cultured utricles. Positively labelled apoptotic hair cell nuclei appeared more in central areas of gentamicin treated utricles. The mean number of apoptotic hair cell nuclei were significantly higher in cultured utricles after 24 hours gentamicin incubation and in those survived for further five days in vitro after 6 hours or 24 hours gentamicin treatment compared with the mean number of other groups, suggesting hair cells degenerated through apoptosis after gentamicin treatment.

Figure 5.1 Fluorescence Microscopy of Normal Inner Ear Tissues

A. Control utricle from an albino animal which received normal saline systemic injection. The utricle is covered with hair cells labelled with actin marker phalloidin under fluorescence microscopy. Bar = 50 μm.

B. Control utricle, actin filaments in hair bundles are specifically labelled by phalloidin. Bar = 30 μm.

C. Control utricle, the cuticular plates (arrowhead) of hair cells are round and larger than the apical parts of the supporting cells. Supporting cells are delineated by labelling of a ring of actin associated with the intercellular junctions and their apical surfaces (arrow) are polygonal in shape without the hair bundle and free of actin staining. Bar = 20 μm.

D. Organ of Corti from a normal pigmented guinea pig, phalloidin staining is found in the cuticular plate of three rows of outer hair cells (OHC) and a single row of inner hair cells (IHC). The stereocilia (arrowhead) of outer hair cells are arranged in "V" shape. The actin staining of the adherens junctions of supporting cells with hair cells displays a mosaic pattern of the organ of Corti. Bar = 20 μm.

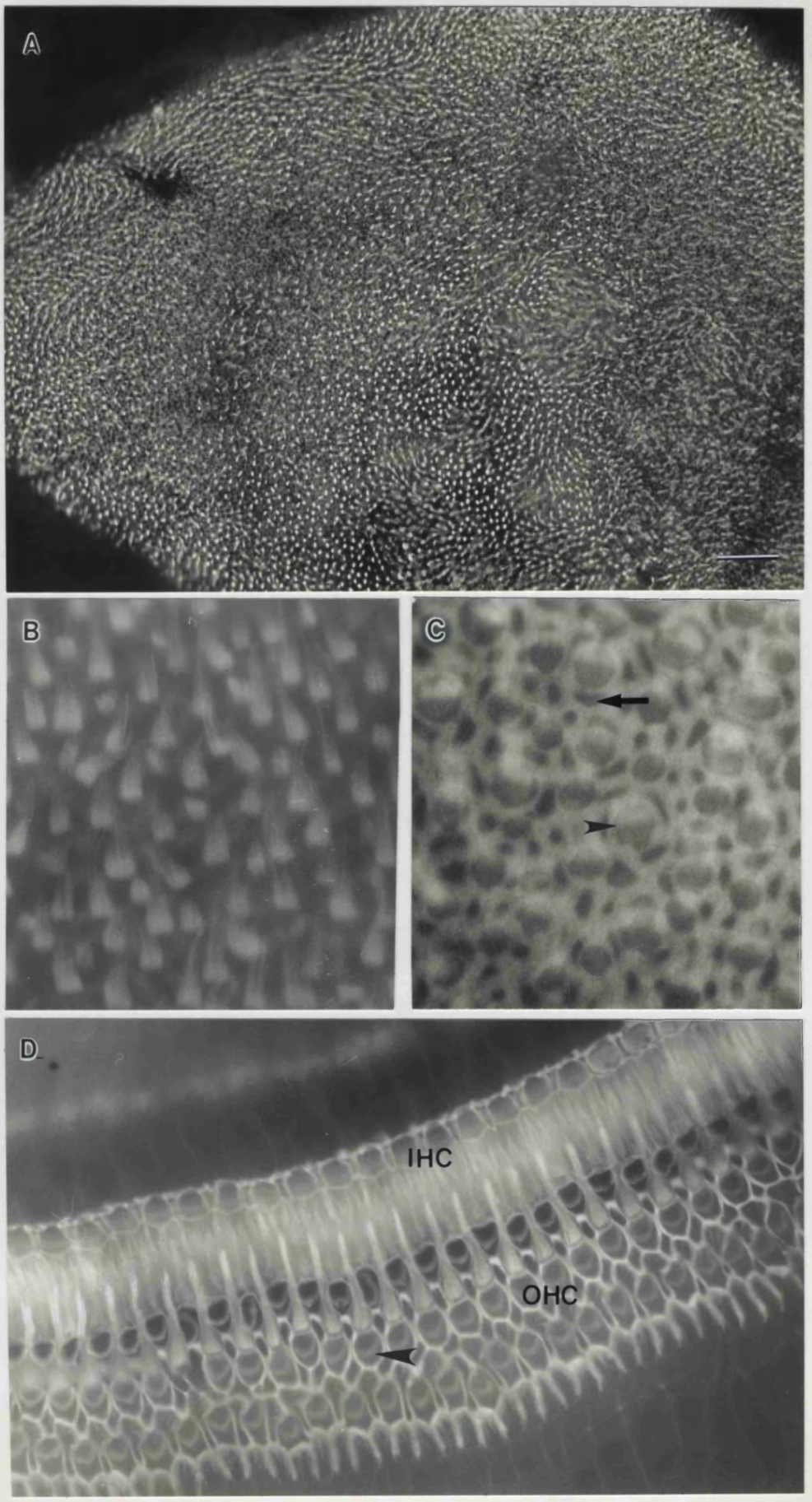
A
B
C
D
IHC
OHC

Figure 5.2 Fluorescence Microscopy of Utricular and Crista Tissues

A. Utricle from a treated guinea pig at two days after 10 days of systemic gentamicin injection. An area weakly stained by phalloidin appears in the striolar region and the remaining peripheral hair cells look normal. Bar = 50 μm.

B. Striolar region of the utricle three days post-treatment. Hair cell loss is apparent and many scars (arrowhead) are present at the sites of the cuticular plates of the lost hair cells. Some surviving hair bundles (arrow) remain and are intensely stained with phalloidin. Bar = 20 μm.

C. Central area of a crista at four weeks after 10 days of systemic gentamicin injection. Enlarged and intensely stained hair bundles (arrow) and scars (arrowhead) are present. The intercellular junction rings are thick and stained intensely. Bar = 10 μm.

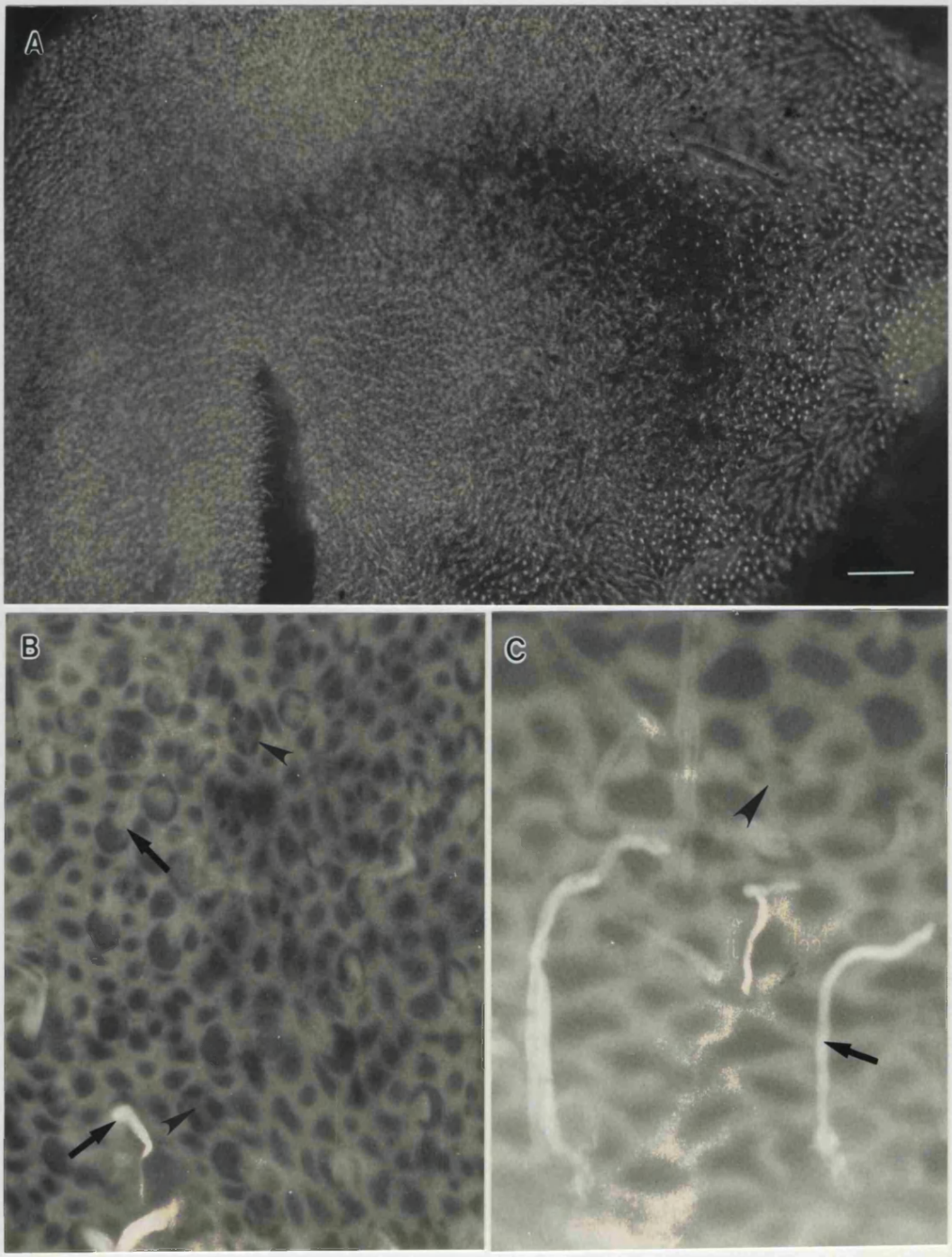
A
B
C

Figure 5.3 Fluorescence Microscopy of the Saccule and the Cochlea

A. Saccule at four weeks after 10 days of systemic gentamicin injection. Hair cell loss is not apparent and the sensory epithelium is evenly covered by hair cells stained with phalloidin. Bar = 50 μm.

B. Organ of Corti at three weeks after 10 days of systemic gentamicin injection. Lost outer hair cells are replaced by the surrounding supporting cells and their actin rings meet together to form "scars" (arrow) at the sites of the lost hair cells. Bar = 10 μm.

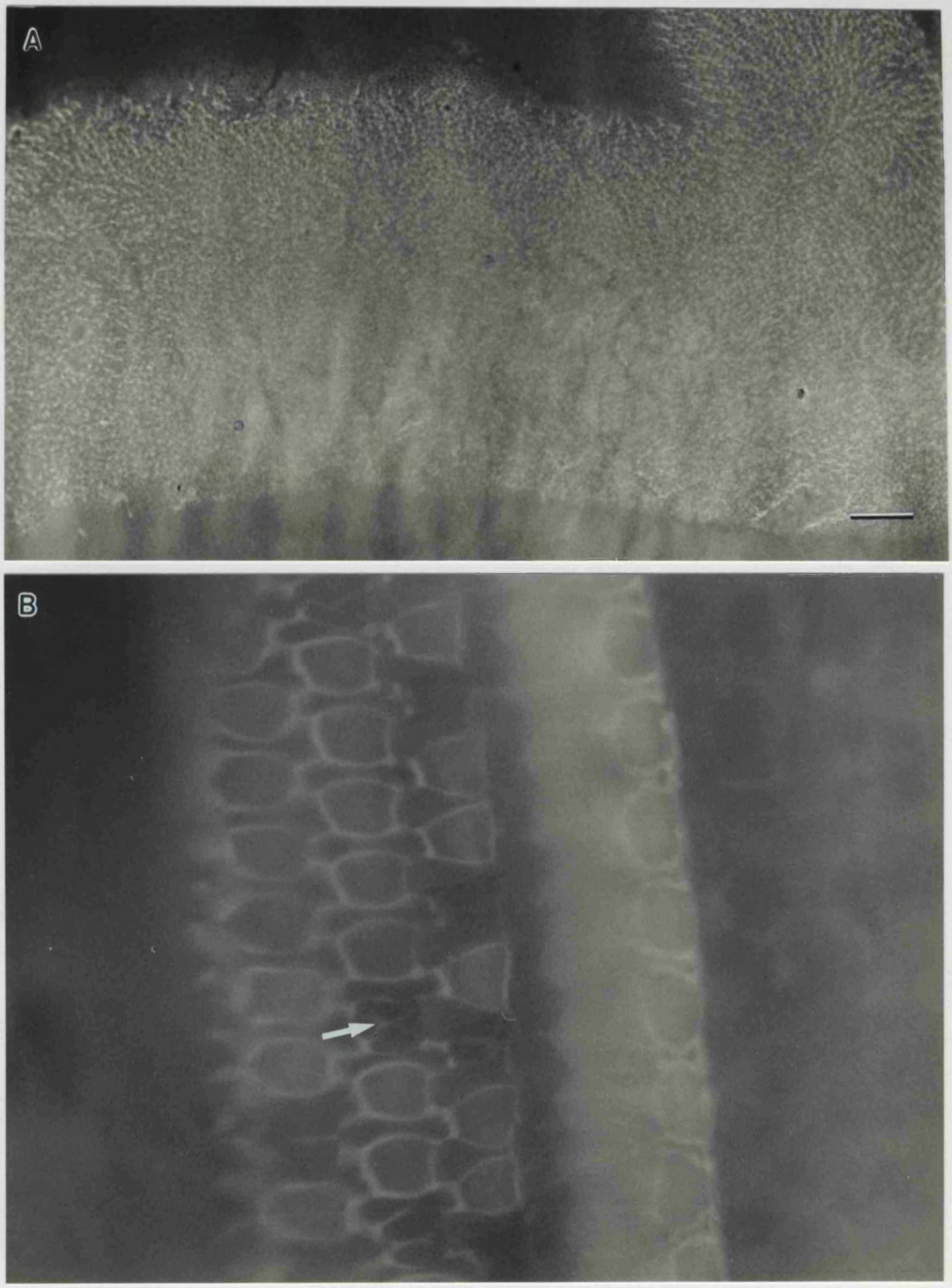
A
B

Figure 5.4 Scanning Electron Microscopy of the Cristae

A. Normal crista. Its sensory epithelium is fully covered by hair cells with long hair bundles.

B. Gentamicin affected crista one week post-treatment. Hair bundle loss is apparent at the central epithelium.

C. Gentamicin affected crista four weeks post-treatment. Extensive hair cell loss extends towards the periphery.

Bar (A-C) = 50 μm.

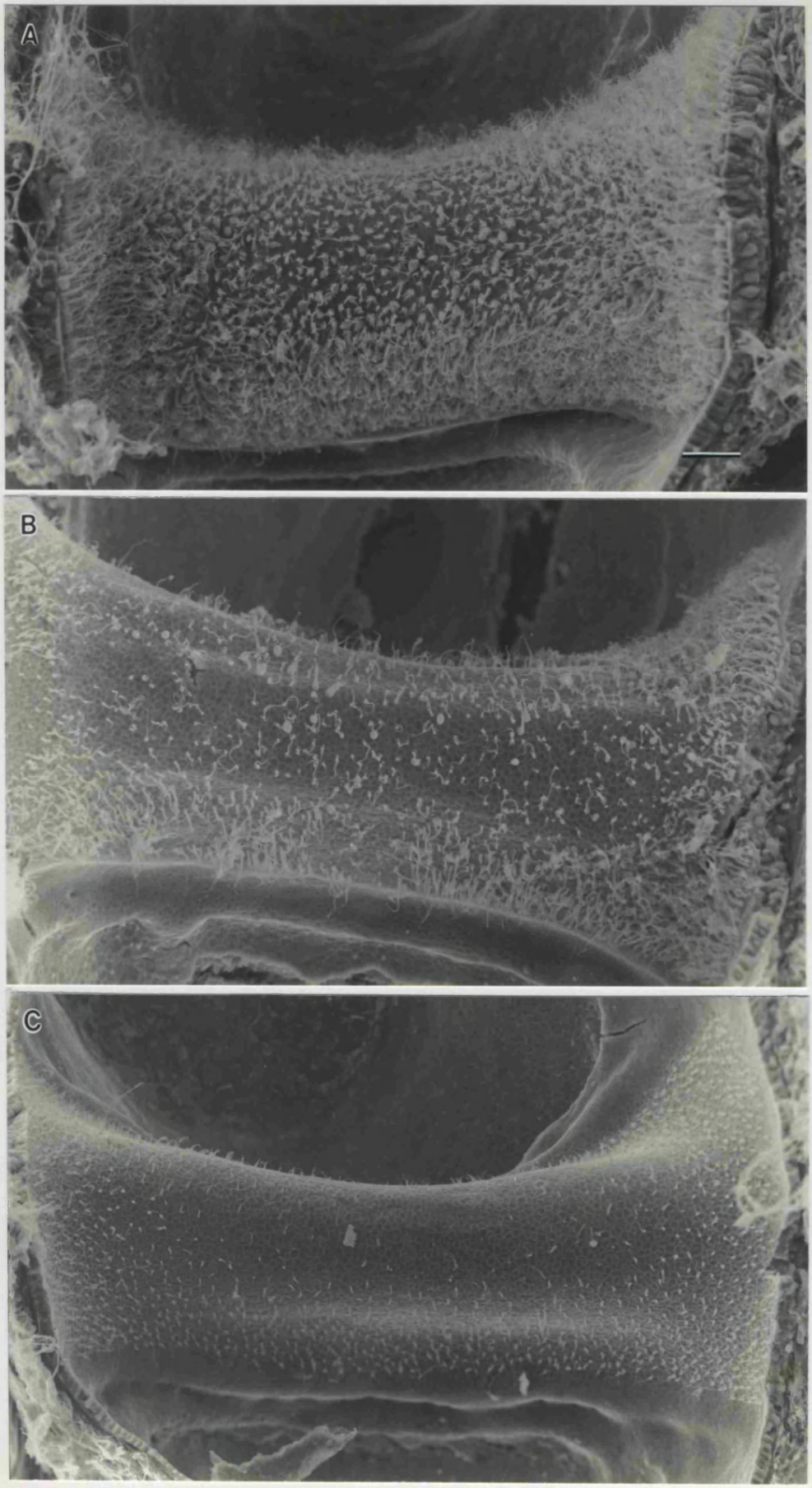
A
B
C

Figure 5.5 Scanning Electron Microscopy of Crista Sensory Epithelia

A. Central area of Figure 5.4 A. Long hair bundles are oriented in the same direction.

B. Central area of Figure 5.4 B. Stereocilia bundles become fused and swollen giants (arrowhead). The epithelial surface appears continuously uninterrupted.

C. Central area of 5.4 C. The expanded supporting cells occupy the sites of the lost hair cells and the enlarged polygonal surfaces of supporting cells form a new meshwork pattern in the epithelium. No mature hair bundles are present in the surfaces.

Bar (A-C) = 4 μm.

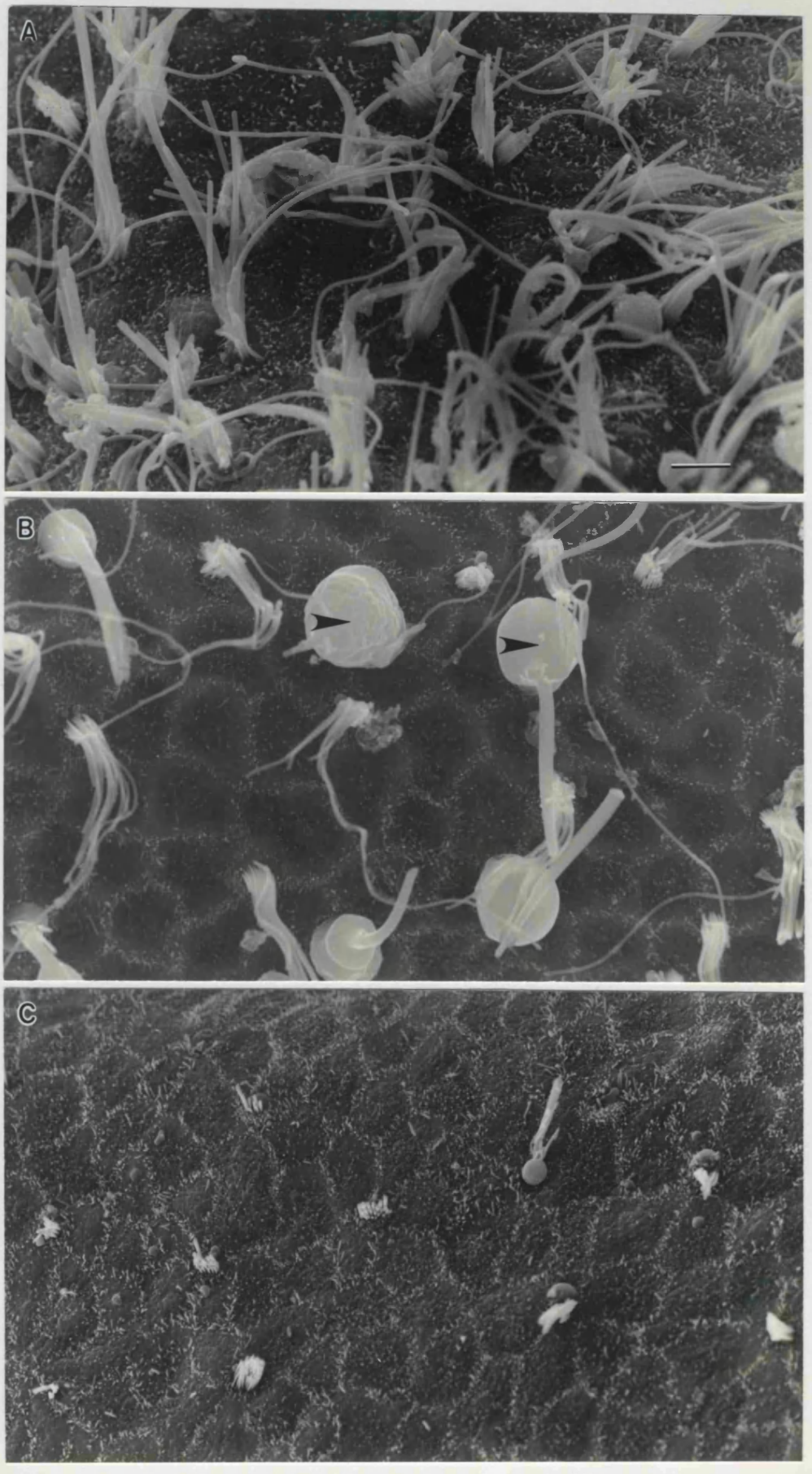
A
B
C

Figure 5.6 Hair Cell Degeneration and Immature Hair Bundles

A. Crista epithelial surface. Hair cells with swollen apical ends appear in the central area of the crista at one week after systemic gentamicin treatment.

B. A shallow depression (arrowhead) of an apex of the lost hair cell is present and it is sealed by supporting cells from the bottom.

C. Scars (arrowhead) appear at three days after systemic gentamicin treatment which are formed by the apical parts of supporting cell expansions.

D. Three weeks post-treatment, the lost hair cell outlines are completely obliterated by the expansion of supporting cells.

E. Crista from an albino guinea pig at three weeks after 10 days systemic gentamicin treatment. Immature hair bundles (arrowhead) are present in the epithelium where hair cell loss is apparent. Bar = 10 μm.

F. High magnification of E. The twin bundles of stereocilia like projections and nearby other two small stereocilia like bundles are the first morphological evidence of mammalian hair cell regeneration.

G. Crista from animals four weeks post-treatment. Immature hair bundles appear in the area of hair cell loss.

Bars (A-D, F, G) = 5 μm.

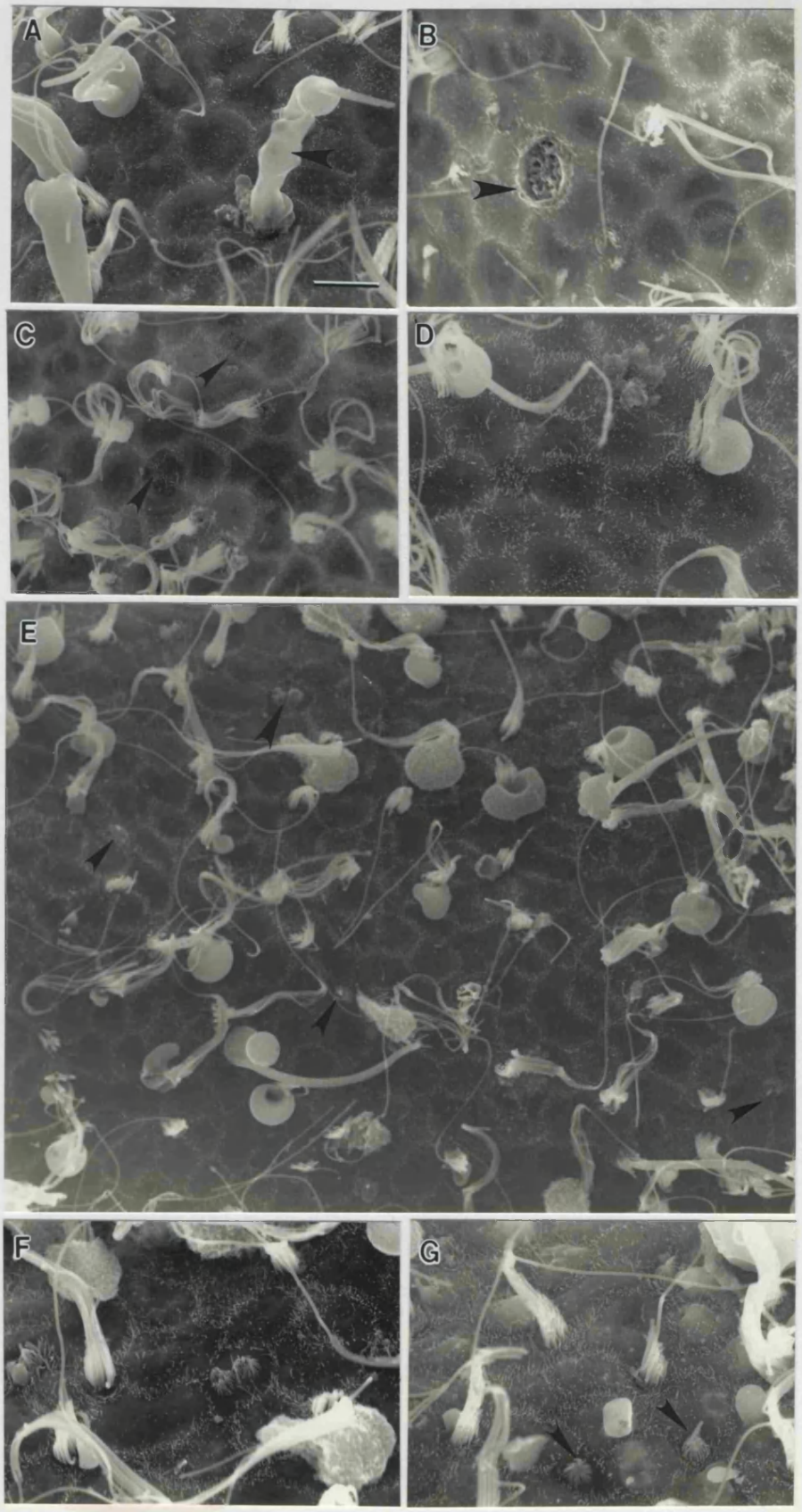
A
B
C
D
E
F
G

Figure 5.7 Scanning Electron Microscopy of Control Utricles and Saccules

A. Control utricle. The epithelium is covered by hair cells. Bar = 120 μm.

B. Control saccule. The epithelial surface is evenly covered by hair cells. Bar = 120 μm.

C. Striolar region of the utricular macula. The orientation (arrowhead) of the tallest stereocilia and the kinocilium of the hair bundles face each other. Bar = 5 μm.

D. Striolar region of the saccular macula. The orientation (arrowhead) of the longest stereocilia and the kinocilium of hair bundles face away from the striola towards the periphery. Bar = 5 μm.

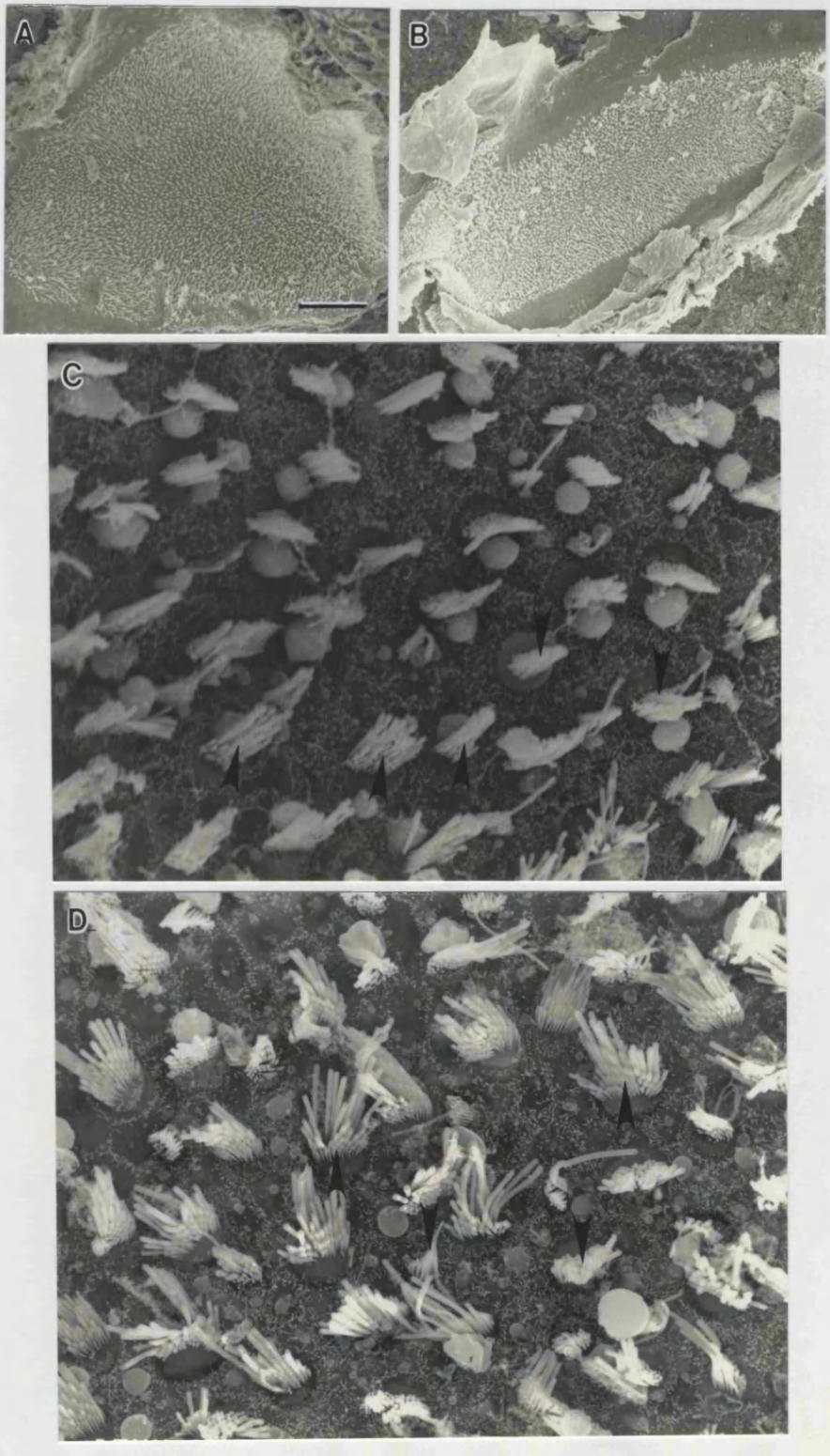
A
B
C
D

Figure 5.8 Early Changes of Gentamicin Affected Vestibular Organs

A. Utricle at four days after 10 days of systemic gentamicin treatment. Hair cell loss appears in the striolar region and hair cells in other parts of the sensory epithelium look normal. Bar = 70 μm.

B. Area of hair cell loss from A. Supporting cells expand to replace the lost hair cells and the epithelial surface maintains uninterrupted. Bar = 5 μm.

C. Saccular macula at one week after 10 days of systemic gentamicin treatment. Hair cells are present with normal distribution. Bar = 100 μm.

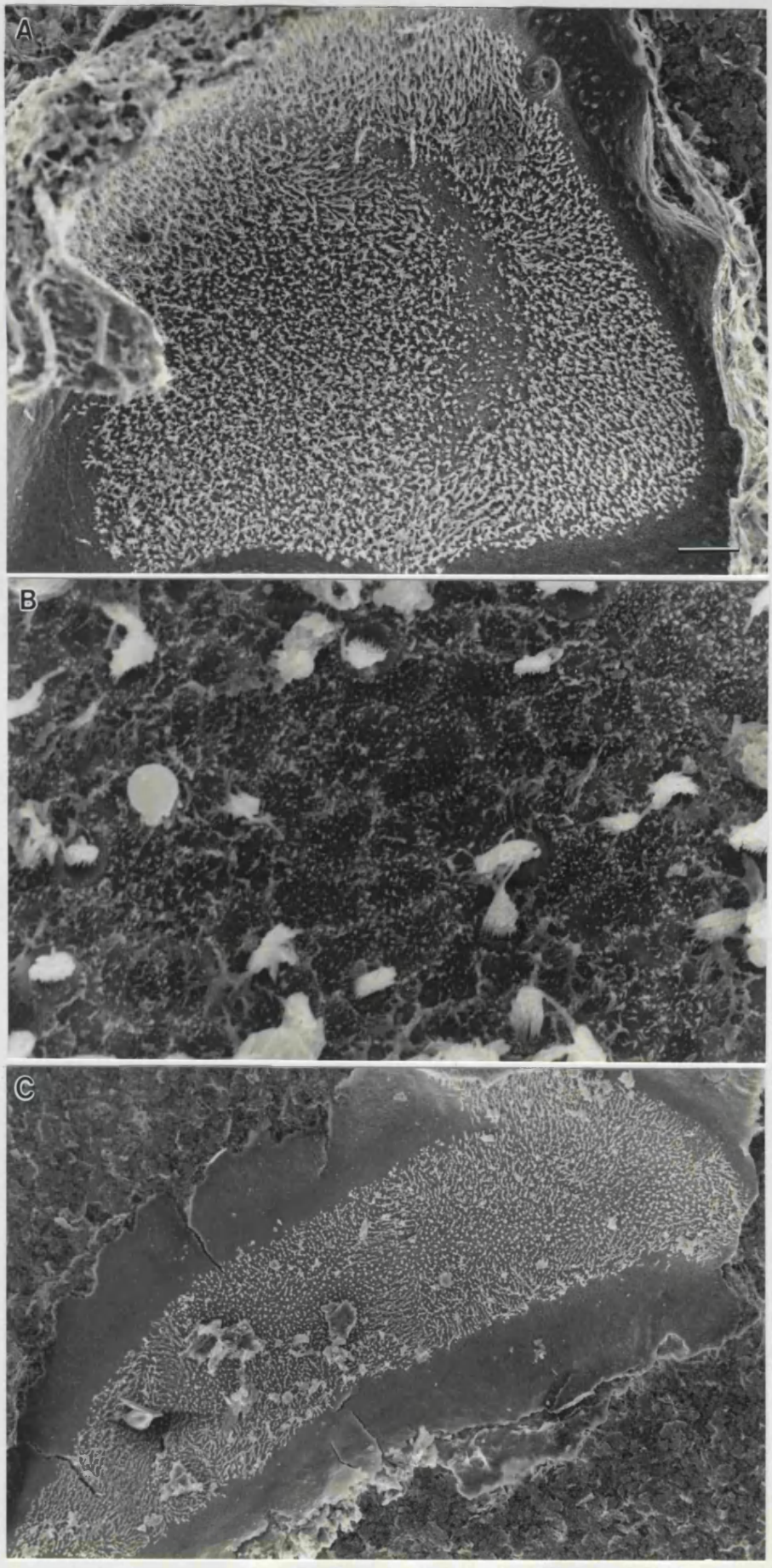
A
B
C

Figure 5.9 Utricles Four Weeks Post-treatment

A. Gentamicin affected utricle. The area of hair cell loss in the striolar region become wider, spreading towards the periphery. Bar = 70 μm.

B. Striolar region of the utricle in A. Some hair cells at their apical surfaces with immature hair bundles (arrowhead) appear in the area of hair cell loss. The immature hair bundles are easily distinguished from the microvilli on the apical surface of supporting cells. Bar = 4 μm.

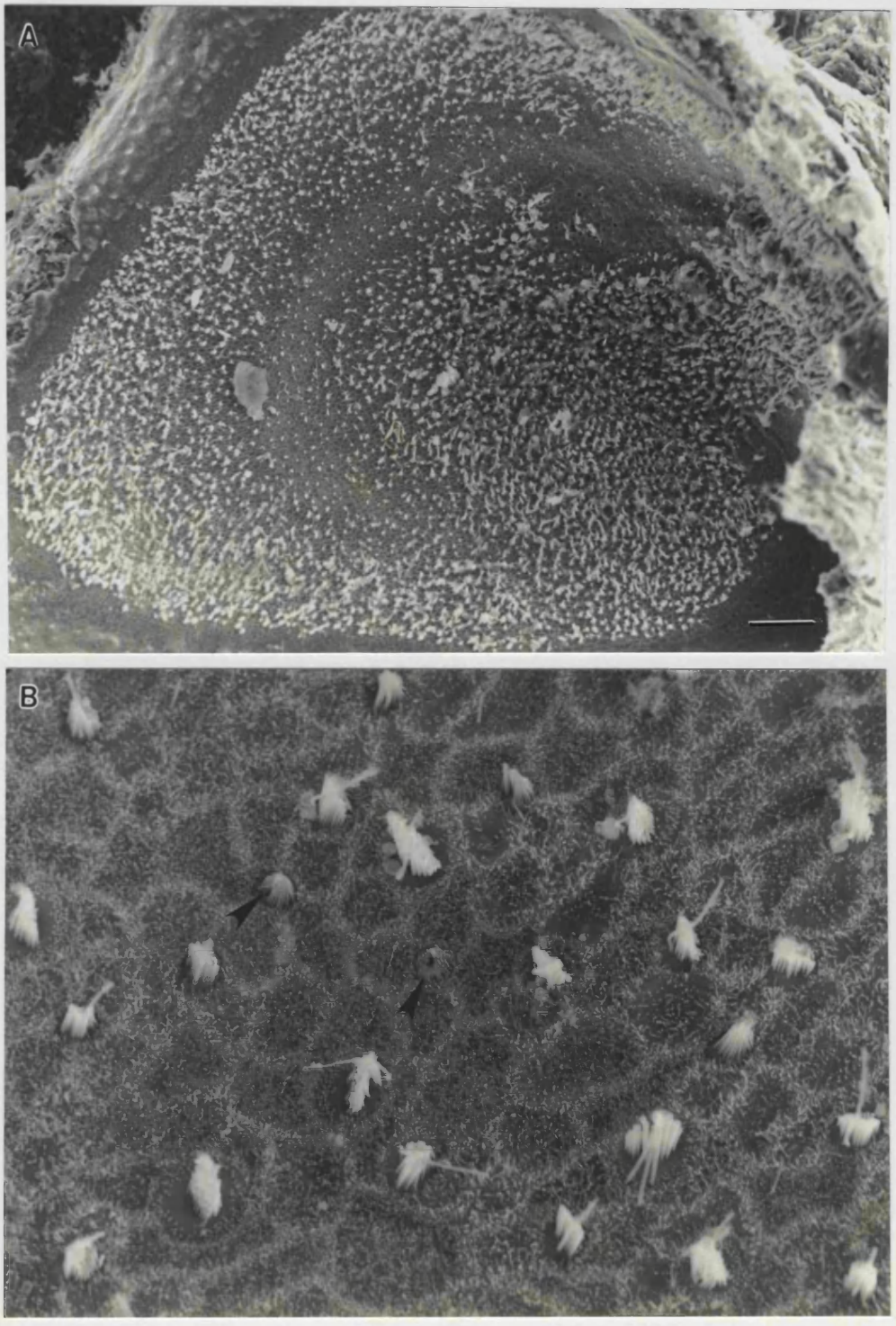
A
B

Figure 5.10 Cristae and Saccules Four Weeks Post-treatment

A. Gentamicin affected crista. More hair cells are lost in the central area and some long hair bundles are present in the periphery. Bar = 50 μm.

B. Central area in A. Short and immature hair bundles (arrowhead) appear together with long and normal hair bundles on the epithelial surface. Bar = 3 μm.

C. Saccular macula. The hair cells are less affected by gentamicin. Bar = 100 μm.

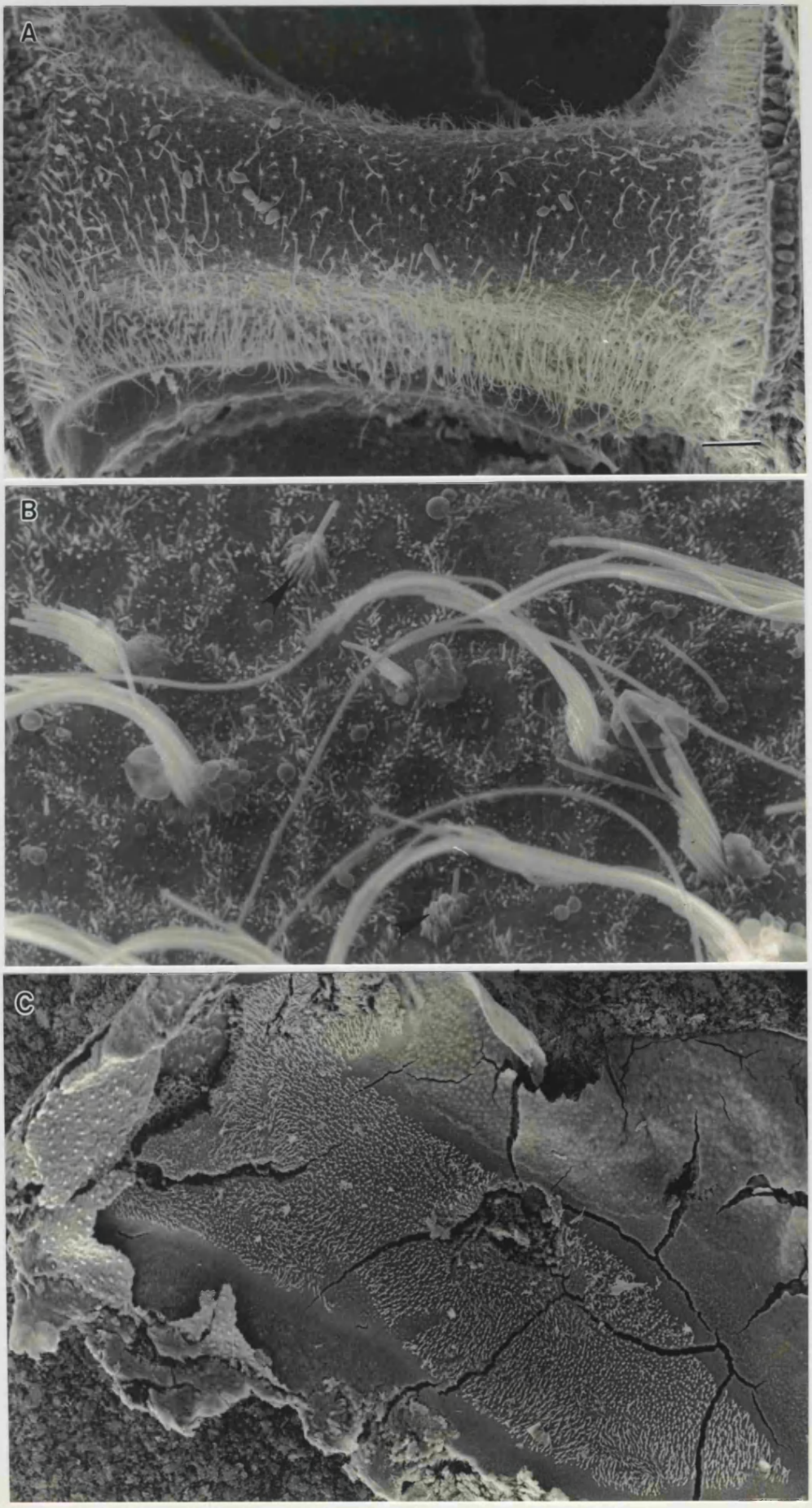
A
B
C

Figure 5.11 Scanning Electron Microscopy of Immature Hair Bundles

A. The immature hair bundle has thin and short stereocilia which covered the whole apical surface of the hair cell. A thicker and taller kinocilium (arrowhead) is located in the centre of the immature stereocilia cluster. Bar = 1 μm.

B. The number of stereocilia varies and this immature hair bundle consists of many stereocilia projecting from the cuticular plate. Bar = 1.5 μm.

C. The short stereocilia are similar in length and angled towards the centre. The kinocilium (arrowhead) is taller and thicker and it is located in the centre of the cell surface surrounded by stereocilia. Bar = 0.75 μm.

D. Stereocilia form a circle and regularly arranged lateral cross-links (arrowhead) are present on the tips of adjacent stereocilia. The kinocilium is inside the stereocilia circle but it is located towards the edge of the stereocilia bundle. Bar = 0.5 μm.

E. The kinocilium is located outside the stereocilia bundle. The space inside the hair bundle disappears and regularly arranged lateral cross-links (arrowhead) are present on the tips of adjacent stereocilia. Bar = 0.75 μm.

F. The kinocilium (arrowhead) is located outside the elongated stereocilia starting to form the staircase shape. Bar = 0.5 μm.

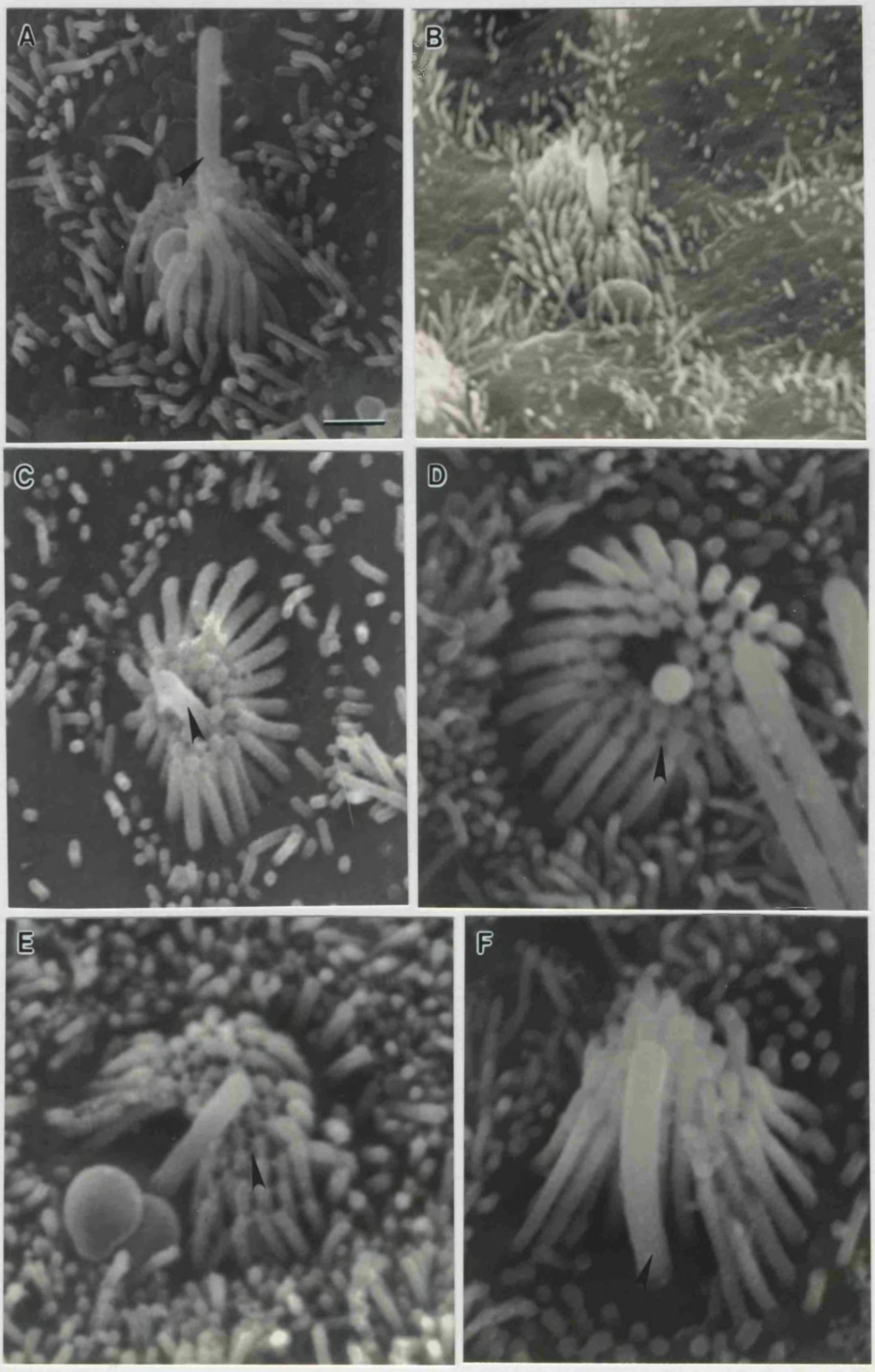
A
B
C
D
E
F

Figure 5.12 Appearance of Immature Hair Bundles

A. Striolar region of a control saccule. One small hair bundle (arrowhead) with same length of stereocilia is found among mature hair bundles which consist of one long kinocilium and many stereocilia arranged in staircase. Bar = 3 μm.

B. Striolar region of an utricle at two weeks after systemic gentamicin treatment. Most hair bundles with immature morphology equivalent to those illustrated in Figure 5.11 are present and hair bundles are in low density. Supporting cells have expanded and sealed the sites of lost hair cells. Bar = 4 μm.

C. Striolar region of an utricle four weeks post-treatment. Immature hair bundles (arrowhead) appear in different developing stages with hair bundles of survived hair cells. Bar = 4 μm.

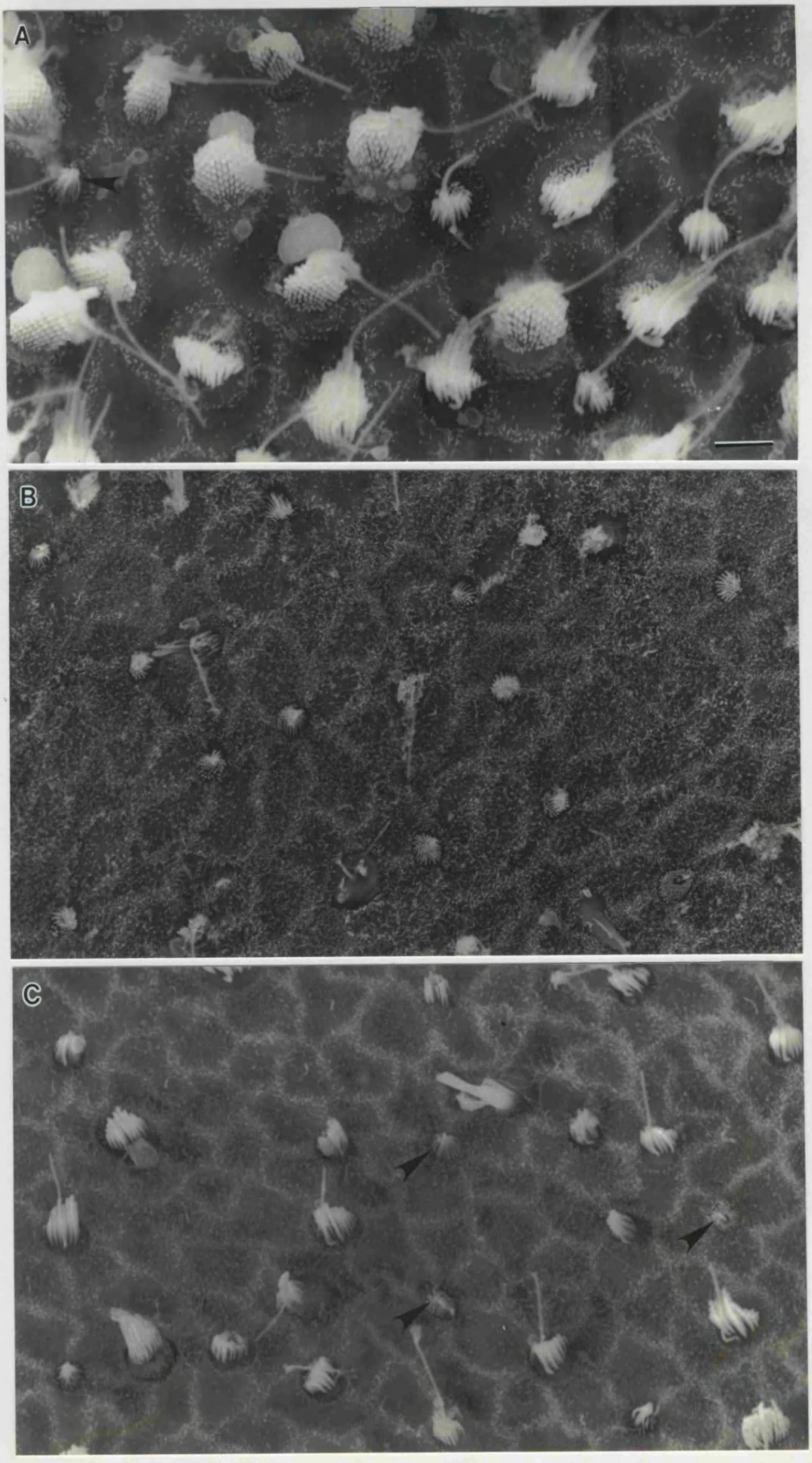
A
B
C

Figure 5.13 Eight to Twelve Weeks after Systemic Gentamicin Treatment

A. Utricle at 8 weeks post-treatment shows the area with low density of hair cells along the striolar region.

B. Utricle at 12 weeks post-treatment shows that the band of decreased hair cells is still recognisable in the striolar region.

C. Saccule from an animal which survived 8 weeks post-treatment shows hair cell loss along the striolar region.

Bar (A-C) = 100 μm.

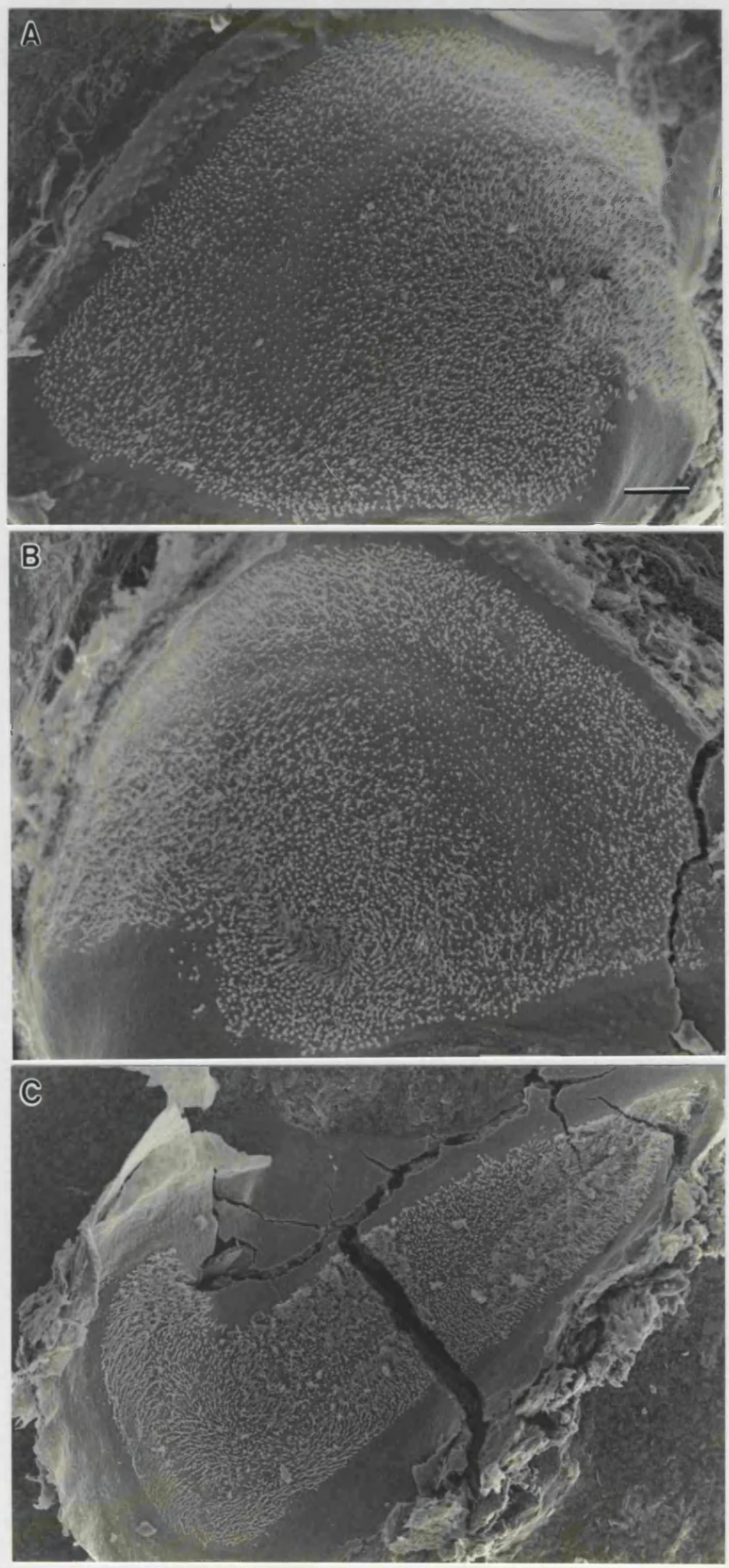
A
B
C

Figure 5.14 Eight to Twelve Weeks after Systemic Gentamicin Treatment

A. Utricular striolar region 12 weeks post-treatment. Most hair bundles show the characteristic of mature hair bundles with stereocilia arranged in staircase of ascending height, but they are noticeably shorter than controls.

B. Utricular striolar region from an age matched control animal. Hair cells have long stereocilia bundles and the density of hair cells is high.

C. Utricular striolar region from the same ear as Figure 5.13 C. The density of hair cells is low and the existing hair bundles are small and short.

Bar (A-C) = 5 μm.

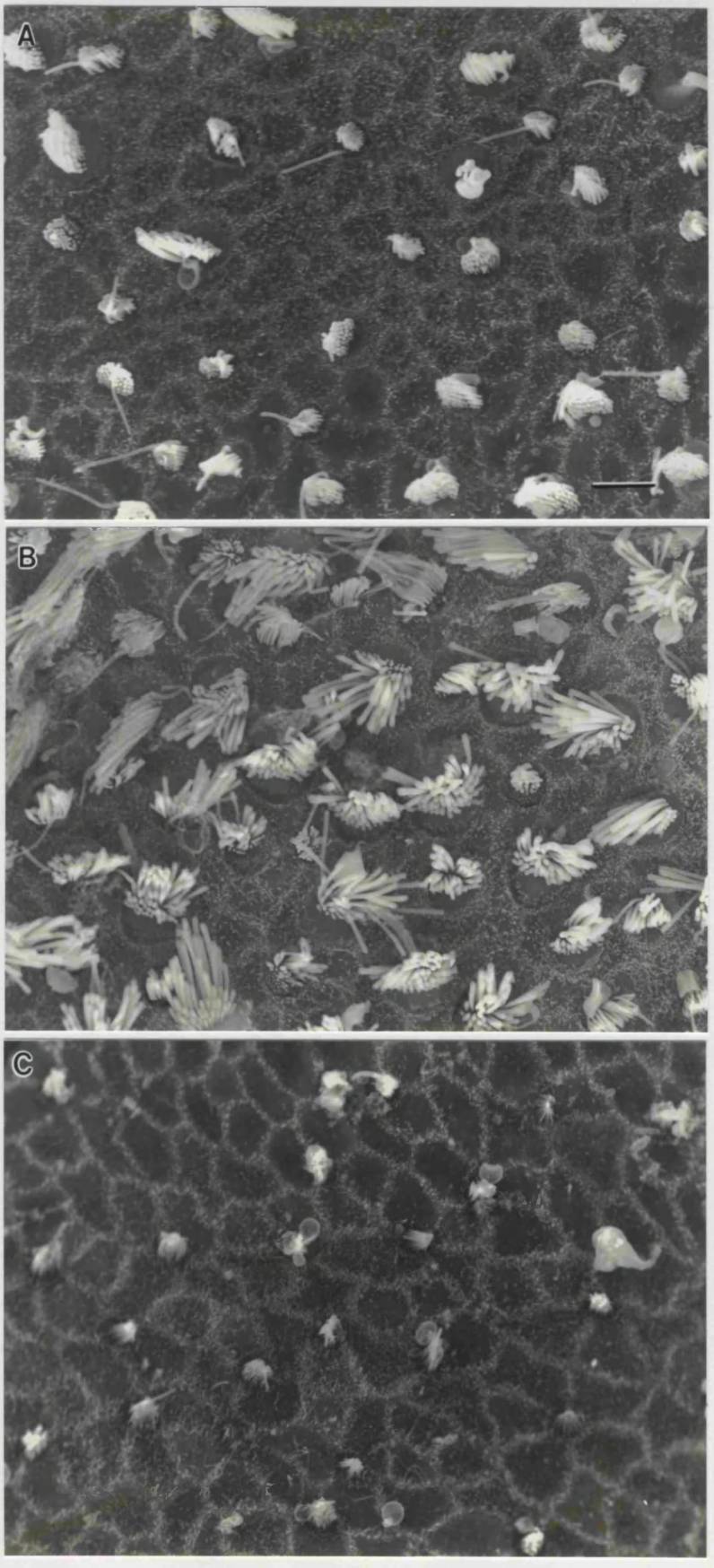
A
B
C

Figure 5.15 Eight Months after Systemic Gentamicin Treatment

A. Gentamicin affected utricle. Low density of hair cell bundles is apparent in the striolar region. Bar = 75 μm.

B. Striolar region of A. The hair bundles are evenly distributed in the striolar region, but they are shorter than peripheral hair bundles. Bar = 35 μm.

C. Organised hair bundles in the striolar region are short and the density is noticeably low. No typical immature hair bundles are found among surviving hair bundles. Bar = 4 μm.

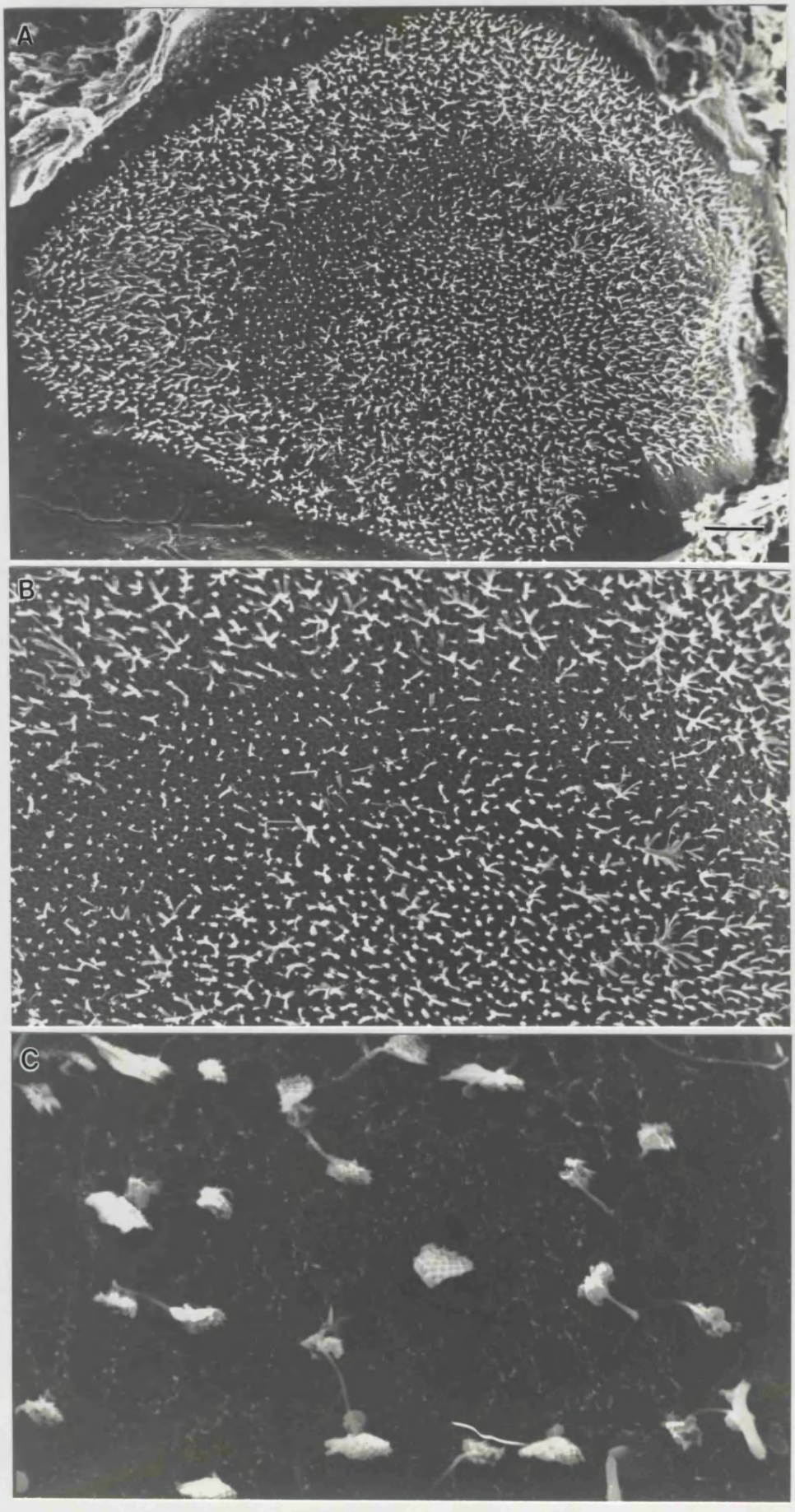
A
B
C

Figure 5.16 Eight Months after Systemic Gentamicin Treatment

A. Striolar region of a gentamicin affected utricle shows many balloon shaped extrusions and peripheral hair cells appear normal. Bar = 30 μm.

B. The hair bundles in the saccular striolar region appear in normal morphology and even distribution. Bar = 4 μm.

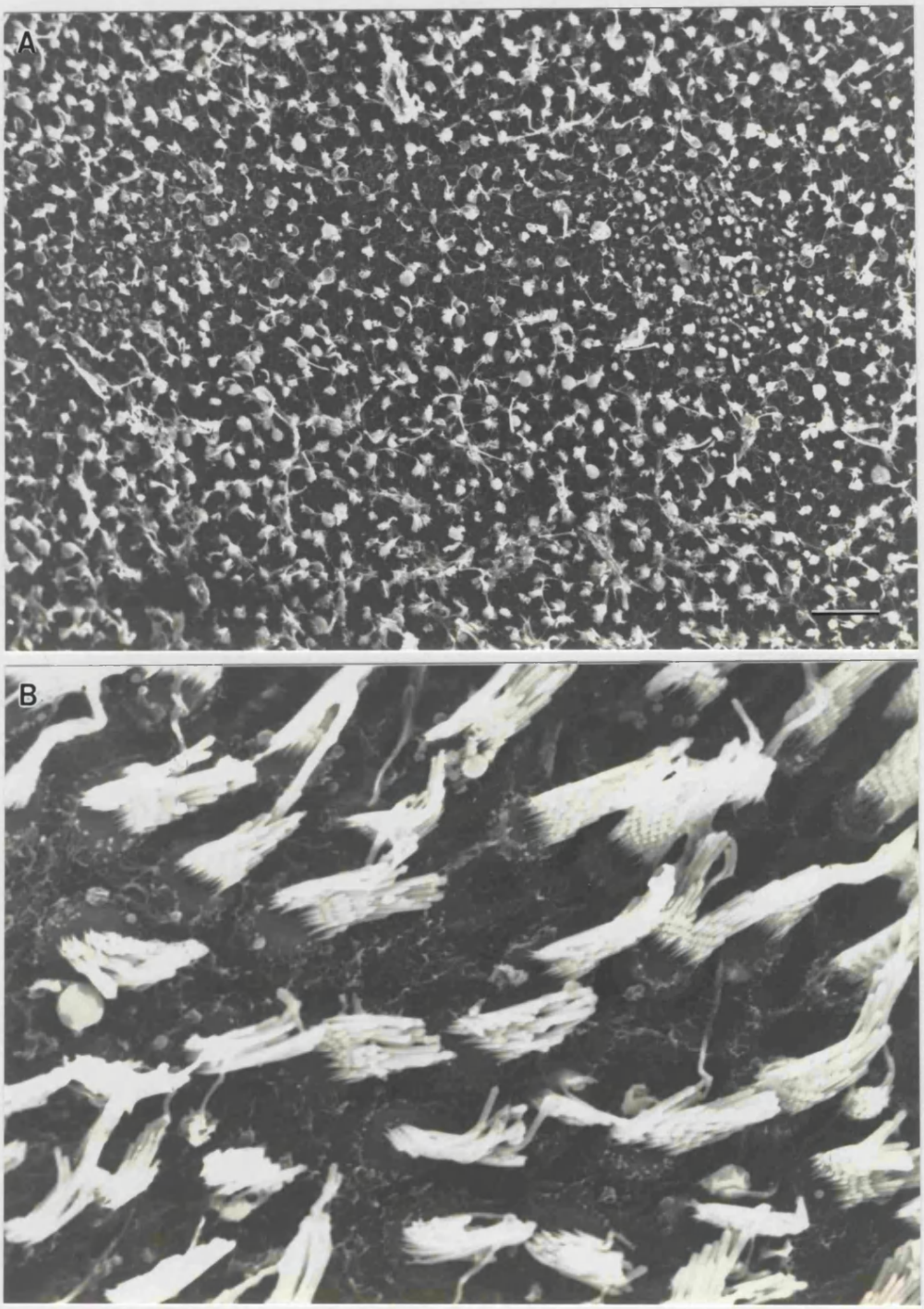
A
B

Figure 5.17 Early Changes after Topical Gentamicin Application

A. Saccule at three days after topical application. Hair cell loss is apparent along the striolar region and hair cells near the edge of the epithelium are less affected.

B. Utricle one week post-treatment. Hair cell loss is apparent in the striolar area similar to the result from the systemic treatment.

C. Saccular macula one week post-treatment. Severe hair cell loss appears along the striolar region.

Bar (A-C) = 100 μm.

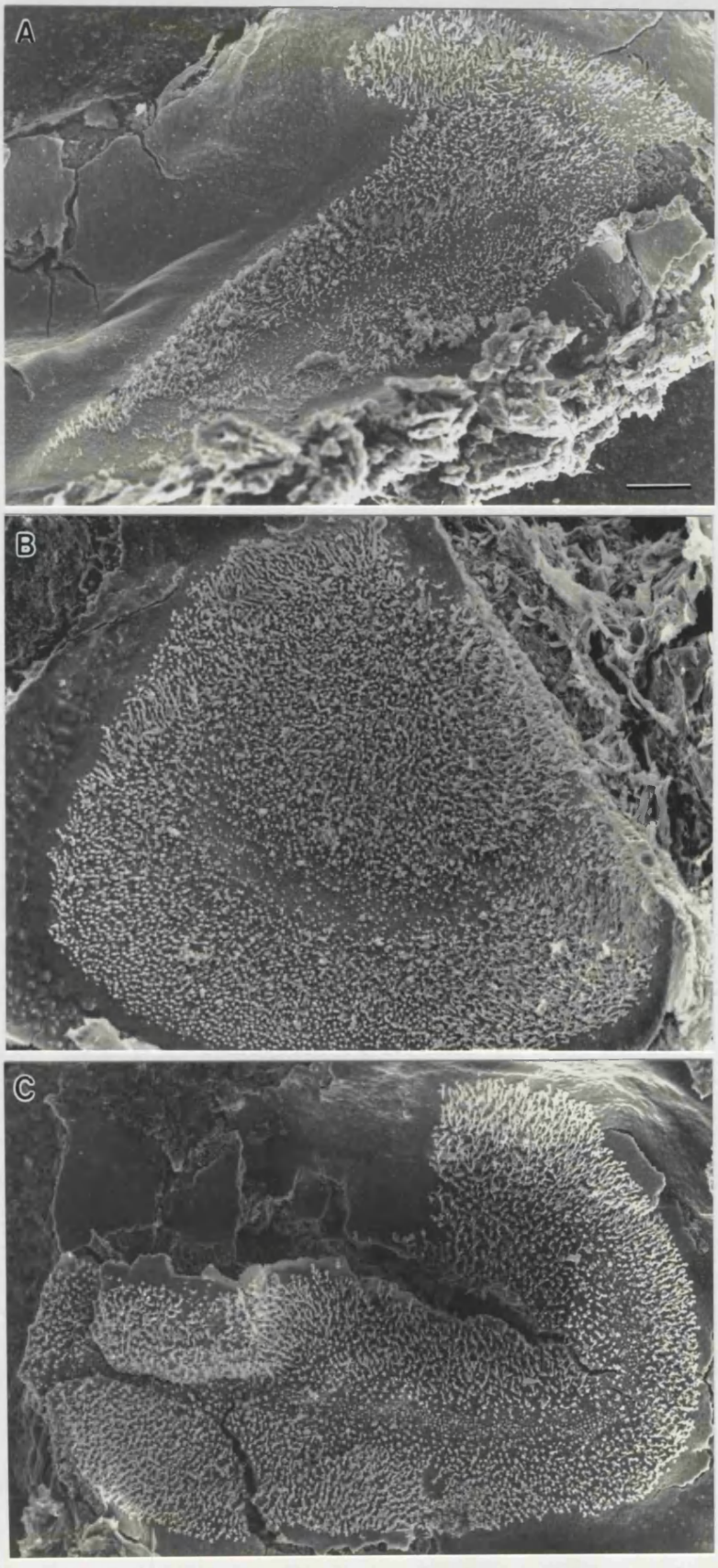
A
B
C

Figure 5.18 Early Changes after Topical Gentamicin Application

A. At high magnification of Figure 5.17 A, the striolar region in the damaged saccule. Lost hair cells are replaced by supporting cells, some normal looking hair bundles are present at the edge of hair cell loss.

B. At high magnification of Figure 5.17 B, the striolar region in the damaged utricle. Hair cells loss is replaced by supporting cells, some normal looking and a small immature like hair bundle (arrowhead) are present at the area of hair cell loss.

C. At high magnification of Figure 5.17 C, the striolar region in the saccular epithelium. Most of the balloon shaped apical ends are present and the microvilli appear fewer on the apical surfaces of the supporting cells.

Bar (A-C) = 6 μm.

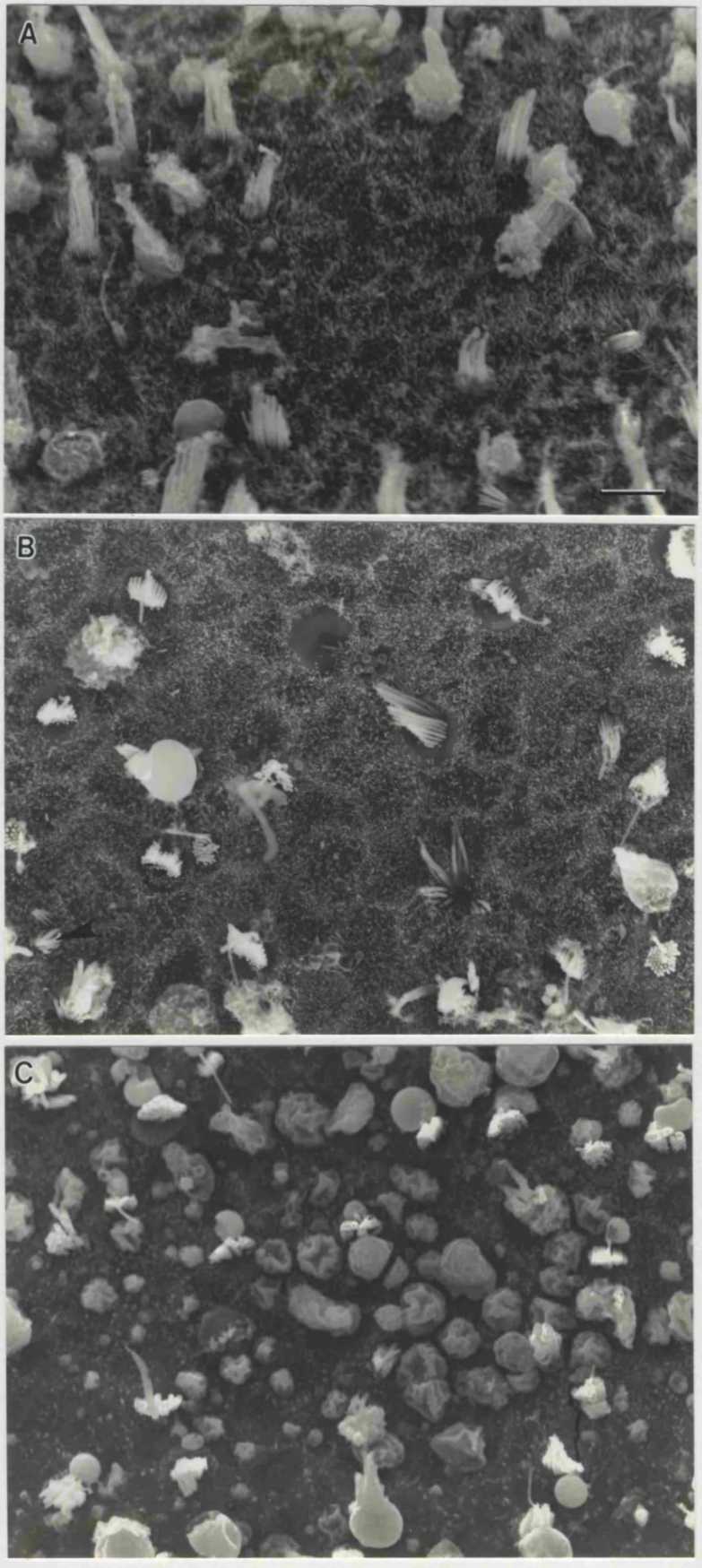
A
B
C

Figure 5.19 One Month after Topical Gentamicin Application

A. Gentamicin affected saccular macula shows that the band of hair cell loss becomes wider in the striolar region. Bar = 100 μm.

B. Gentamicin affected utricular macula shows that the band with low density of hair cells is apparent in the striolar region. Bar = 100 μm.

C. High power view of A. Immature hair bundles (arrowhead) with characteristics of development appear in the area of hair cell loss in the saccular macula. Bar = 6 μm.

D. High power view of B. Immature hair bundles (arrowhead) are short and the density of hair cells is low in the striolar region of the utricular macula. Bar = 6 μm.

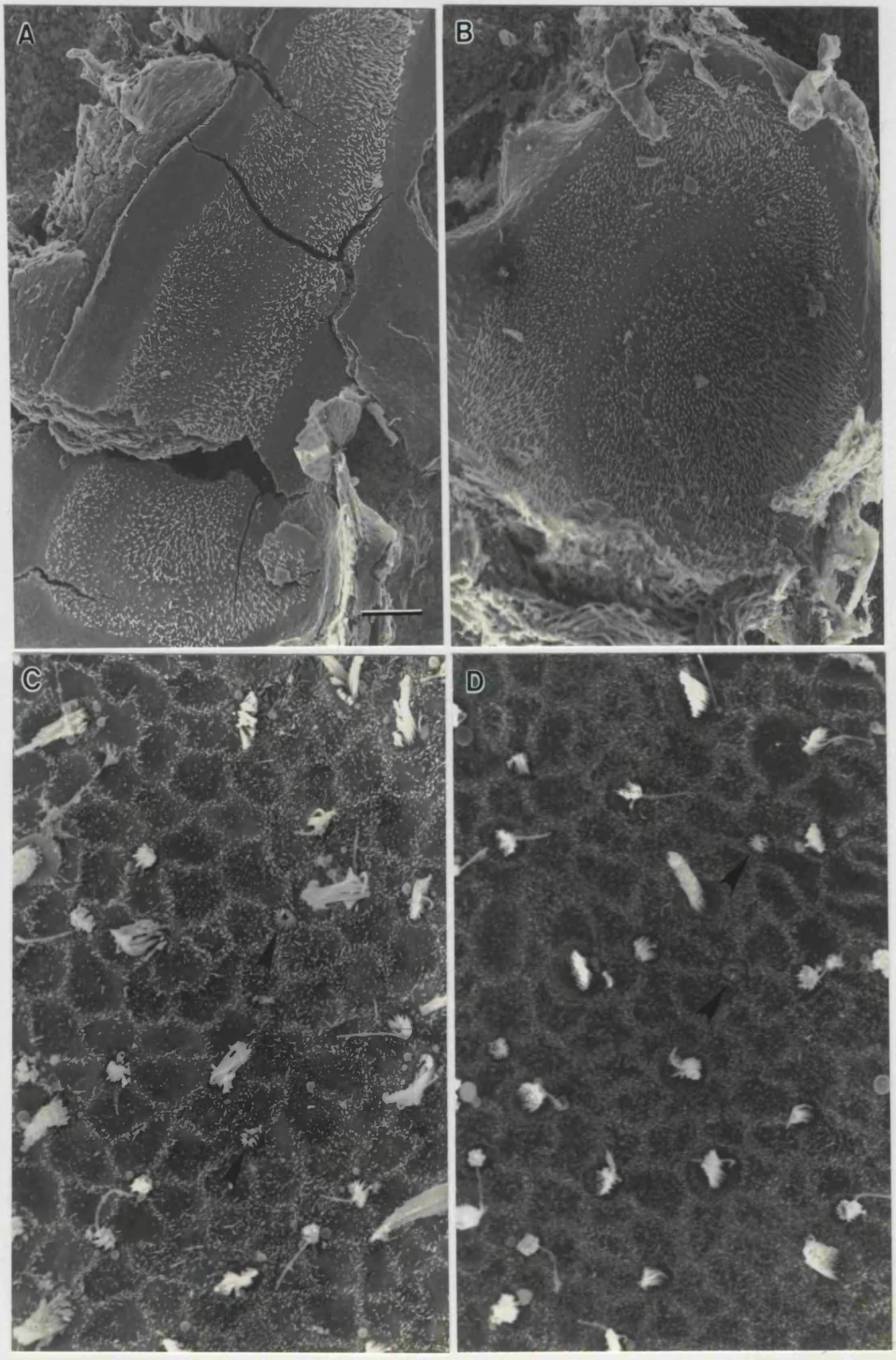
A
B
C
D

Figure 5.20 Twelve Weeks after Topical Gentamicin Application

A. Treated saccular macula shows a narrow band of hair cell loss along the striolar region. Bar = 100 μm.

B. High power view of A. Mature looking hair bundles are apparent in the area of hair cell loss, but immature hair bundles (arrowhead) are still present and the density of hair cells is low in the striolar region. Bar = 6 μm.

C. Utricle from a normal saline treated ear. Hair cells are not affected and their distributions are normal. Bar = 120 μm.

D. Cochlear basal turn at 12 weeks after saline treatment. Inner and outer hair cells appear normal. Bar = 12 μm.

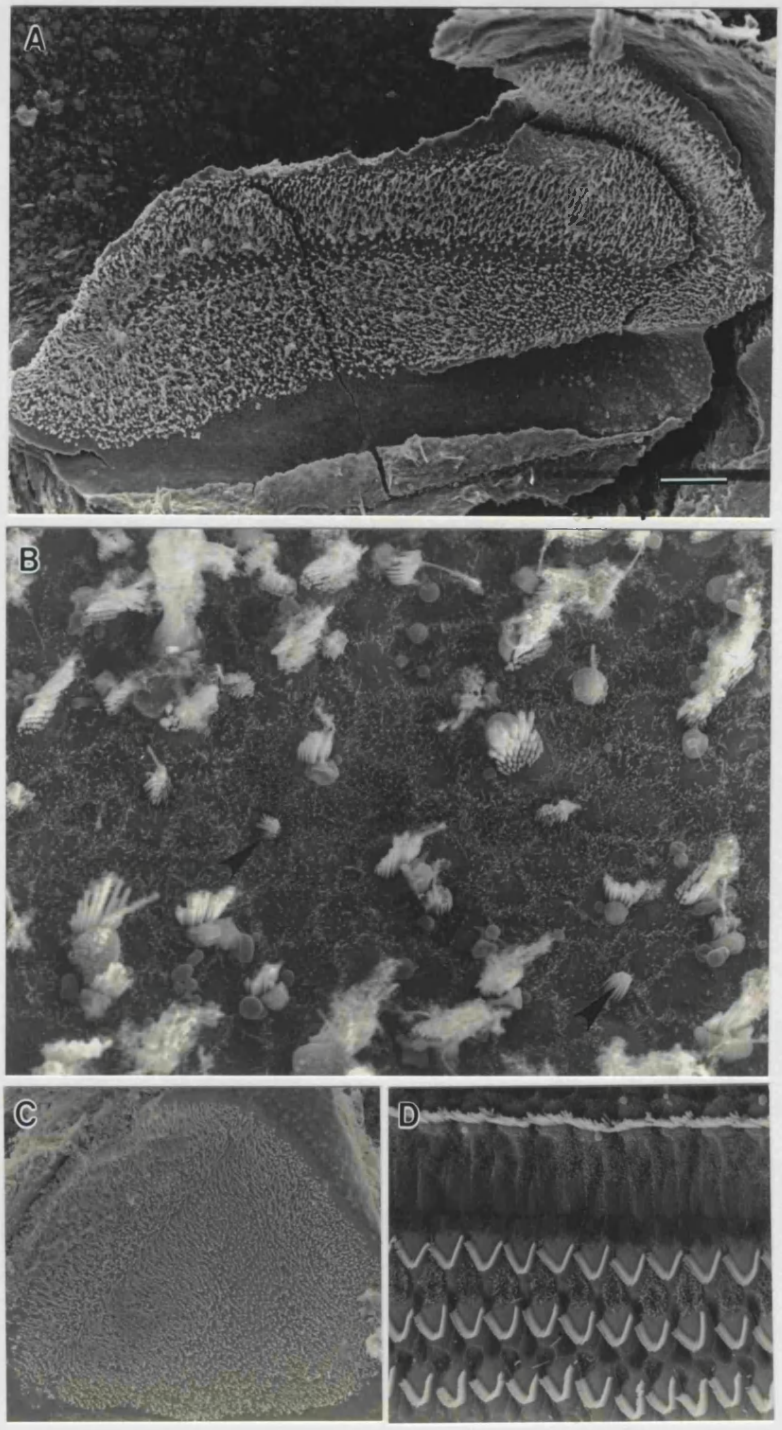
A
B
C
D

Figure 5.26 Cochlear Damage after Systemic Gentamicin Treatment

A. One week post-treatment, outer hair cells are extensively damaged and inner hair cells appear intact in the basal turn. Degenerated hair cell debris is scattered on the cuticular lamina. Bar = 7 μm.

B. At 12 weeks post-treatment, outer hair cells are lost and some of the inner hair cells still remain. Supporting cells expand to replace the lost hair cells and scars are present (arrow) on the reticular lamina. At any time point examined, no evidence of immature hair bundles is found in the cochlear sensory epithelium by SEM. Bar = 7 μm.

C. Cochlear basal turn from the same animal as Figures 5.13 C; 5.14 C 8 weeks post-treatment. Both inner hair cells and outer hair cells are completely lost and the mosaic pattern of the organ of Corti is formed by epithelial cells (arrow). Bar = 60 μm.

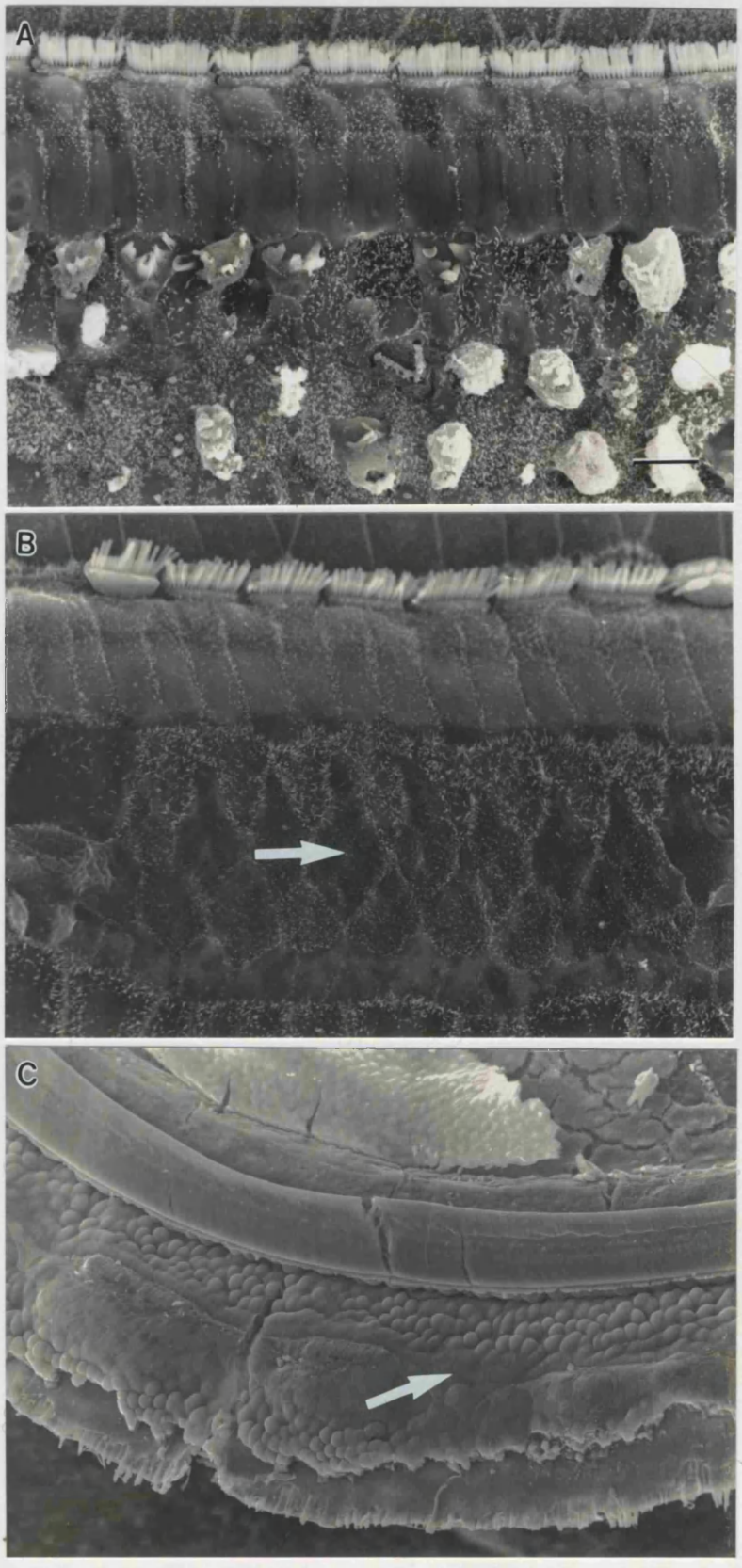
A
B
C

Figure 5.27 Cochlear Damage after Topical Gentamicin Application

A. One week post-treatment, cochlear hair cells are severely damaged and degenerated hair cell debris is present on the surface of the distorted reticular lamina. Bar = 7 μm.

B. Four weeks post-treatment, the second turn of the cochlea shows intensive hair cell loss. Both outer and inner hair cells disappear and the epithelial surface of the organ of Corti becomes narrow. Bar = 7 μm.

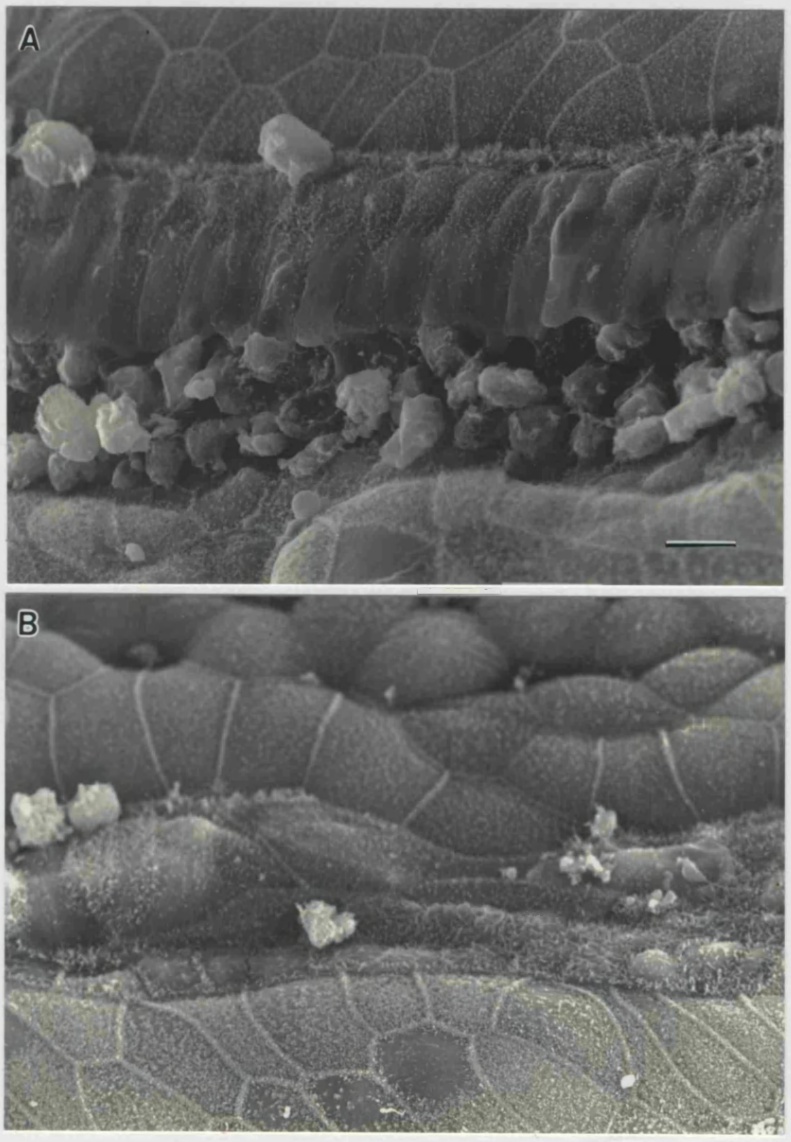
A
B

Figure 5.28 Thin Sections of Gentamicin Affected Vestibular Tissues

A. Crista sensory epithelium three weeks post-treatment. Degeneration and loss of vestibular type I (arrowhead) and type II hair cells (arrow) are present in the central part of the crista. Lost hair cells are replaced by supporting cells and there is no lesion through the epithelium associated with hair cell loss. Bar = 5 μm.

B. Crista sensory epithelium at three days after systemic gentamicin treatment. Degenerated hair cells shrink and fragment inside the sensory epithelium. A thick bundle of microfilaments is present at the apical part of each supporting cell at the level of the adherens junctions (arrowhead). Bar = 1 μm.

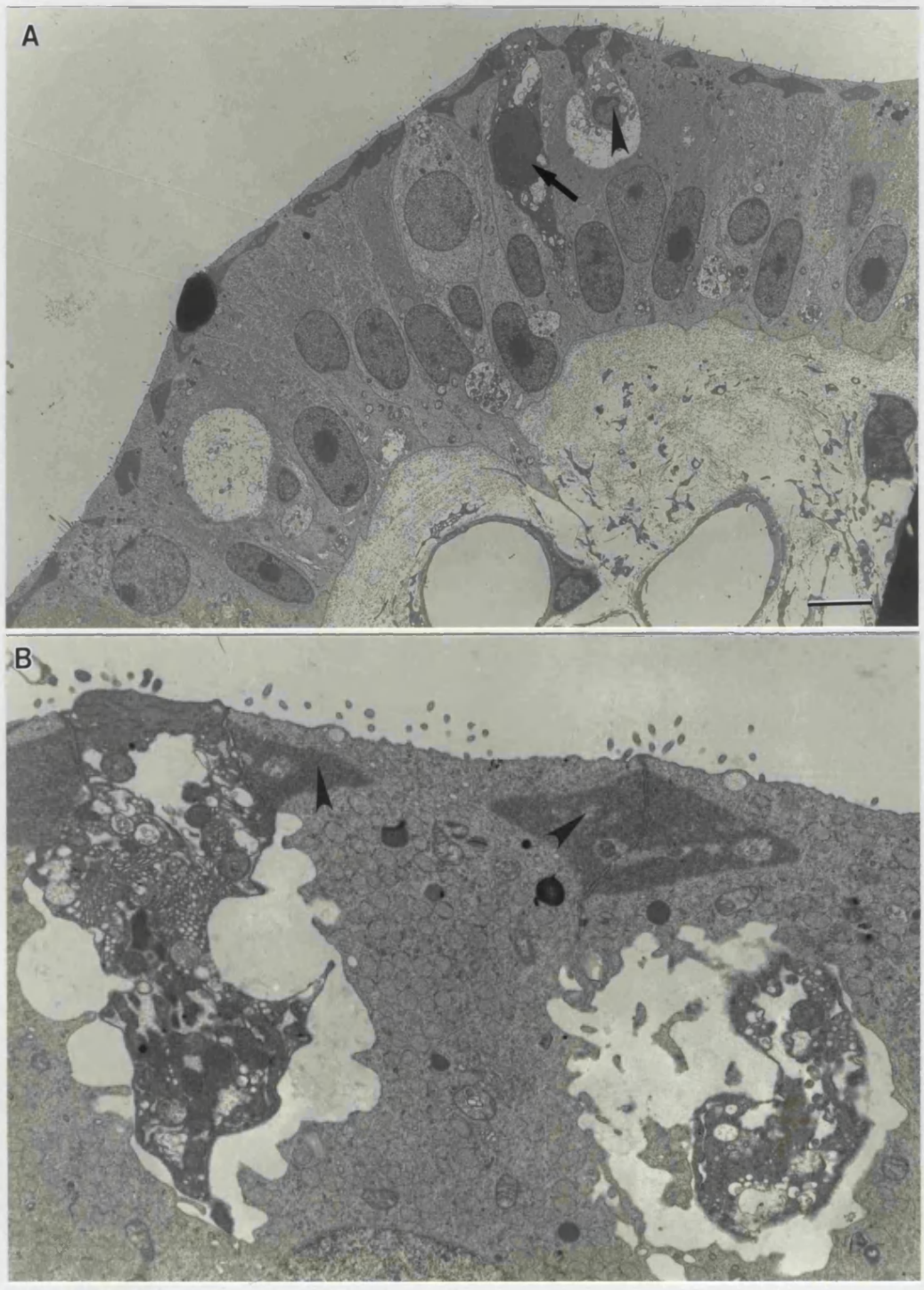
A
B

Figure 5.29 Thin Sections of Degenerated Hair Cells

A. A darkly stained hair cell is found in the utricular sensory epithelium one week post-treatment. The nuclear chromatin becomes condensed and marginated (arrow), but the cuticular plate and hair bundle remain normal. Bar = 2 μm.

B. A degenerated type II hair cell in gentamicin affected utricular macula. The cuticular plate is attached to the adjacent supporting cells by tight junctions and the intracellular structures appear intact.

C. The apical surface of the degenerated hair cell is being pushed out.

D. A condensed hair cell shows cytoplasmic shrinkage and nuclear fragmentation (arrow) in the crista sensory epithelium four weeks post-treatment. The apical parts of the supporting cells expand and come to enclose the degenerated hair cell.

Bar (B-D) = 1 μm.

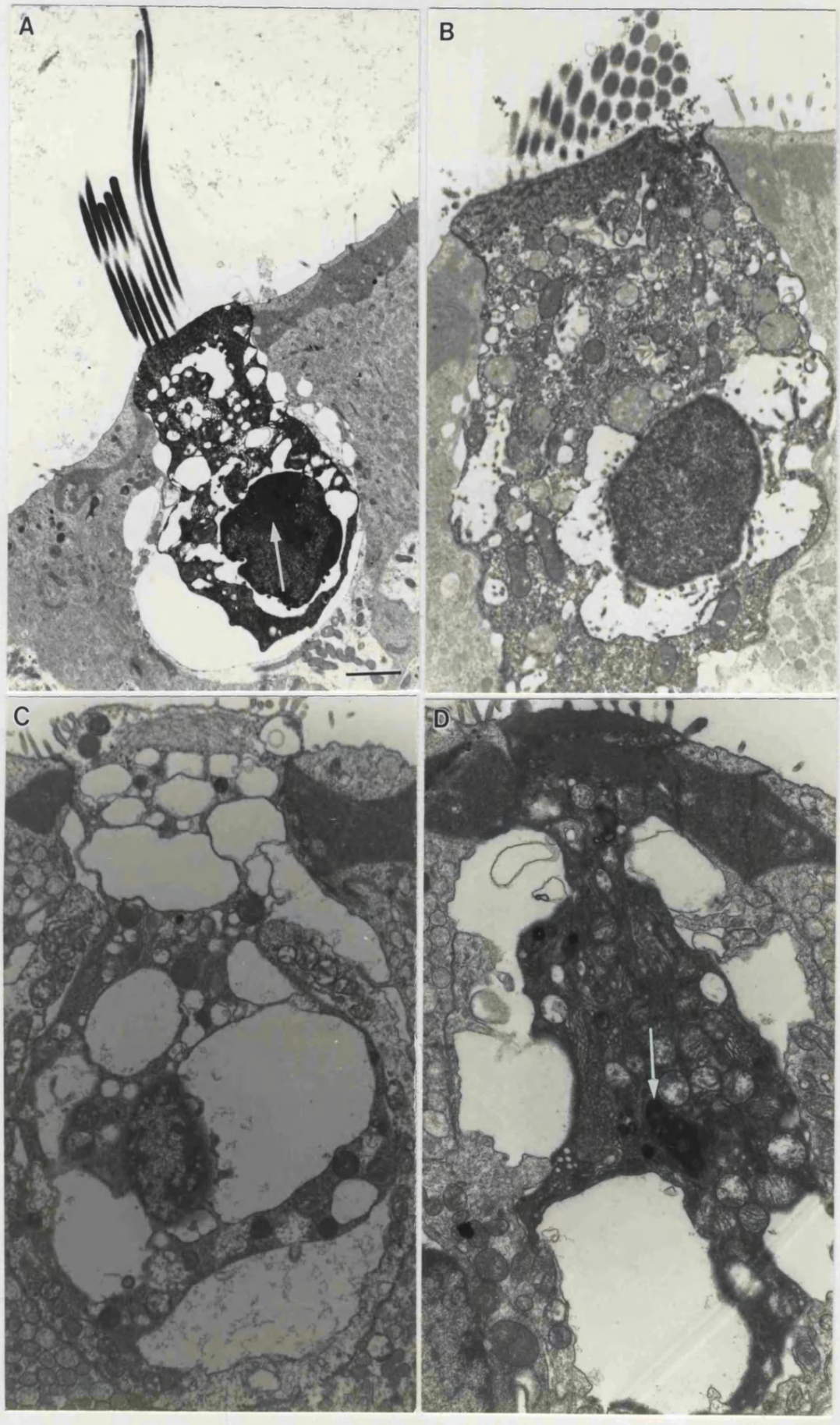
A
B
C
D

Figure 5.30 Apoptotic Hair Cells in Gentamicin Affected Utricles

A. A degenerated hair cell shows the features of apoptosis with nuclear chromatin condensed and marginated, packed into smooth masses against the nuclear membrane. The apoptotic hair cell is at the lower level of the epithelium. Bar = 1.5 μm.

B. A degenerated type II hair cell is darkly stained showing cytoplasmic shrinkage and condensation. The degenerated cell is enclosed inside the sensory epithelium and other hair cells and supporting cells appear normal. Bar = 1.5 μm.

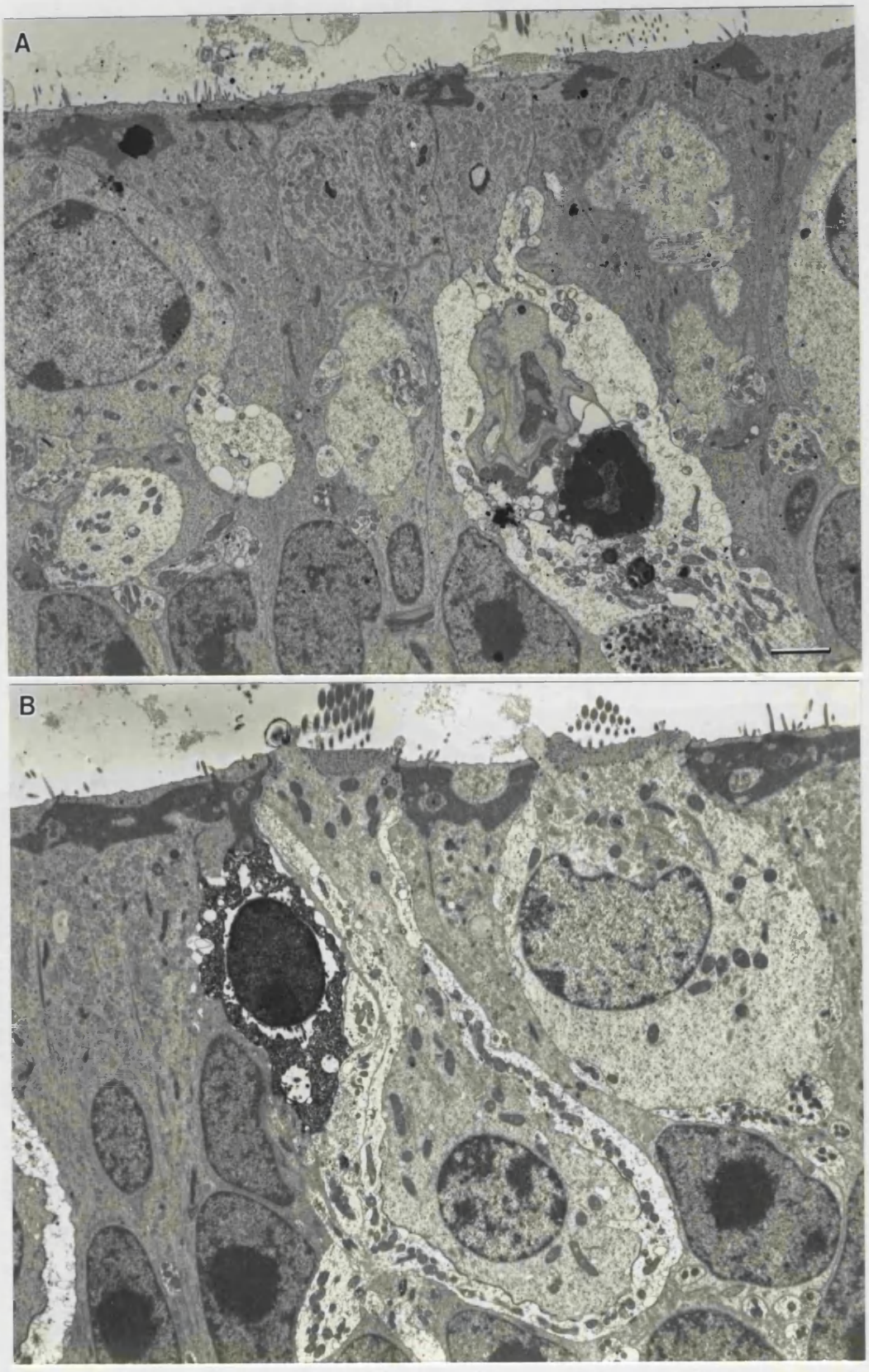
A
B

Figure 5.31 Thin Sections of Extrusion of Hair Cells

A. A hair cell with a fused and expanded protrusion at the apical surface is present in a crista at three days after systemic gentamicin treatment. Bar = 2 μm.

B. The cuticular plate (arrowhead) is ruptured and cytoplasm has extruded from the cell body into the protrusion. Bar = 3 μm.

C. The cuticular plate (arrowhead) of the hair cell could be seen inside the extrusion in the utricular macula.

D. In thin sections of partially ejected hair cells, the tight junctions between the hair cell and the adjacent supporting cells appear intact.

E. Larger and rounded protrusion containing the cell nucleus is attached at the apical surface of the epithelium. The density of cytoplasm and the nucleus morphology in the protrusion appear relatively normal. The space left by the ejected hair cell is gradually closed by the expansions of the adjacent supporting cells.

F. Tight junctions (arrow) have formed between supporting cells beneath the extruded hair cell and the space left by the ejected hair cell is sealed by supporting cells.

Bar (C-F) = 4 μm.

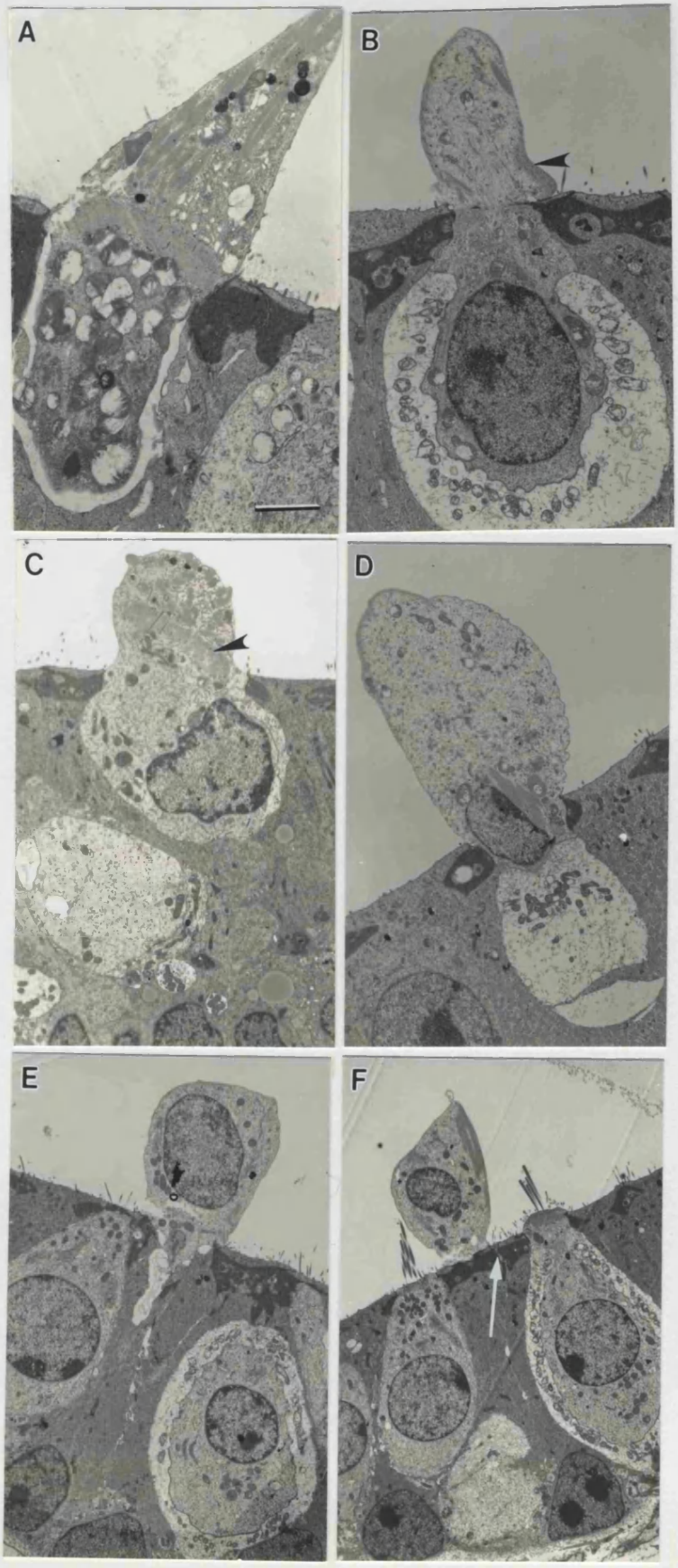
A
B
C
D
E
F

Figure 5.32 Thin Sections of Gentamicin Affected Utricles

A. Utricular striolar region at four weeks after systemic gentamicin treatment. The macular epithelium is thinner and the existing hair cells are shorter and type I hair cells have disappeared. The spaces of lost hair cells are occupied by expansion of supporting cells. Bar = 5 μm.

B. Utricular epithelium 12 weeks post-treatment. More hair cells are present in the striolar region and most of them are cylindrical in shape, like type II hair cells. Bar = 5 μm.

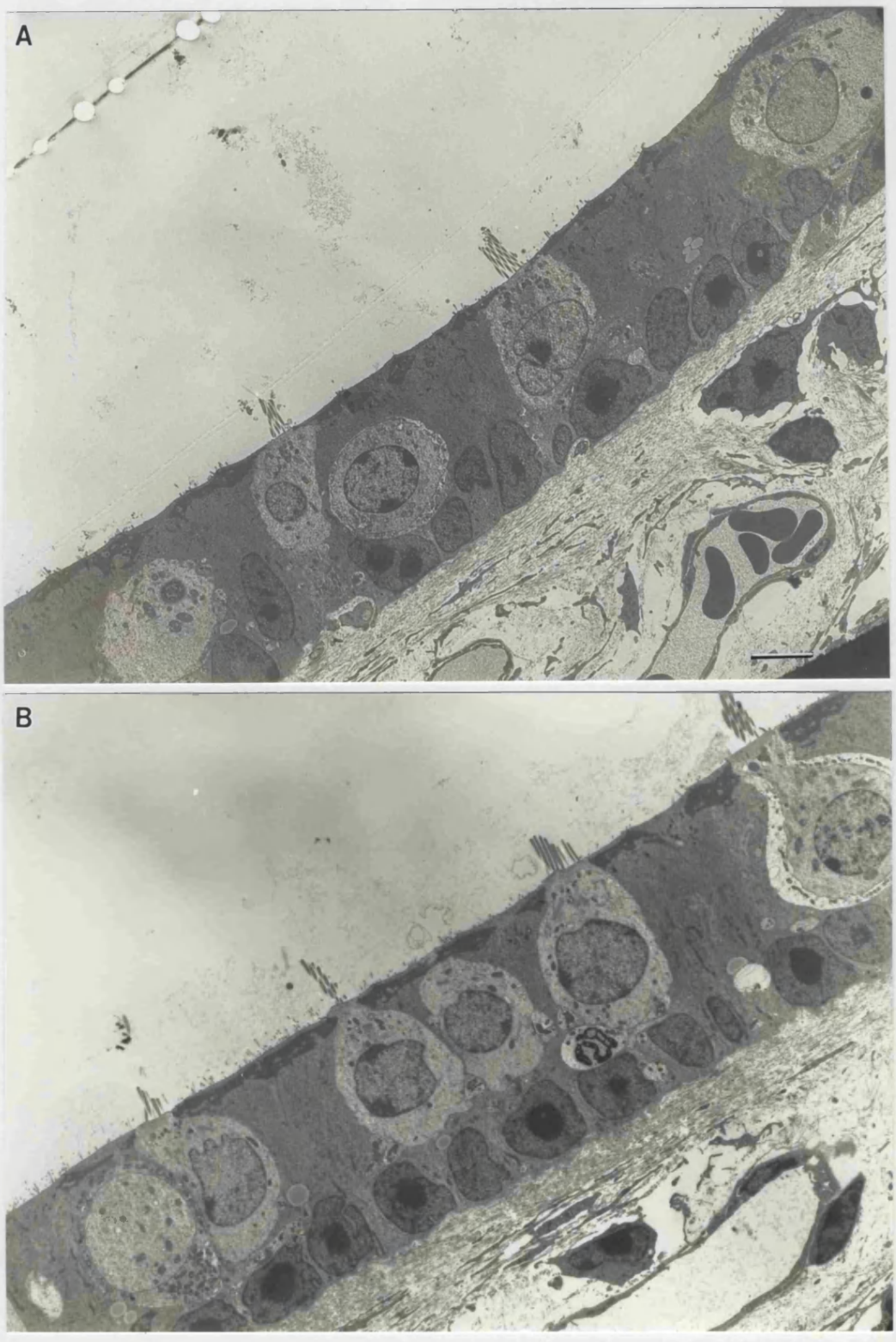
A
B

Figure 5.33 Thin Sections of Immature Hair Cells

A. Mature hair cells have the organised stereociliary bundles in staircase arrangement on a developed cuticular plate and are innervated at the cell body. Bar = 3 μm.

B. An immature hair cell has small, not well defined apical cuticular plate and no nerve endings at the cell body. The immature hair bundle contains a single kinocilium (arrowhead) and many thin stereocilia in same length with relatively low density of microfilaments. Bar = 2 μm.

C. Hair cells with relatively mature looking stereocilia and a thick cuticular plate show bouton shaped nerve endings (arrowhead) at the cell body. Bar = 4 μm.

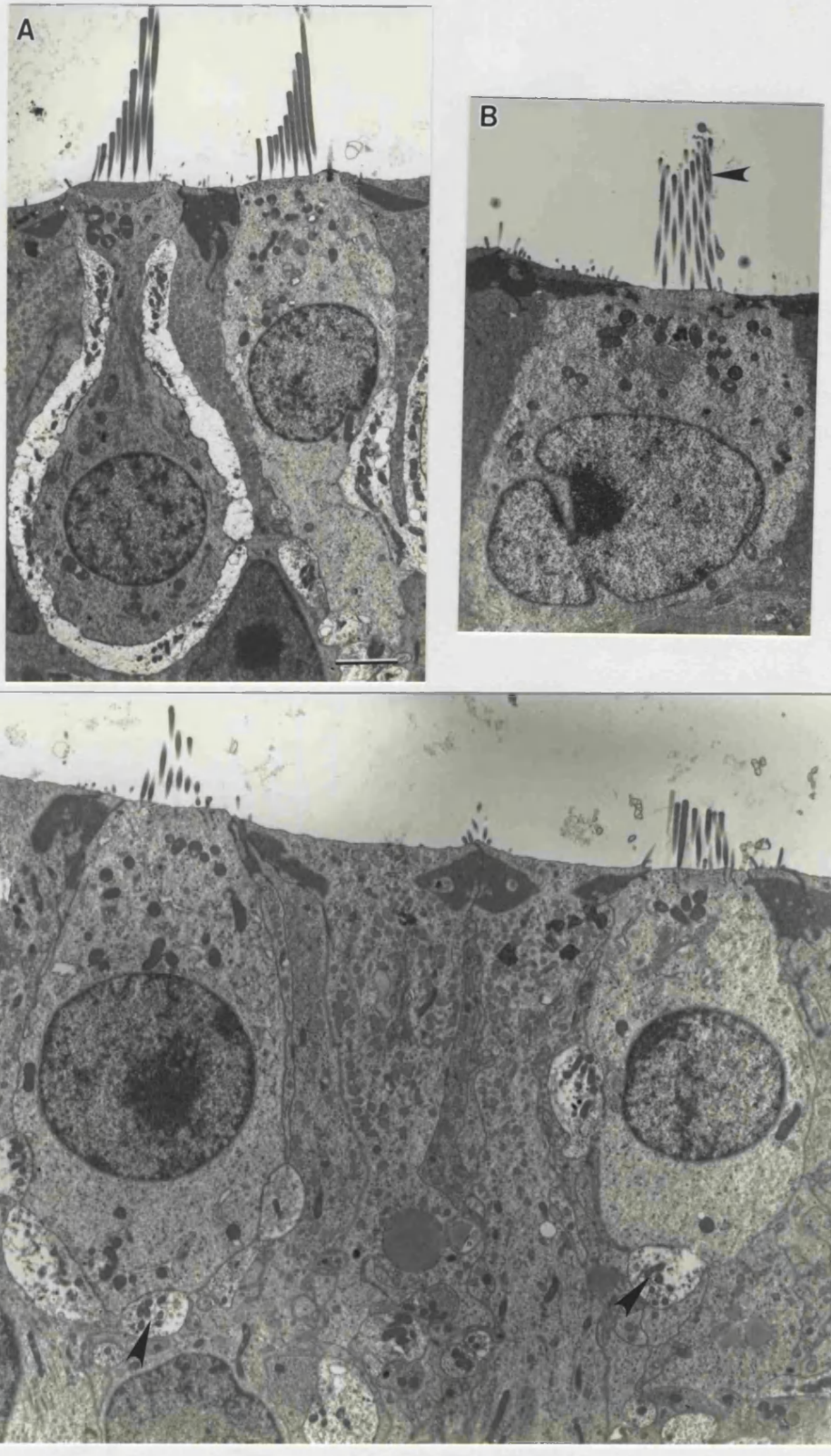
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Figure 5.34 Thin Sections of the Utricular Macula 33 Weeks Post-treatment

Many type II like hair cells are present in the striolar region of an utricular macula from a gentamicin treated animal 33 weeks post-treatment. The hair cells are innervated at the lower part of the cell body with small bouton shaped nerve endings and no type I like hair cells can be seen. Bar = 4 μm.

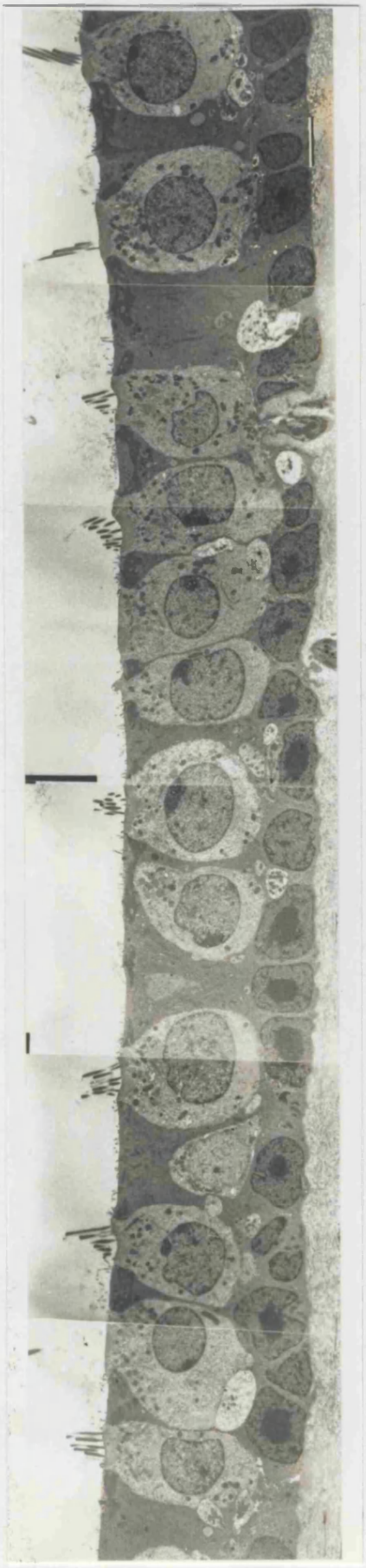

Figure 5.35 BrdU Labelling in Whole Mount Preparation

A. Utricle of an animal at two weeks after systemic gentamicin treatment shows BrdU labelling in the supporting cells of the sensory epithelium. Bar = 7 μm.

B. Utricle of an animal at four weeks after systemic gentamicin treatment. BrdU positive labelling appears to be in the connective tissue.

C. Utricle of another animal at four weeks after systemic gentamicin treatment. BrdU positive labelling appears in the sensory epithelium.

The animals in (A-C) were given BrdU by intraperitoneal injections every 6 hours over 24 hours and the last injection was made one hour before sacrifice.

D. Right treated utricle of an animal at 6 weeks after topical application with gentamicin . A pair of labelled nuclei are present in the connective tissue underlying the sensory epithelium.

The animal was given BrdU by intraperitoneal injection once at dosage 100 mg/kg on day 7 after topical application with gentamicin.

E. Same utricle as D, a pair of labelled supporting cell nuclei are present in the centre of the epithelium.

Bar (B-E) = 17 μm.

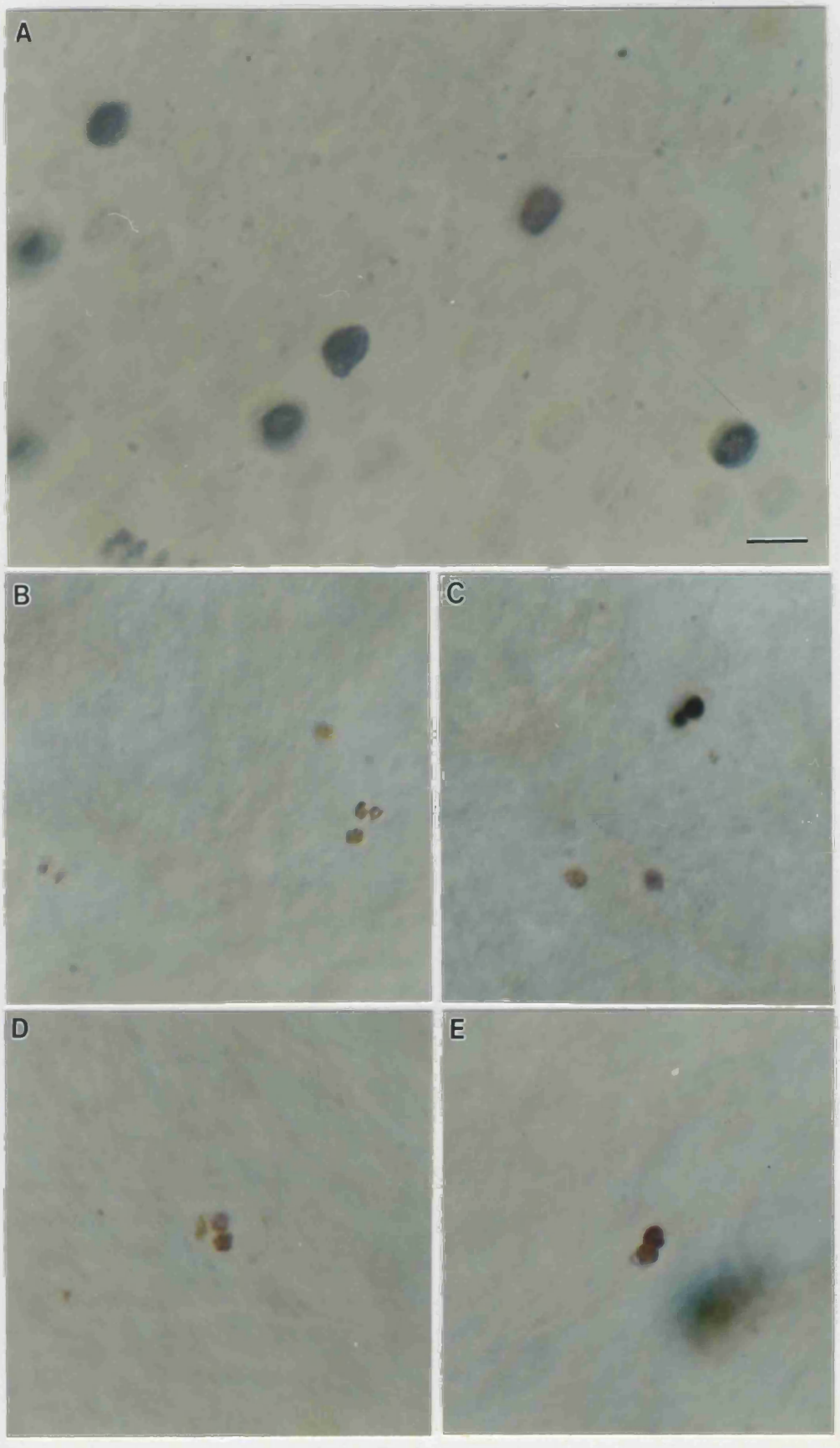
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Figure 5.36 BrdU Labelling in Wax Sections of Control Tissues

A. Wax section of the small intestine counterstained by haemotoxylin shows BrdU positively labelled nuclei in brown colour along the epithelial layer of the villi as well as in the underlying connective tissue. Bar = 26 μm.

B. BrdU positively labelled nuclei are present in the wax section of liver tissue counterstained by haemotoxylin. Labelled dividing nuclei of the hepatocytes (arrowhead) are in closely adjacent pairs and labelled stroma nuclei (arrow) of the connective tissue are irregularly shaped. Bar = 17 μm.

C. Method control section of liver tissue without BrdU primary antibody incubation, all nuclei are stained blue by haemotoxylin. Bar = 17 μm.

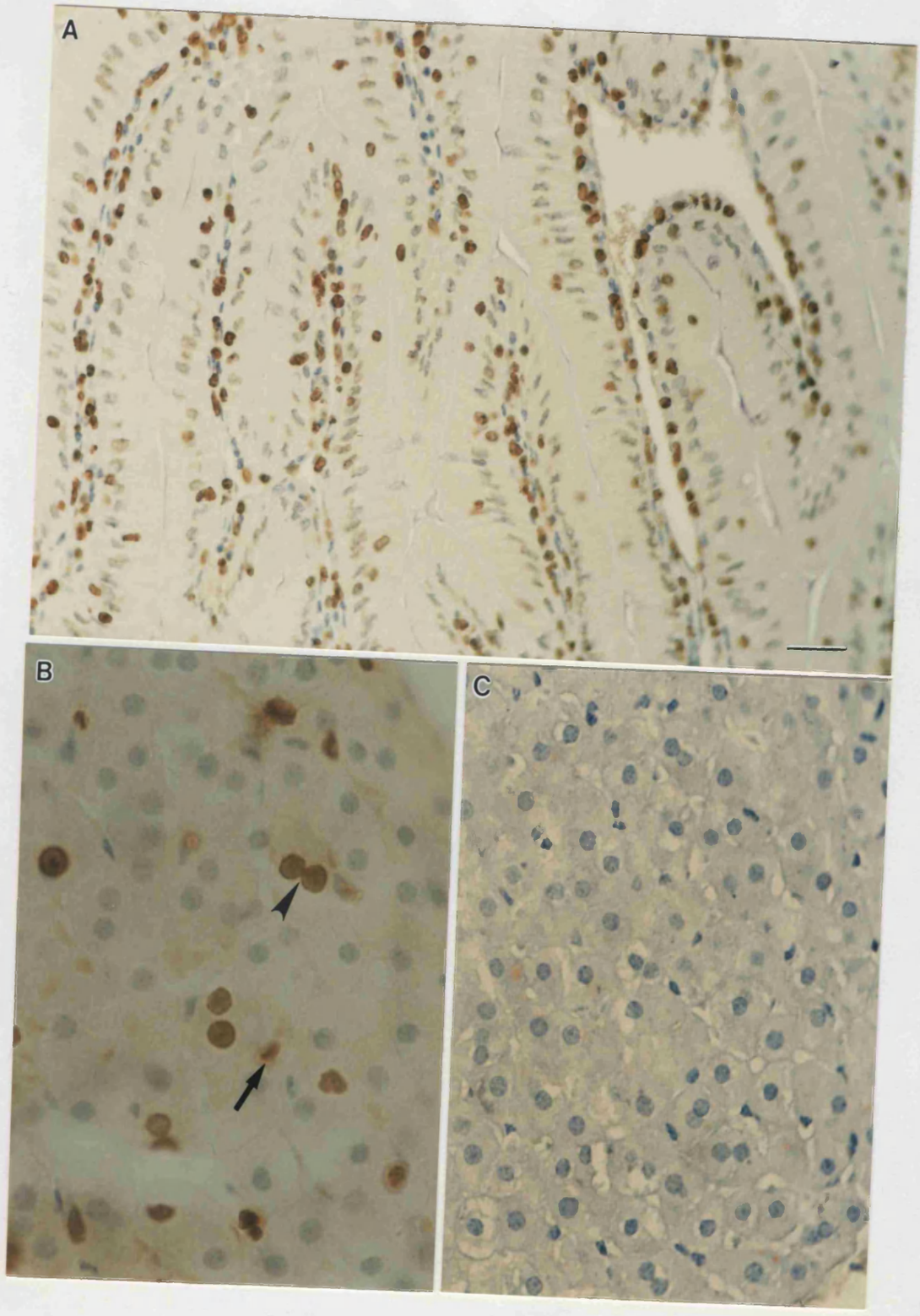
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Figure 5.37 BrdU Labelling after Two Weeks Osmotic Pump Implantation

A. Hair cell loss across the striolar region is apparent in the damaged utricular macula after topical application with gentamicin. The epithelium is thinner and most cell nuclei are stained blue by haemotoxylin. One nucleus in brown colour appears to be labelled by BrdU antibody in the sensory epithelium.

B. Labelled nucleus is in brown colour at the position of supporting cell nuclei in the utricular sensory epithelium after topical application with gentamicin.

C. BrdU labelled supporting cell nucleus is located more lumenally.

D. BrdU positively labelled nucleus is present at the hair cell level in the utricular sensory epithelium and most hair cells have been lost after topical application with gentamicin.

Bar (A-D) = 7 μm.

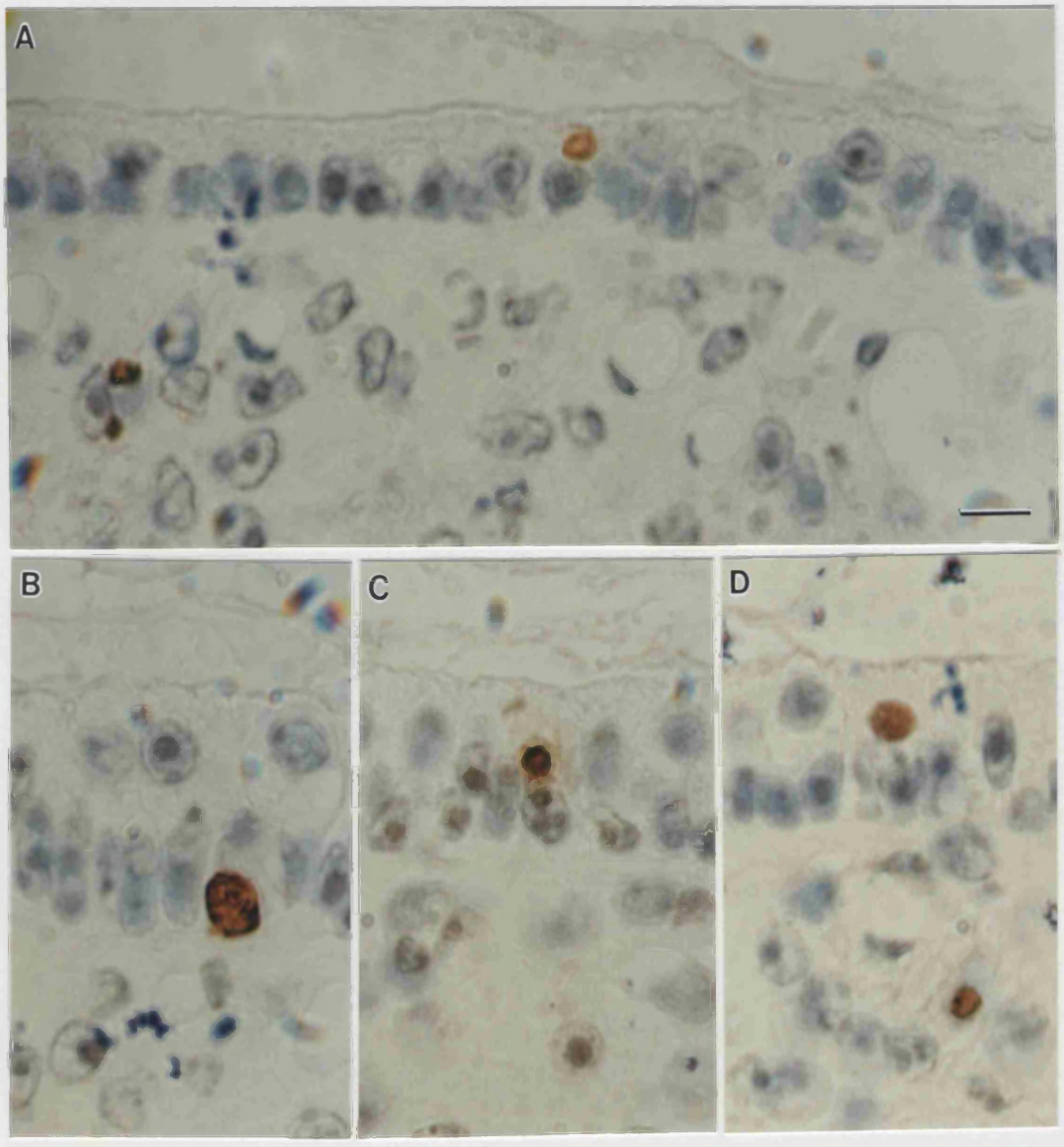
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Figure 5.38 BrdU Labelling in Wax Sections of Utricular Maculae

A. Section of the left control utricular maculae shows regularly arranged hair cells and BrdU positive labelling is absent in the sensory epithelium. Bar = 17 μm.

B. Treated utricle shows intensive BrdU labelling in nuclei of the connective tissue underlying the sensory epithelium where hair cell loss is apparent. Bar = 7 μm.

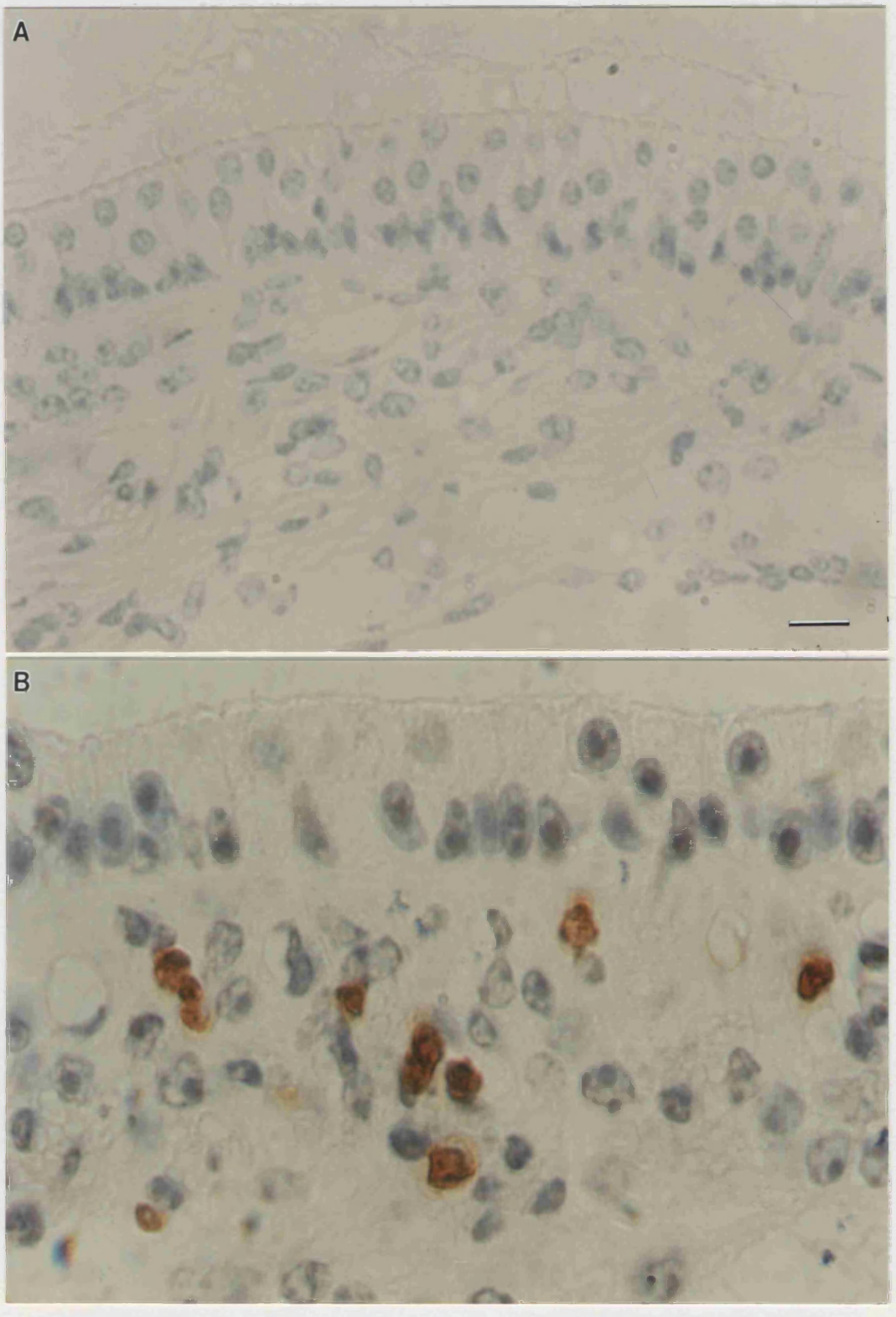
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Figure 5.39 Light Microscopy of Outgrowth of Cultured Utricles

A. Cultured utricle shows that fibroblasts begin to grow within 24 hours in vitro.

B. Cultured utricle shows an early outgrowth (arrow) at two days in vitro.

C. The outgrowth tissue of the cultured utricle has spread out and attached to the collagen covered coverslip at one week in vitro.

Bar (A-D) = 50 μm.

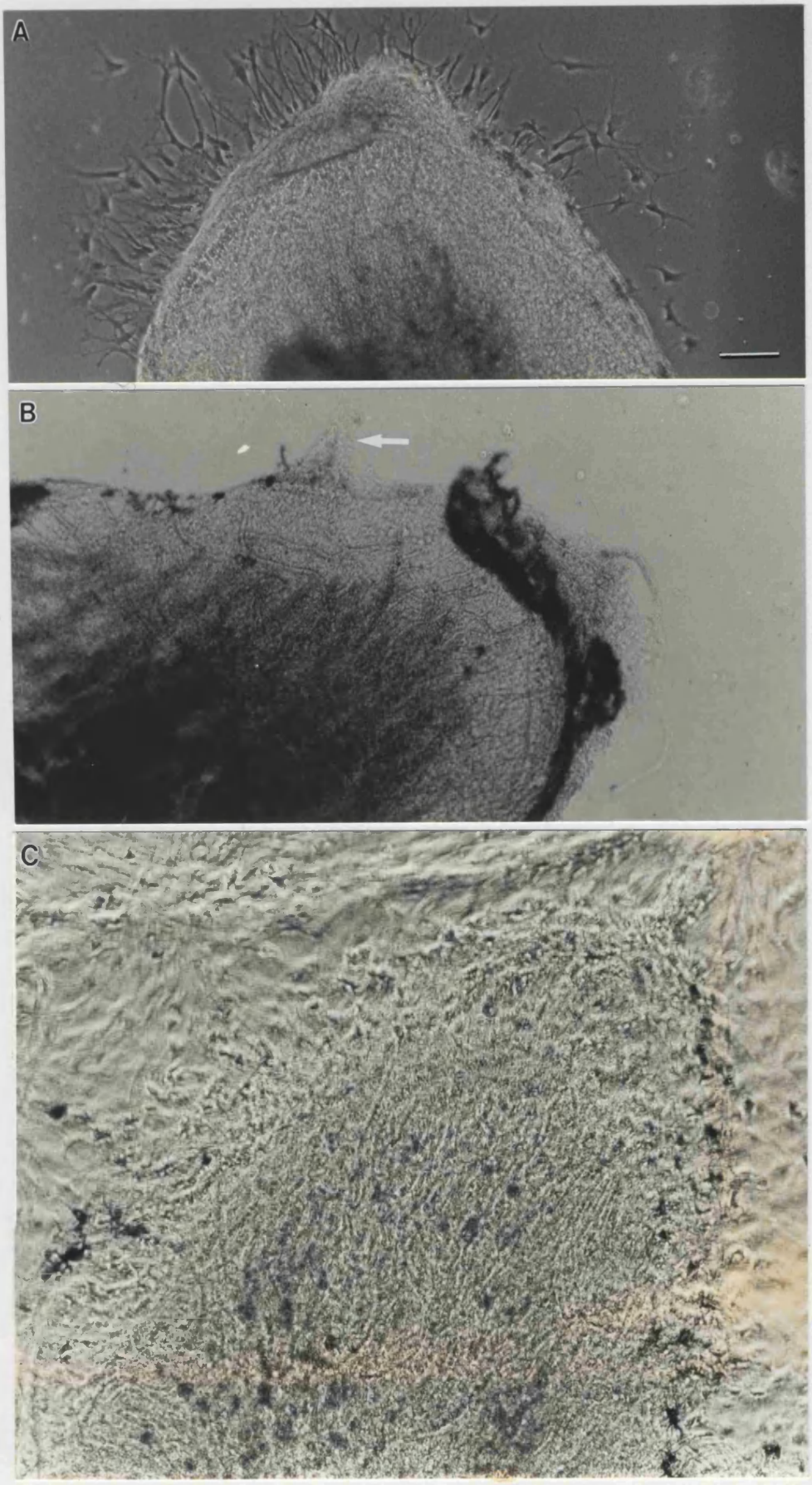
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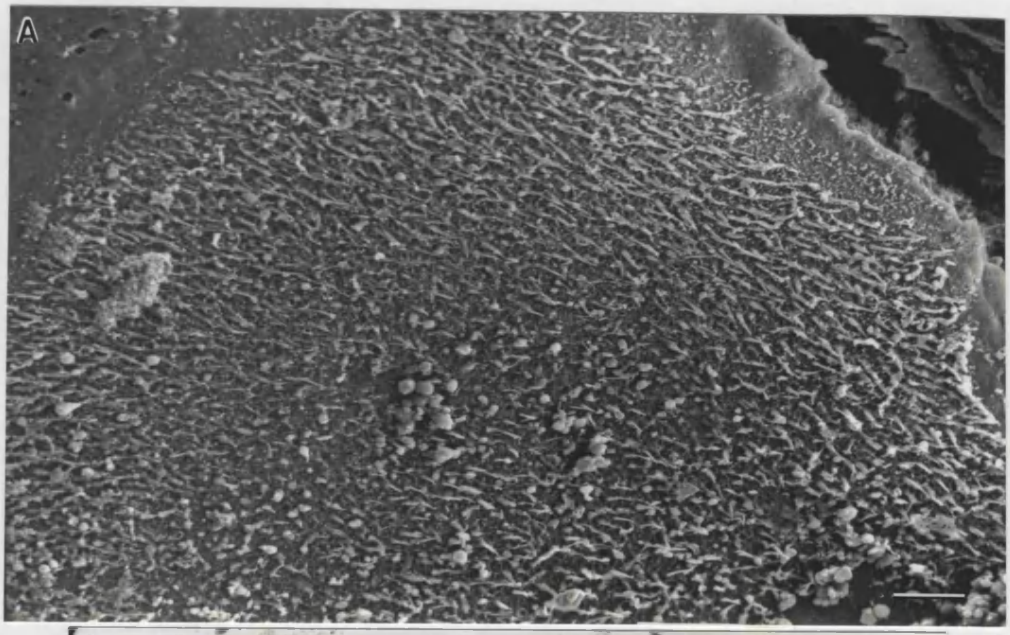
Figure 5.40 Control Guinea Pig Utricular Cultures

A. SEM of control guinea pig vestibular utricle at one week in vitro. Hair cells are evenly distributed in the utricular epithelium. Bar = 40 μm.

B. Thin sections of a control utricle at 16 days in vitro. Type I hair cell has pear shaped body and type II cells are cylindrical in shape. The innervation of both types of hair cells is absent. Bar = 2.5 μm.

C. Hair cell stereocilia at 16 days in vitro show preservation of closely packed microfilaments and extensive cross links (arrowhead). Bar = 0.3 μm.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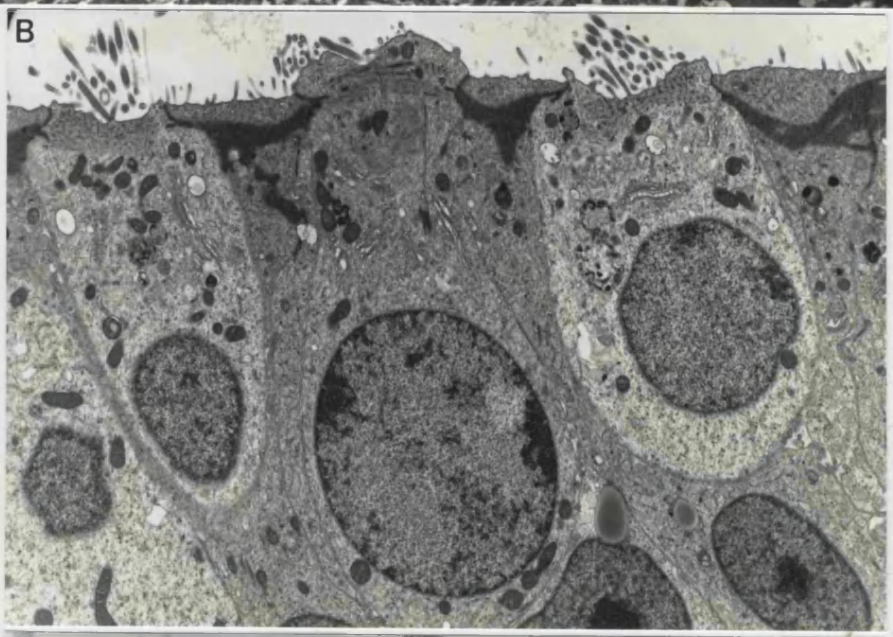
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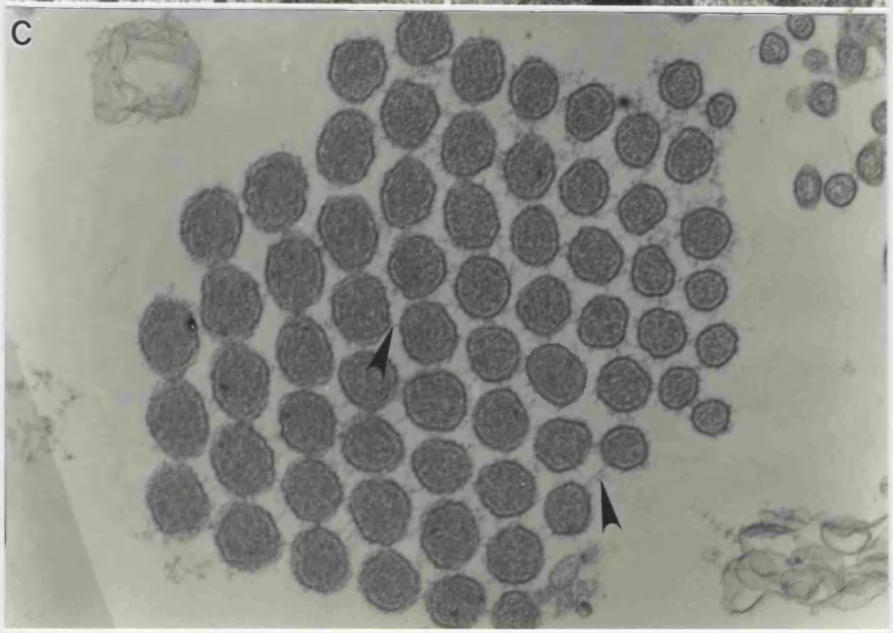
C

Figure 5.41 Hair Cell Loss in Cultured Guinea Pig Utricles

A. Cultured utricle at 14 days in vitro after 1mM gentamicin incubation for 24 hours. Hair cell loss is extensive and there are no obvious lesions through the epithelium after hair cell loss. Bar = 60 μm.

B. Central epithelium of the same utricle of A. Lost hair cells are replaced by expansion of supporting cells. A short and small hair bundle is present (arrow). Bar = 30 μm.

C. Peripheral area of the same utricle of A. Some normal looking hair cells remain. Bar = 30 μm.

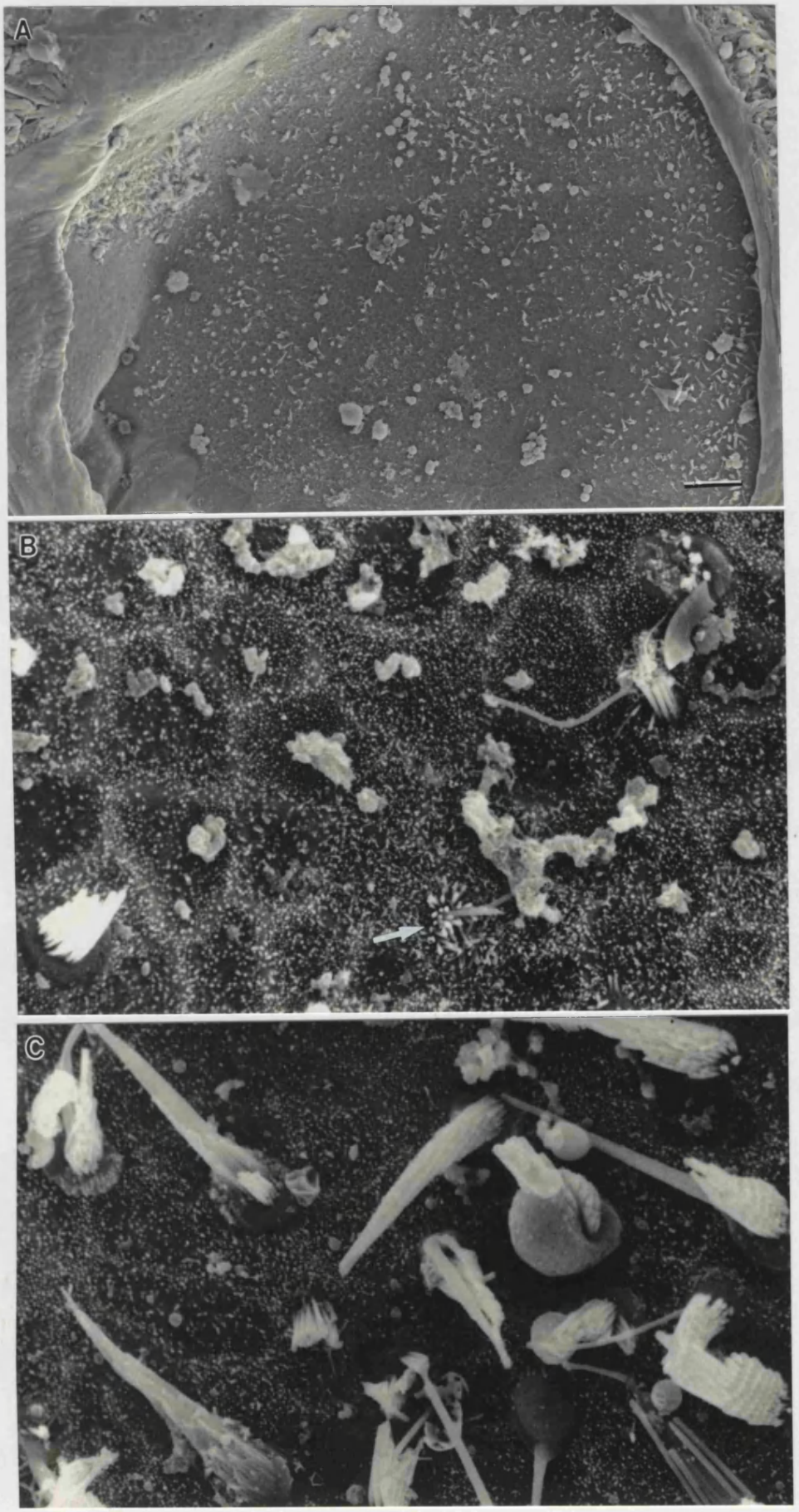
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Figure 5.42 Thin Sections of Treated Guinea Pig Utricular Cultures

A. The extrusion containing the cuticular plate (arrowhead), stereocilia (arrow), the nucleus and cytoplasm organelles is attached at the apical surface of the sensory epithelium in a gentamicin treated utricle at 7 days in vitro. Bar = 0.8 μm.

B. Cuticular plate (arrowhead) and stereocilia are enclosed inside the sensory epithelium. New tight junction is formed by supporting cells at the apical surface of the epithelium (arrow). Bar = 1.5 μm.

C. Striolar region of a cultured utricular macula at 16 days in vitro after 1 mM gentamicin treatment for 24 hours. Neural elements are absent in the sensory epithelium. The nuclei of supporting cells in the area of hair cell loss become elongated and some of them are located more lumenally. New tight junctions (arrowhead) between supporting cells are formed in the apical part of the sensory epithelium. Bar = 10 μm.

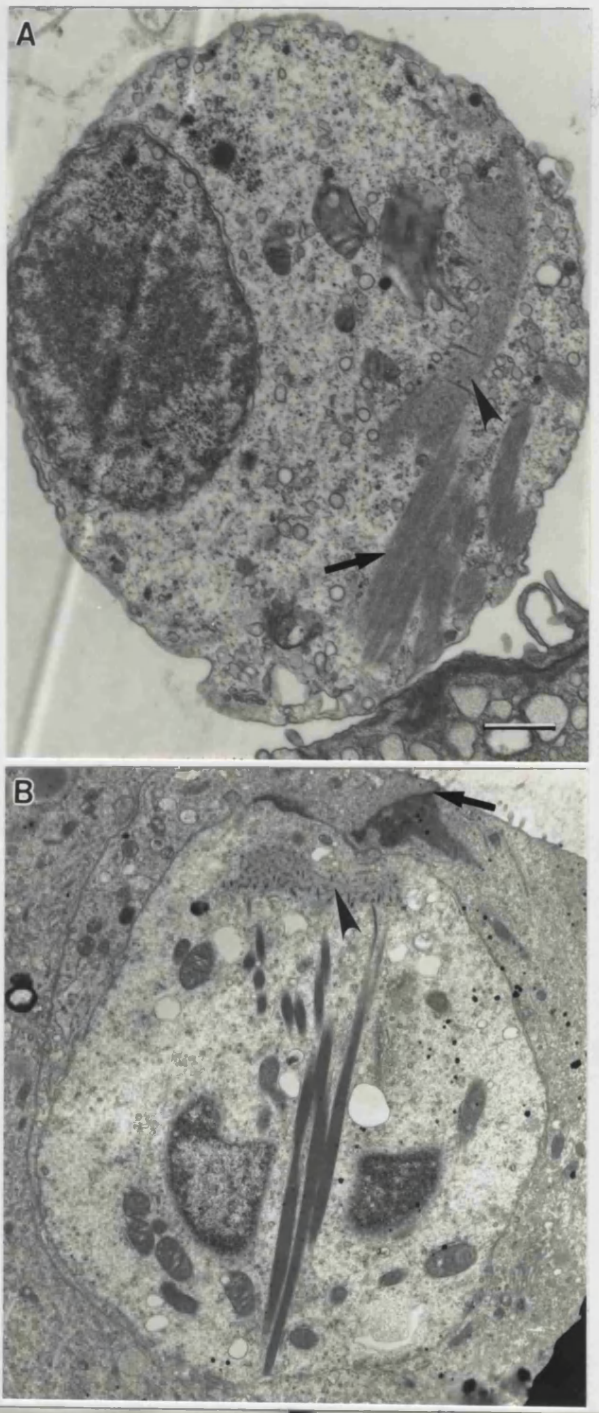
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Figure 5.43 Apoptotic Hair Cells in Cultured Guinea Pig Utricles

A. Hair cell nucleus shows condensation of chromatin and nuclear membrane blebbing. Mitochondria (arrowhead) appear intact. Bar = 2.5 μm.

B. Hair cell nucleus shows condensed and marginated chromatin. Bar = 0.7 μm.

C. Gentamicin treated utricle at 13 days in vitro. Apoptotic hair cell remains in situ (arrow) or becomes to be enclosed inside the supporting cell (arrowhead). Bar = 2 μm.

D. Apoptotic hair cell showing nuclear fragmentation (arrowhead) is at the level of supporting cell nuclei and surrounded by supporting cells. Bar = 2 μm.

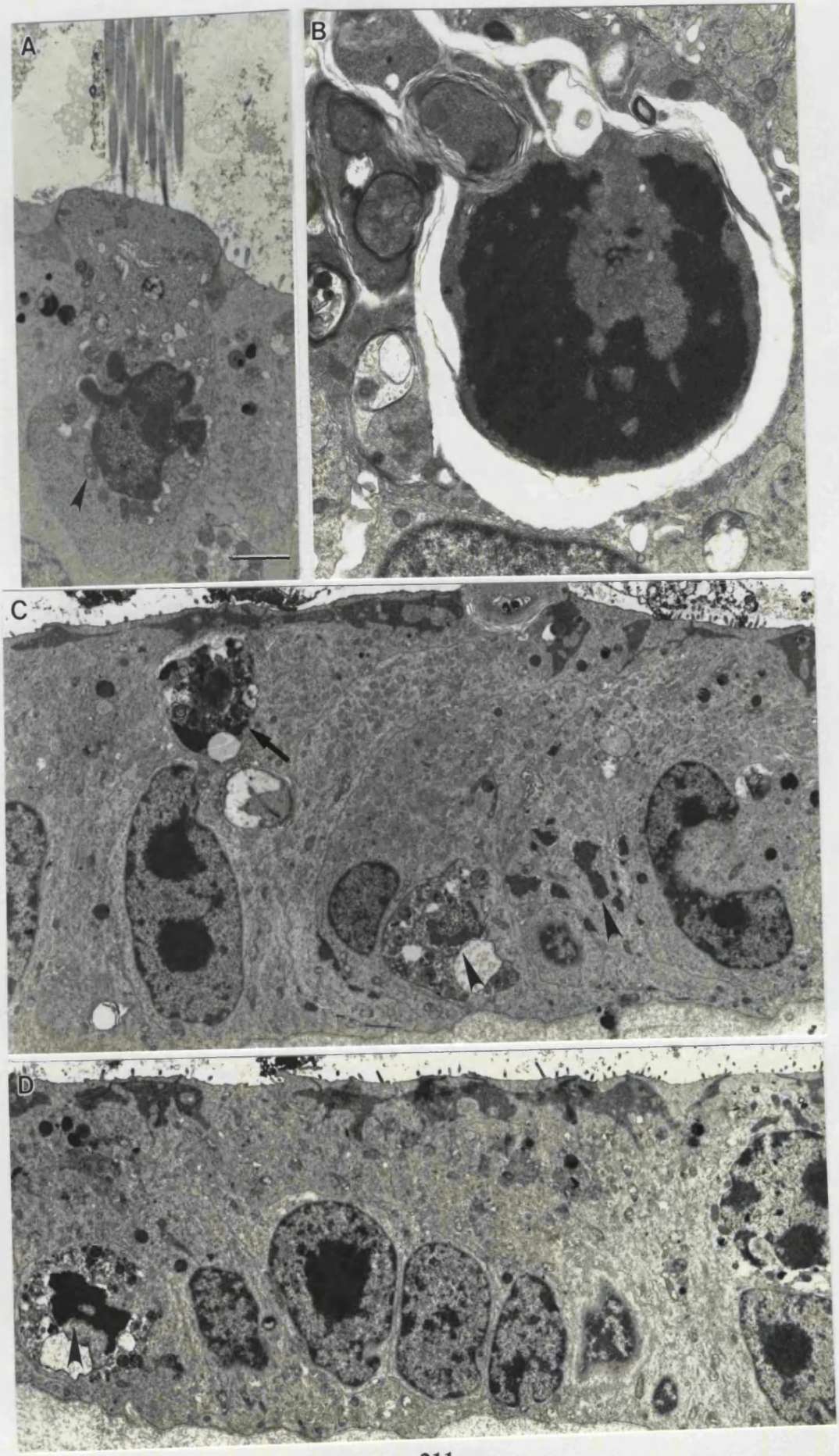
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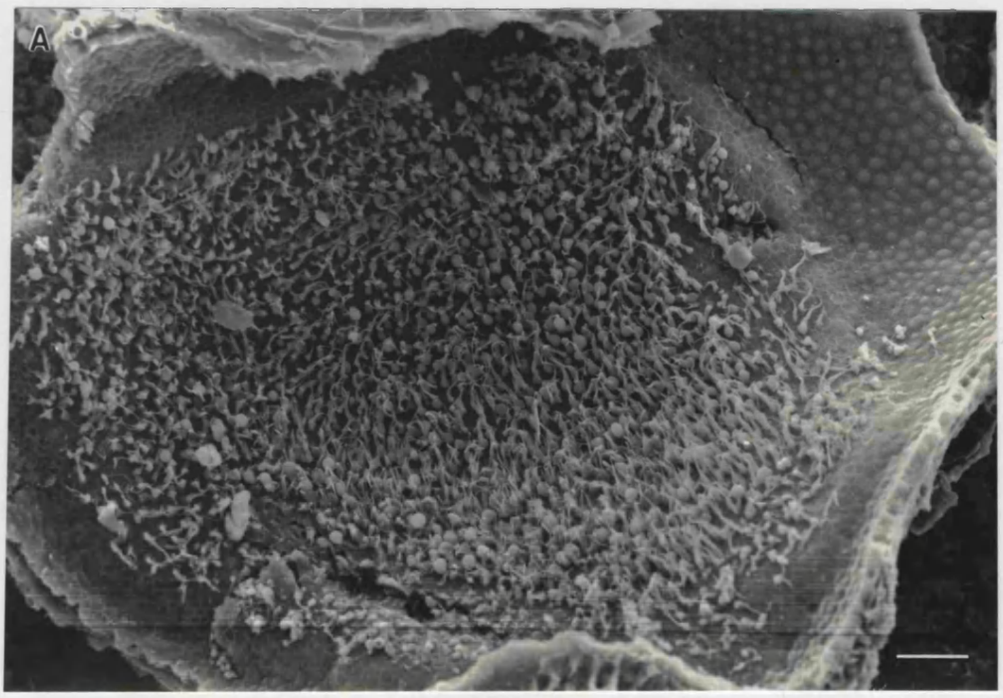
Figure 5.44 Cultured Gerbil Utricles

A. Control gerbil utricular culture. Hair cells fully cover the epithelium examined by SEM. Bar = 35 μm.

B. Control gerbil utricle at 13 days in vitro. The hair cells are well preserved and surrounded by supporting cells. Hair cell bundles and the cuticular plates appear normal although neural elements are lost in vitro. Bar = 4 μm.

C. Cultured gerbil utricle at 13 days in vitro after 1 mM gentamicin for 6 hours. Extensive hair cell loss is apparent and supporting cells are present in the thin epithelium in the striolar region. New tight junctions (arrowhead) between supporting cells are formed in the apical part of the sensory epithelium. An apoptotic hair cell is enclosed inside the supporting cell (arrow) and some intact hair cells are present peripherally. Bar = 4 μm.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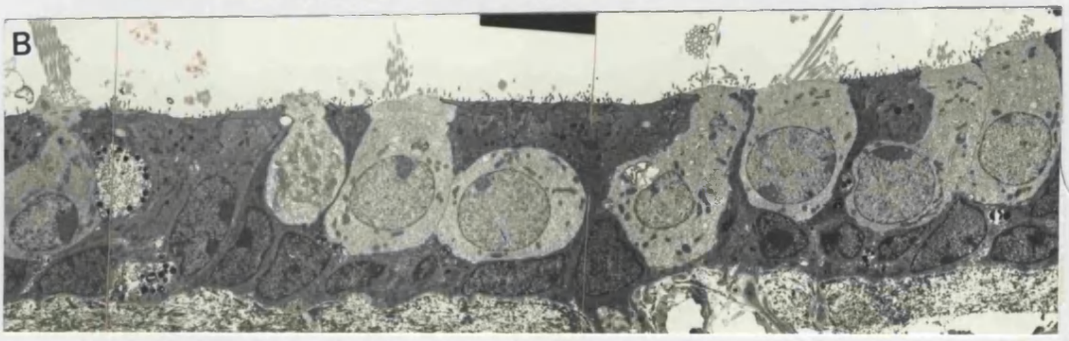
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Figure 5.45 Apoptotic Hair Cells in Cultured Gerbil Utricles

A. Cultured gerbil utricle at three days in vitro after 1 mM gentamicin treatment for 6 hours. The individually located apoptotic nuclei (arrowhead) of hair cells show compaction of chromatin and margination. Bar = 2 μm.

B. Cultured gerbil utricle at three days in vitro after 1 mM gentamicin treatment for 6 hours. Apoptotic body like structures (arrowhead), containing electron-dense inclusions, could be seen in the area of hair cell loss and they are ingested by surrounding supporting cells. Bar = 2 μm.

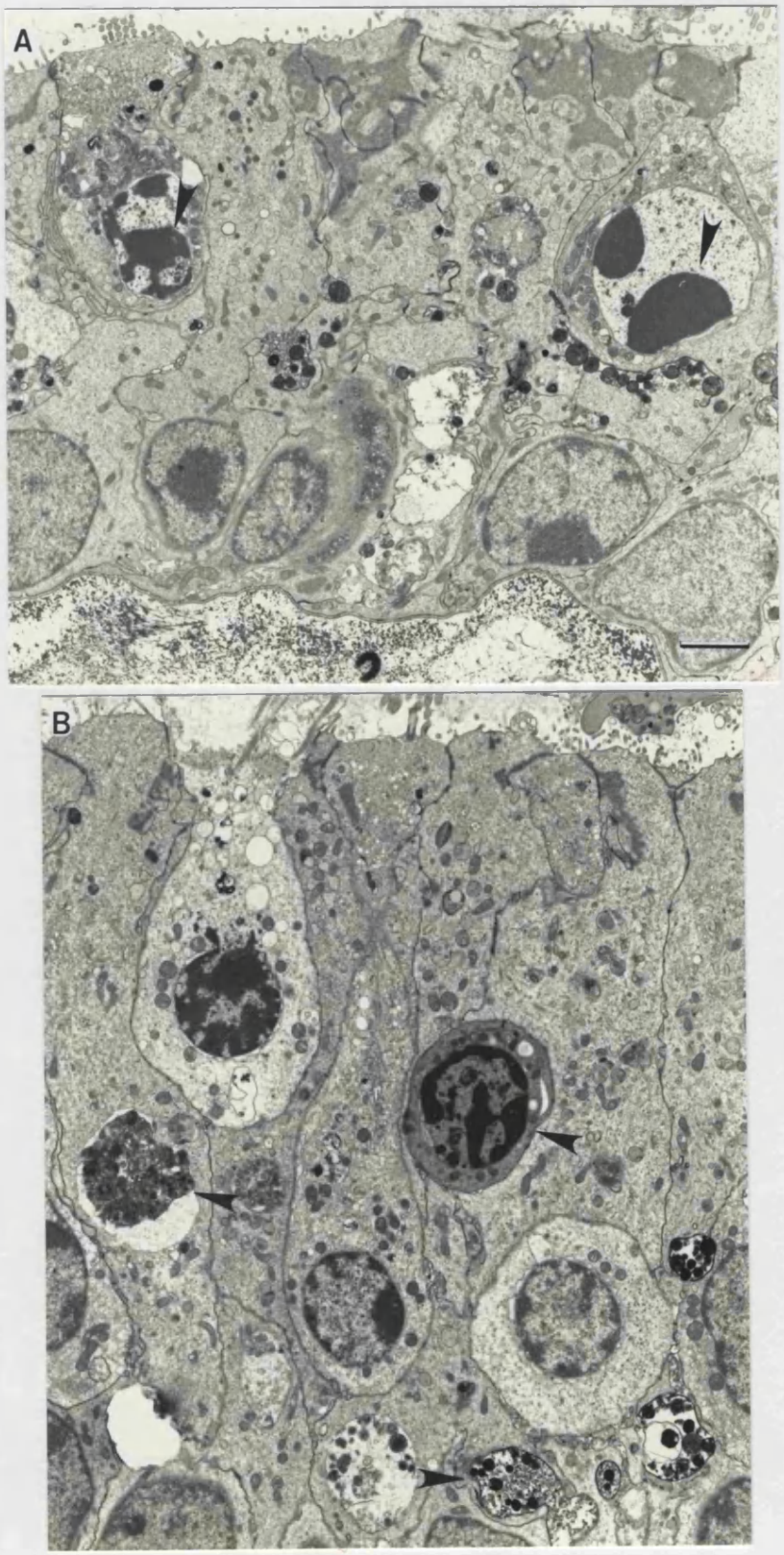
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Figure 5.46 BrdU Labelling of Cultured Utricles

A. Cultured guinea pig utricle at 16 days in vitro after 1 mM gentamicin for 24 hours. Fluorescence microscopy shows BrdU labelling in the nuclei of the outgrowth tissue and some nuclei are in pairs (arrowhead). Bar = 20 μm.

B. A cultured gerbil utricle at 13 days in vitro after 1 mM gentamicin for 6 hours. The nucleus of a transitional cell is labelled by gold particles, which appear as small black spots densely present over the nucleus, in the thin section for immunoelectron microscopy. Bar = 0.15 μm.

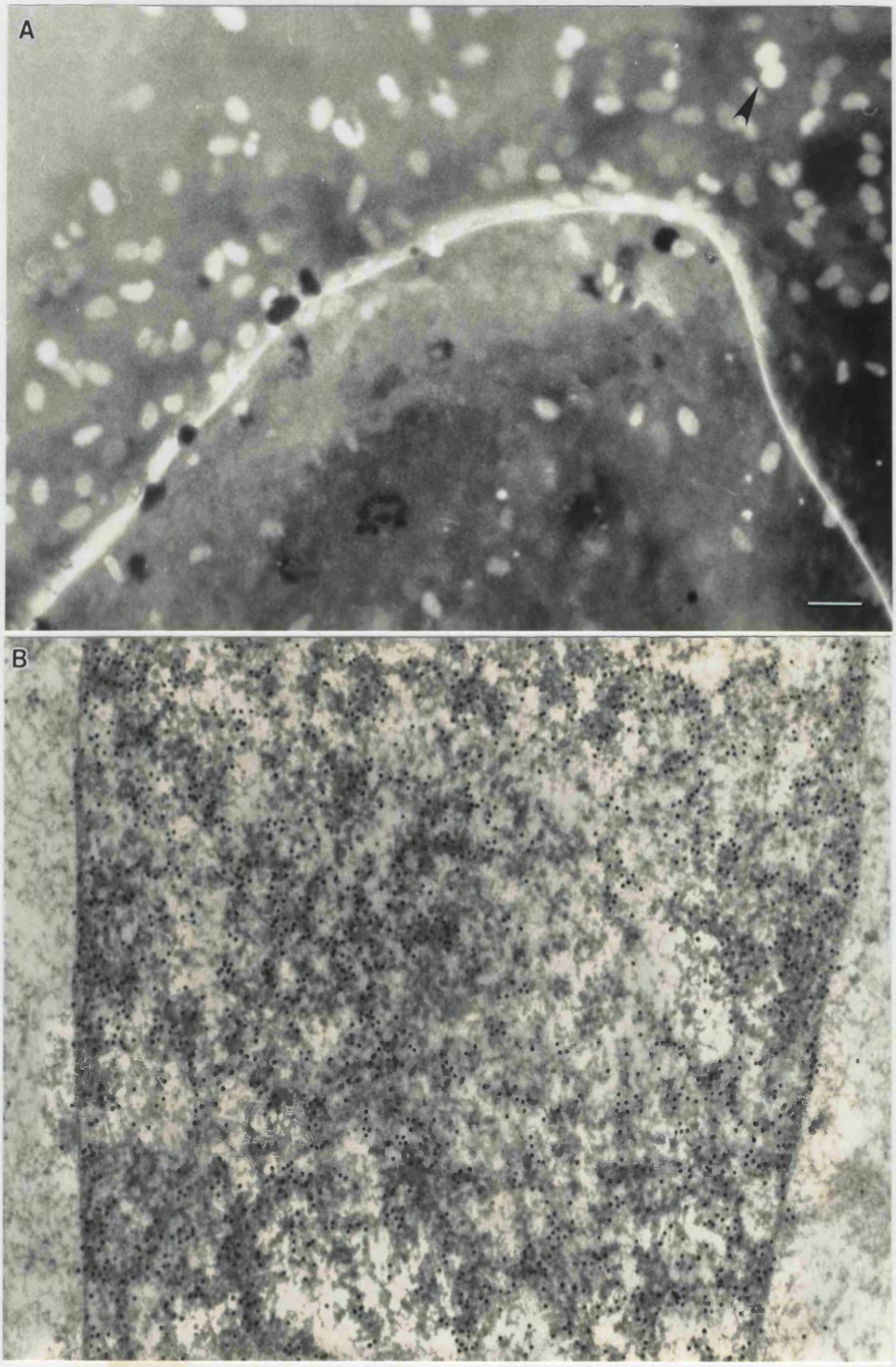
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Figure 5.47 Propidium Iodide Labelling of Control Cultured Utricles

A. Untreated control utricle at 8 days in vitro. The nuclei of hair cells stained by propidium iodide are round in shape forming an even layer under fluorescence microscopy.

B. Supporting cell nuclei are smaller in diameter and are located beneath the layer of hair cell nuclei.

C. Peripherally located hair cell nuclei appear normal and round in shape.

Bar (A-C) = 7 μm.

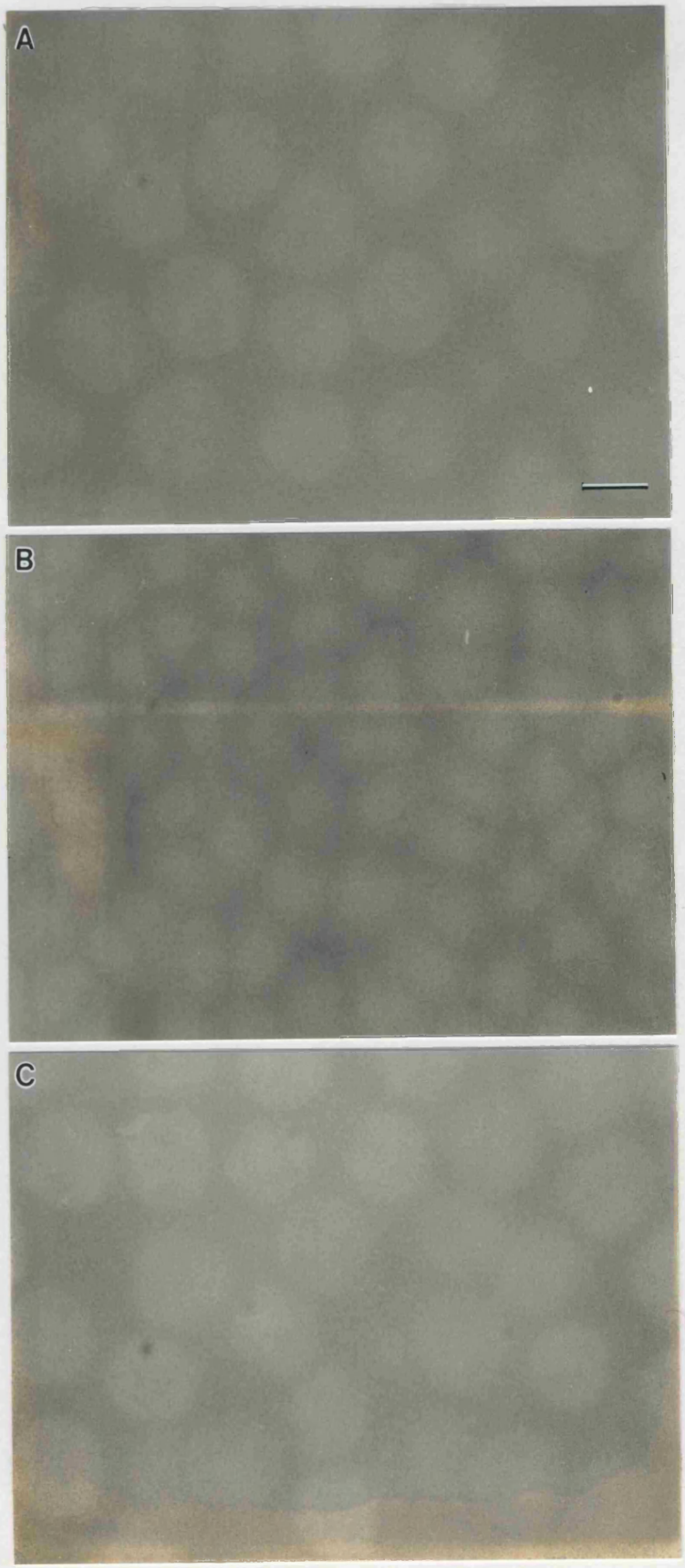
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Figure 5.48 Double Labelling of Gentamicin Affected Utricles

A. Immediately after 2 mM gentamicin treatment for 6 hours. Propidium iodide labelling shows that condensed nuclei of degenerated hair cells (arrowhead) are brightly stained by propidium iodide. The apoptotic nuclei are smaller than normal hair cell nuclei (arrow). Bar = 7 μm.

B. Same field from A. Most hair bundles labelled by phalloidin are intact, but scars (arrowhead) are formed at the site of hair cell loss. Bar = 7 μm.

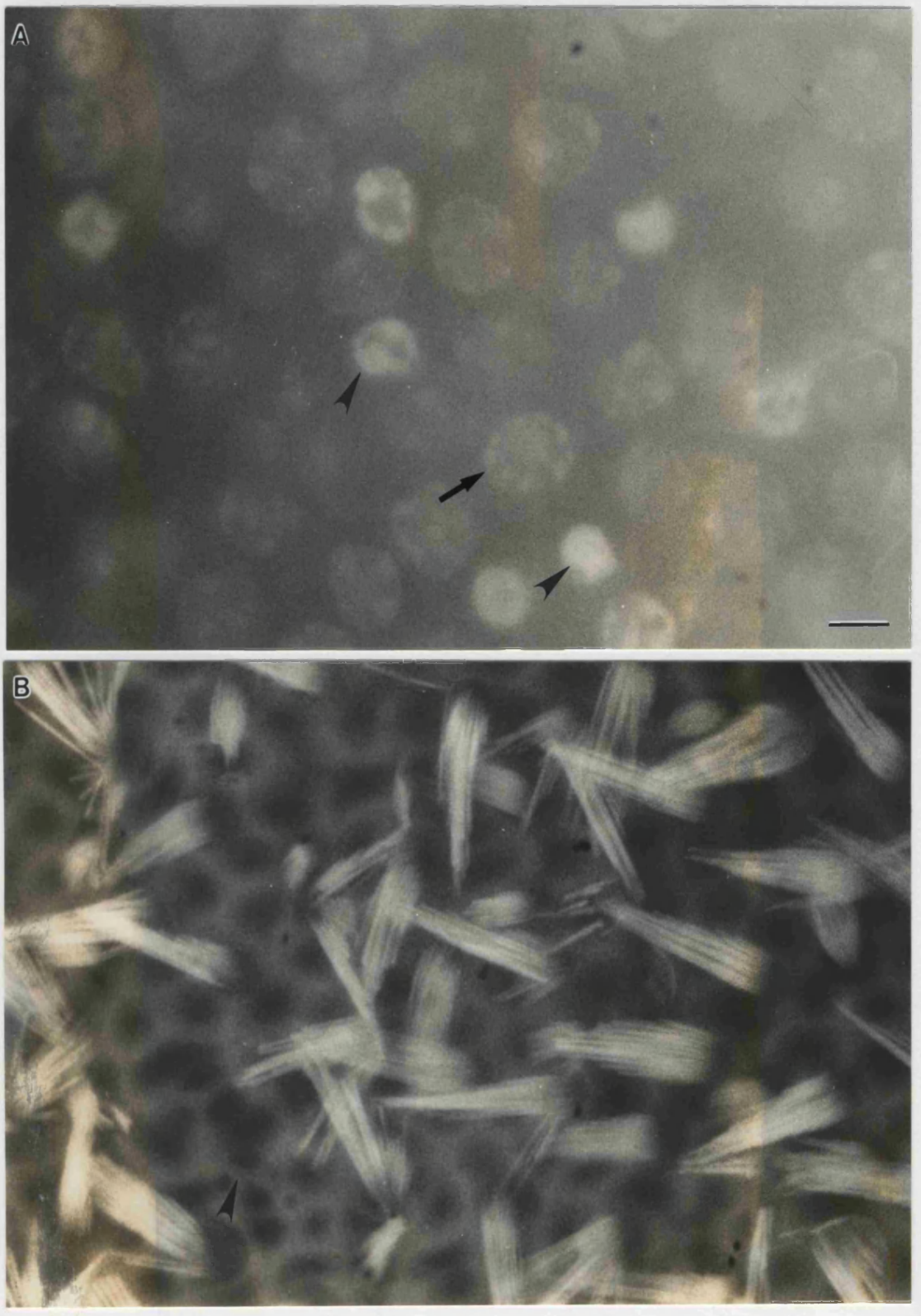
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Figure 5.49 Double Labelling of Gentamicin Affected Utricles

A. Immediately after 24 hours incubation with 2 mM gentamicin. More hair cell nuclei stained by propidium iodide become condensed and fragmented (arrow) in the central area of the utricular culture. Bar = 7 μm.

B. Same field from A. Phalloidin labelling of actin shows that hair bundles are absent from the apical surfaces of the hair cells and many scars (arrowhead) are present in the area of hair cell loss. Bar = 7 μm.

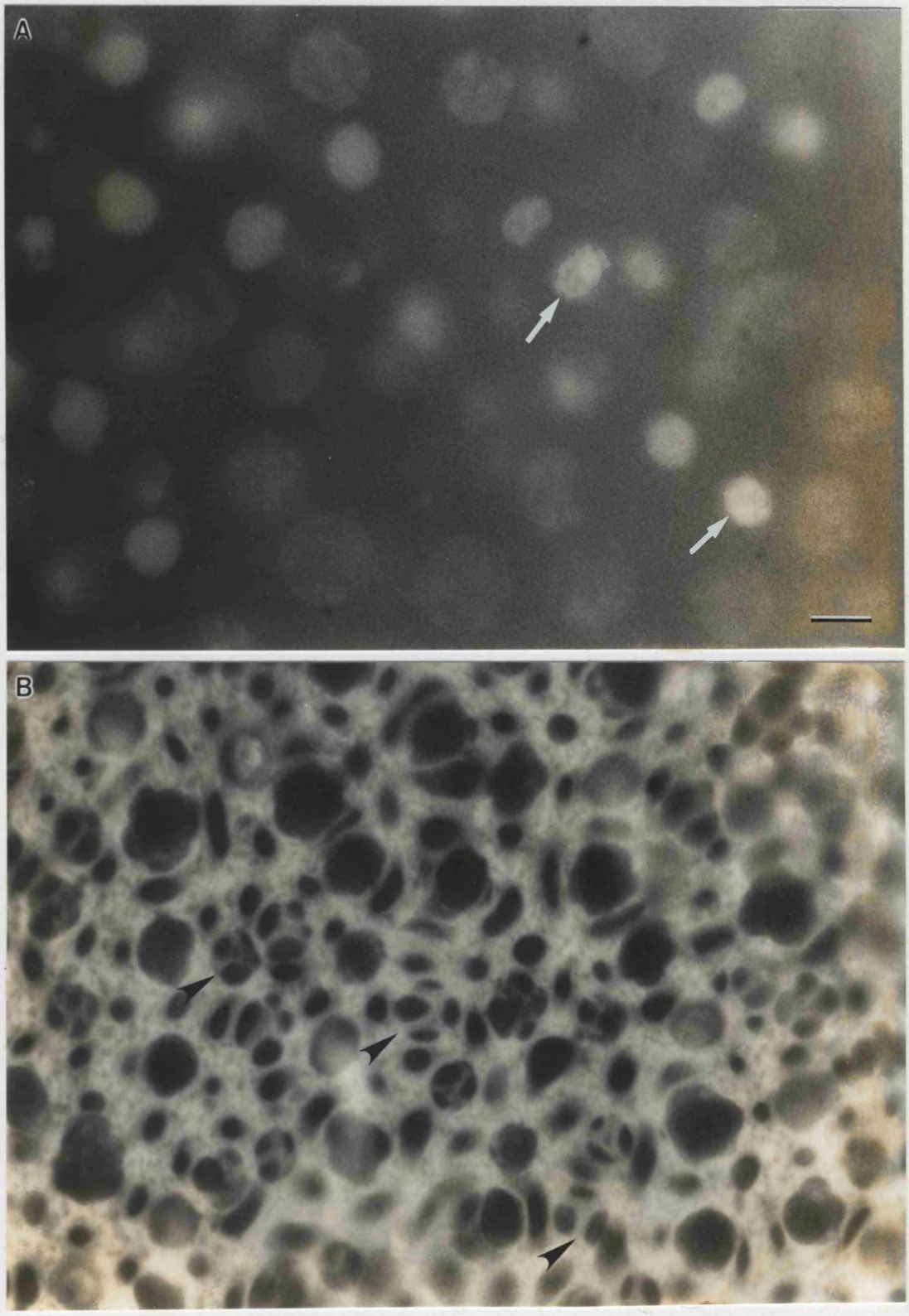
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Figure 5.50 Double Labelling of Gentamicin Affected Utricles

A. Five days after 2 mM gentamicin treatment. Small and short hair bundle stained by phalloidin (arrowhead) could be identified under fluorescence microscopy. Many hair bundles disappear in the cultured utricle and scars (arrow) are present in the apical surface.

B. Same field from A. Some hair cell nuclei stained by propidium iodide have been lost in the gentamicin treated utricle and the number of hair cell nuclei decreases.

C. Nuclei of supporting cells appear less affected by gentamicin treatment.

Bar (A-C) =7 μm.

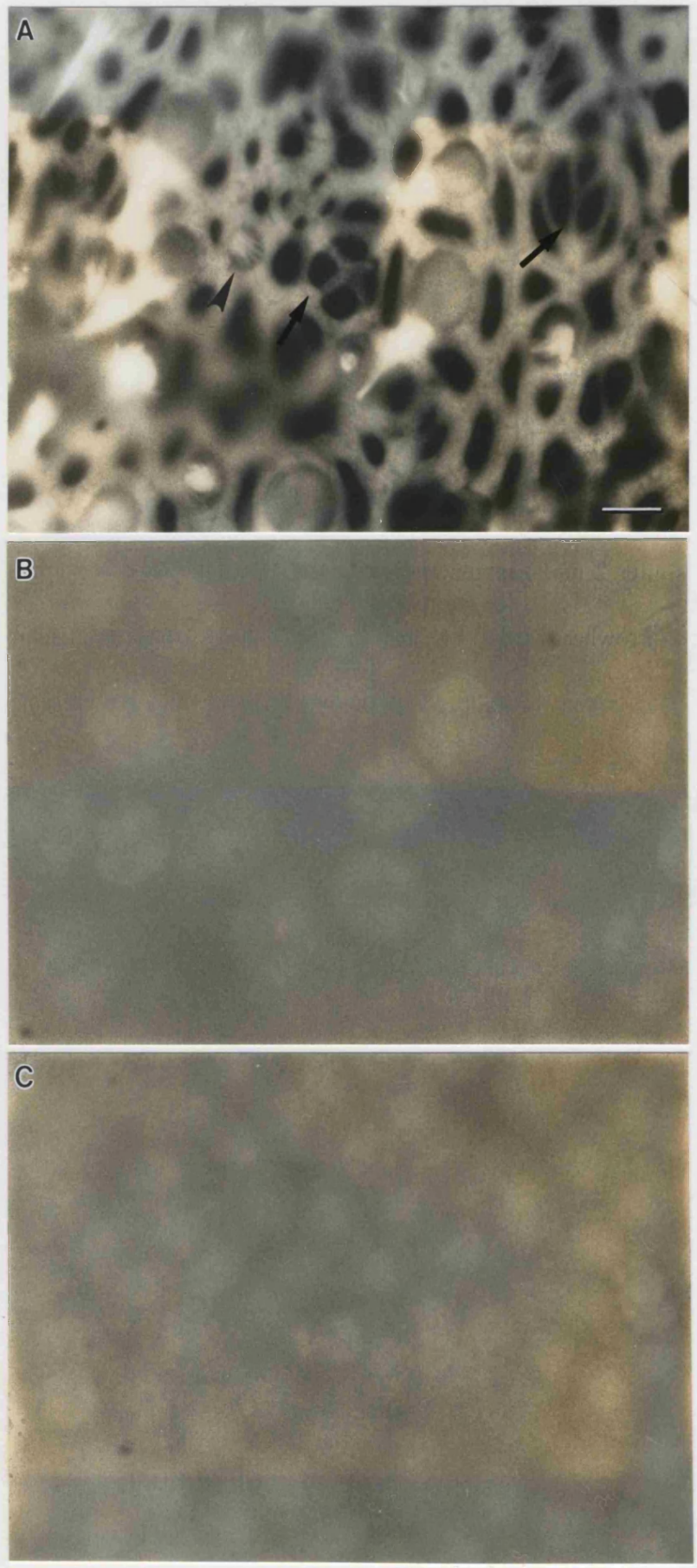
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Figure 5.51 In Situ End Labelling Apoptosis

A. Hair cell nuclei in a control utricle at 8 days in vitro. Under DIC microscope, hair cell nuclei of similar size appear in an even layer.

B. After 2 mM gentamicin incubation for 24 hours. Labelled apoptotic hair cell nuclei (brown colour) are found in the central area of the treated utricle.

C. In method control preparation, there is no labelling in the gentamicin treated utricular culture.

Bar (A-C) =7 μm.

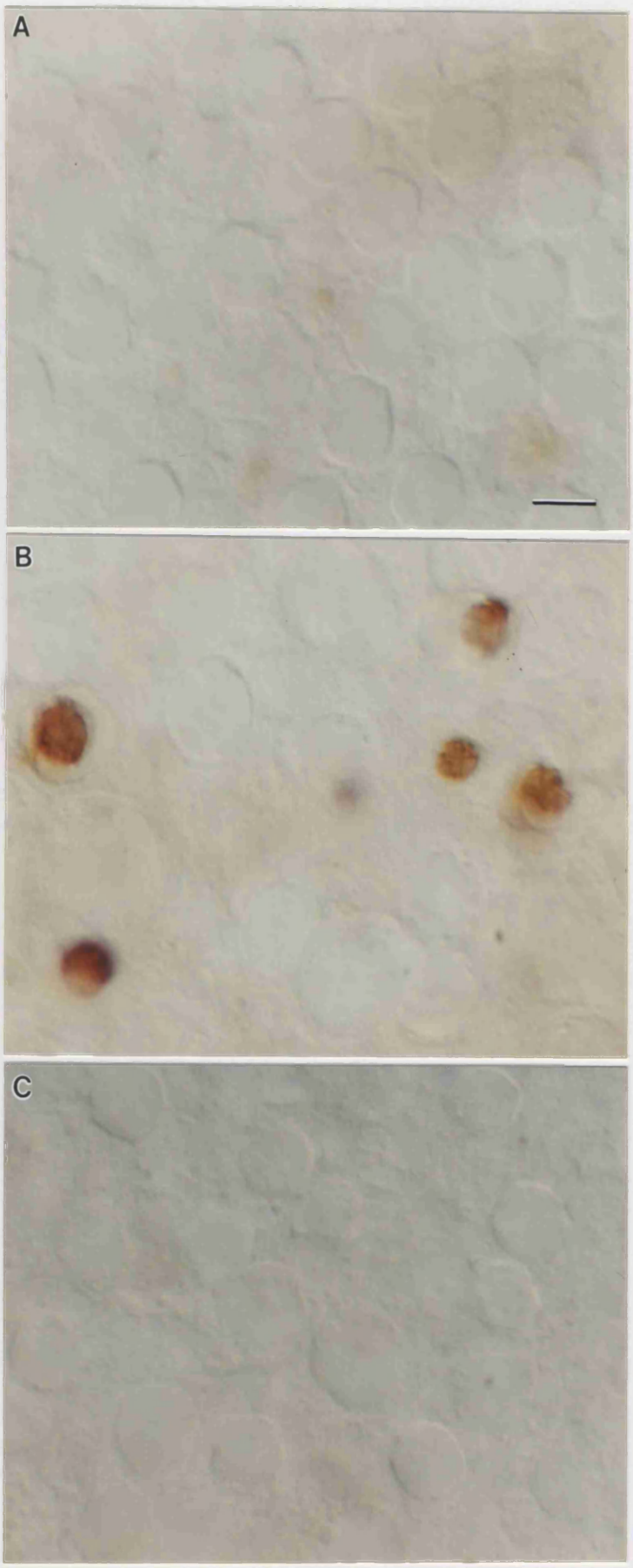
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Figure 5.52 Apoptotic Hair Cells Five Days Post-treatment in Vitro

A. Utricular culture after 2 mM gentamicin for 24 hours. Positively labelled hair cell nuclei show nucleus fragmentation and chromatin margination. Bar =7 μm.

B. Same field of A. Hair bundles are present on the cuticular plates of the surviving hair cells. Bar =7 μm.

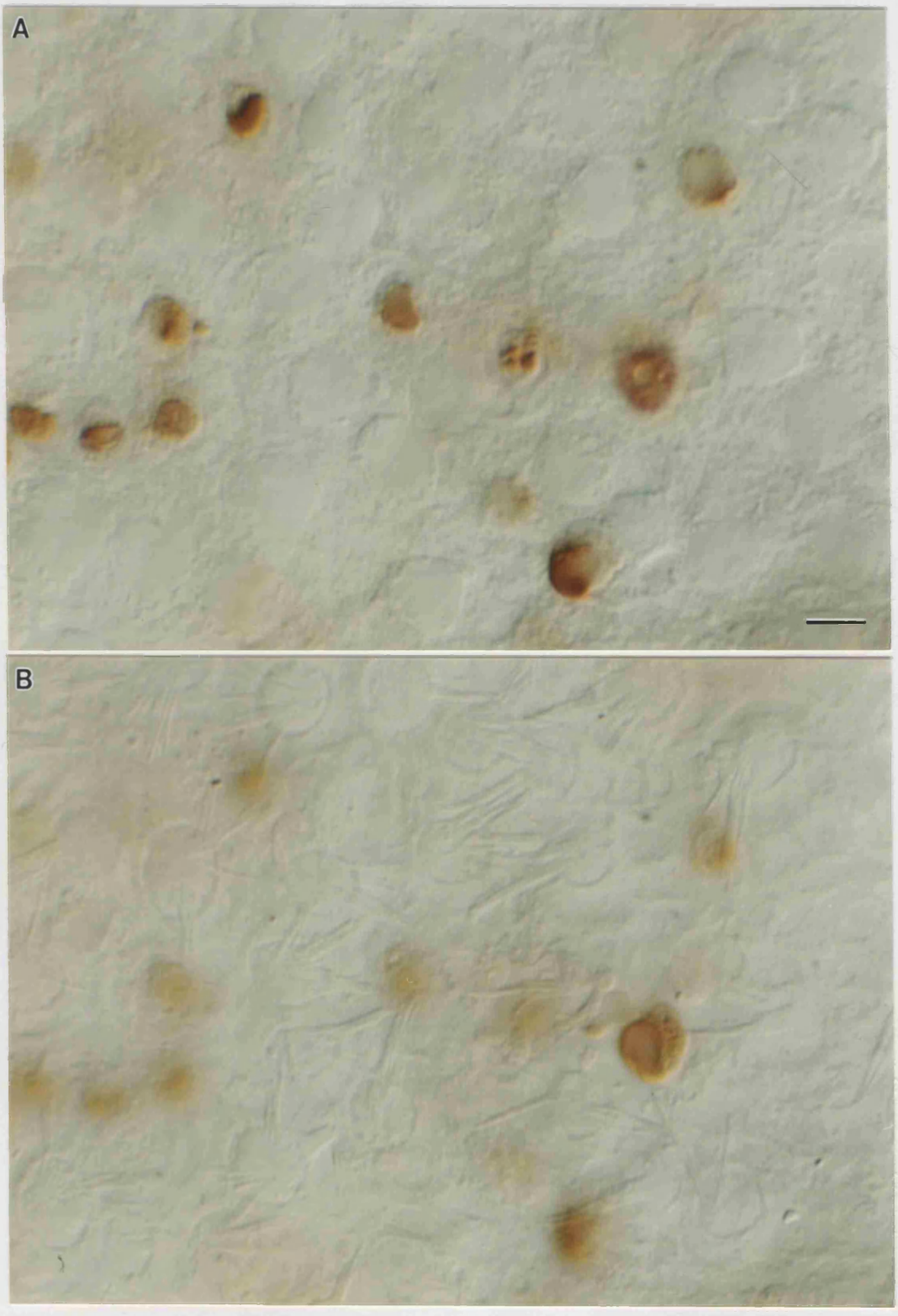
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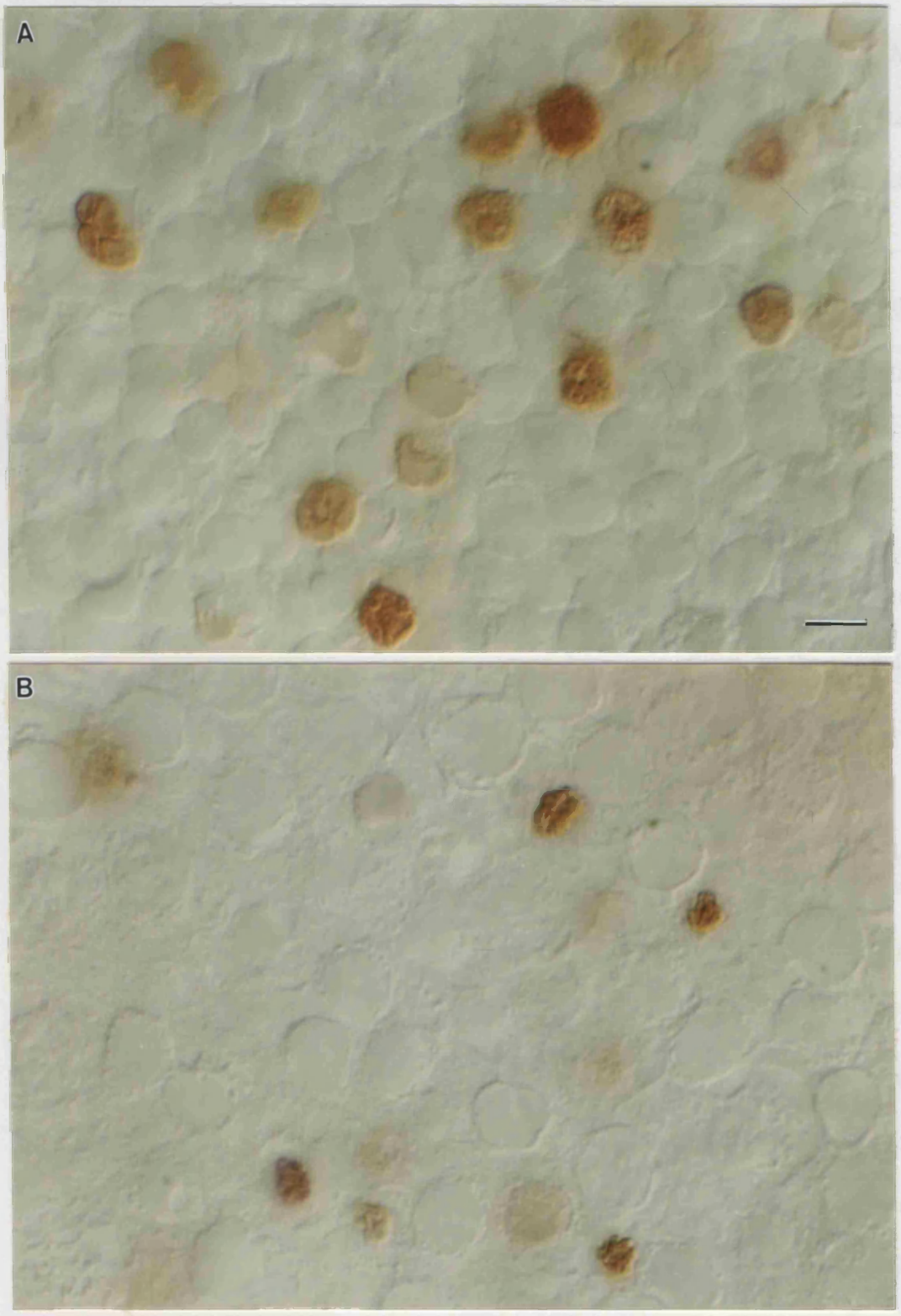
Figure 5.53 Apoptotic Hair Cells Five Days Post-treatment in Vitro

A. Apoptotic nuclei of centrally located hair cells are found at the lower level of the epithelium and overlap on the layer of supporting cell nuclei. The nuclei of supporting cells appear normal. Bar =7 μm.

B. Peripheral hair cells are less affected by gentamicin and a small number of apoptotic hair cell nuclei are positively labelled. Bar =7 μm.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CHAPTER 6: DISCUSSION

6.1 BRIEF SUMMARY OF THE RESULTS

Examination of the vestibular sensory epithelia of the mammalian inner ear using SEM, TEM, fluorescence microscopy and immunohistochemical labelling demonstrated: 1) hair cell degeneration induced by gentamicin treatment is mainly through apoptosis; 2) hair cell recovery occurs in the mammalian vestibular sensory epithelia following hair cell loss; 3) the organotypic culture system is a suitable model for examination of ototoxicity and the process of hair cell loss, epithelial repair and hair cell regeneration in the mature mammalian vestibular sensory tissues.

6.2 HAIR CELL DEGENERATION IN THE VESTIBULAR EPITHELIA

In this series of animal experiments, aminoglycoside gentamicin has been used to induce hair cell degeneration in the mammalian inner ear and its toxic effects on the vestibular sensory epithelia have been examined at various times both in vivo and in vitro.

6.2.1 Damage Pattern of Vestibular Sensory Epithelia by Gentamicin

Selective damage to the different sensory epithelia of the inner ear and the differential sensitivity in the same tissue of different hair cell types have been demonstrated in a number of different animal experiments (Wersäll and Hawkins, 1962; Lindeman, 1969a; Watanuki et al., 1972; Twine, 1985). Animal models serve a useful purpose. Although variation among animal species and among individual animals in response to ototoxic drugs is known, it is generally true that the pattern of damage in animals is very similar to the changes in the human inner ear following aminoglycoside exposure (Harpur, 1982;

Wright, 1986). In this work, progressive hair cell loss induced by gentamicin treatment occurs over a prolonged period and appears to show different sensitivity in the cristae, the utricles and saccules after chronic systemic gentamicin injection. Hair cell loss is also present in the organ of Corti examined by SEM, consistent with other studies concentrating on the cochlea (Forge, 1985; McDowell et al., 1989). However, the saccular maculae appear to be less affected by the systemic gentamicin treatment when other inner ear tissues from the same ear demonstrate hair cell loss. This result agrees with the reported results that the saccular maculae in mammalian inner ears are less sensitive to aminoglycosides than other vestibular organs (Lindeman, 1969a; Twine, 1985; Aran et al., 1995). There was only one guinea pig which survived 8 weeks after systemic gentamicin treatment, where hair cell loss was also found in the saccular sensory epithelia, however, the damage was much less than in other sensory epithelia. The different sensitivities could arise for a number of reasons: drug distribution could be different to the inner ear tissues; different anatomical factors, in particular the absence of dark cells in the saccular maculae; or an intrinsic difference in the susceptibility of hair cells in the different locations, in different sensory epithelia, and in different animals.

Using a different dosing route, topical application with gentamicin to the middle ear cavity, the saccular sensory epithelium suffered hair cell loss in a similar pattern to that in the utricular maculae, that is mainly in the striolar region. There was not complete destruction of hair cells in the vestibular and cochlear sensory epithelia after topical application examined by SEM and wax sections. Other studies of single or multiple topical application with drugs to the ear caused extensive hair cell loss, and showed that the ototoxic effect appeared more severe by topical application than systemic treatment (Dupont et al., 1993;

Kimura and Hashimoto, 1988; Rubel et al., 1995). The explanations may be due to the different aminoglycosides used, different drug dosages and different techniques. Considerable variability between individual animals makes it impossible to judge how much gentamicin enters the inner ear by systemic treatment. Topical application of the drugs to the ear represents an almost direct application to the inner ear sensory epithelia in vivo. This technique has been used by others (Rubel et al, 1995) and it is safe to animals and efficiently damages one inner ear sensory epithelia within a short time and allows the other ear to be used as a control.

Gentamicin also appears to selectively induce hair cell loss in the striolar regions of the maculae, in the central part of cristae and the basal turns of the cochlea by either systemic or topical gentamicin treatment in vivo. This regional difference to aminoglycoside gentamicin has been suggested to be related to the distribution of different hair cell types (Lindeman, 1969a; Wersäll et al., 1973), the centrally located type I vestibular hair cells being more susceptible to the aminoglycoside treatment than type II vestibular hair cells (Watanuki et al., 1972). However, it has been found in this work that after gentamicin treatment, degeneration and loss of both hair cell types occurred in the striolar regions of the maculae and in the central part of cristae. Both type I and type II vestibular hair cells in the periphery are also affected, but to a much lesser extent. Thin sections of the inner ear vestibular tissues clearly demonstrate morphological evidence of degeneration of type II hair cells in the central or striolar regions. In some cases, the hair cells completely disappeared from the area of hair cell loss in the vestibular sensory epithelia after gentamicin treatment.

The different susceptibility of the vestibular hair cells in the striolar regions of maculae and in the central part of the cristae to aminoglycosides is not very clear. The hair cell distribution in the vestibular sensory epithelia is not as regular as the cell distribution in the organ of Corti, where inner and outer hair cells are clearly separated according to their locations. Cochlear outer hair cells in the basal turn which are more susceptible to the aminoglycoside damages appear preferentially to uptake aminoglycosides (De Groot et al., 1990; Hiel et al., 1992) and the drug is detected by immunolabelling to be localized below the cuticular plate of outer hair cells (Aran et al., 1995). Freeze fracture analysis demonstrated that there are more endocytotic vesicles at the apical membrane of the outer hair cells in the basal turn (Forge and Richardson, 1993) which may determine the differential sensitivity of apical and basal turn hair cells to aminoglycosides. However, both vestibular type I and type II hair cells have been seen to uptake aminoglycosides (Aran et al., 1995). These studies suggest that there may be a direct result of intrinsic differences in the hair cells which vary with their types and locations in the cochlea and the vestibular sensory organs. It has been documented that a determining factor in the development of ototoxicity in the inner ear epithelia is a specific binding interaction between the aminoglycoside and phosphatidylinositol 4'5' bisphoaphate ($PhIP_2$) of hair cell membrane (Schacht, 1986; Schacht and Weiner, 1986; Henley and Schacht, 1988). There is other evidence that an active metabolism process is involved in the progression of ototoxic response to aminoglycosides in vivo (Huang and Schacht, 1990; Schacht, 1993). The different distribution of metabolizing enzymes involving the synthesis of the cytotoxin and its detoxification in different tissues and cells may determine the different sensitivity of ototoxicity. The toxicity of gentamicin may result from the chelation of iron and the consequent release of free radicals (Priuska and Schacht, 1995). Attenuation of gentamicin

ototoxicity by glutathione, iron chelators and other free radical scavengers have been reported and it may be possible that these agents either inhibit the formation of the cytotoxin or enhance its detoxification to effectively provide intervention against aminoglycoside toxicity (Garetz et al, 1994a; 1994b; Song and Schacht, 1996).

6.2.2 Modes of Hair Cell Loss

The morphological and immunolabelling results from the vestibular sensory epithelia of the mammalian inner ear showed that hair cells were lost inside these epithelia through apoptosis after gentamicin treatment. Hair cell extrusion to the apical surface, another mode of hair cell loss, has also been observed in the vestibular sensory epithelia. The extrusion of the unwanted hair cells from the vestibular epithelia involved the whole hair cell, including the cuticular plate, the hair bundle, the cytoplasm and the nucleus. It is interesting that those hair cells seen to be undergoing extrusion showed relatively little degenerative changes, based on TEM examination. When the entire hair cell is expelled from the sensory epithelium, the space left by the lost hair cell is sealed by the expansion of adjacent supporting cells. In all mammalian inner ear sensory organs examined by TEM in this study, the extrusion mode was only found in the vestibular sensory epithelia, not in the organ of Corti (Forge, 1985), although some debris remained on the reticular lamina of the organ of Corti from gentamicin treated animals. The structural organisation of the mammalian vestibular tissues is close to that of the inner ear tissues of lower vertebrates. These sensory epithelia are closely packed with hair cells and supporting cells, and the supporting cells closely surround each hair cell. In the avian inner ear and other hair cell containing epithelia of lower vertebrates, the extrusion mode of hair cell loss may be a predominant means to remove damaged hair cells (Cotanche, 1987; Marean et al, 1993;

Yan et al., 1991; Baird et al, 1993; Weisleder and Rubel, 1993). But, whether the morphological features of the extrusion process in the present study correspond to those observed in the lower vertebrates is not known. There do not appear to be any detailed studies of hair cell extrusion processes in the ear of the lower vertebrates.

In some vestibular tissues from control animals, both mature vestibular type I and type II hair cells (not the immature hair cells) showed cytoplasmic blebbing at their apical surfaces when examined by SEM and TEM. The surface blebs of the vestibular hair cells in control tissues were always found behind the hair bundle where the cuticular plate is free due to the existence of the kinocilium. This is a post-mortem artefact, similar to the cochlear hair cells when fixation is suboptimal (Forge et al., 1992). The vestibular organs are enclosed inside the vestibule and the fixative is difficult to get into the vestibular tissues if the site is not widely opened.

In the mammalian organ of Corti, the bodies of the damaged hair cells degenerate within the sensory epithelium, and the apical parts of the hair cells retained in the reticular lamina until the supporting cells expand, and replace the sites of the damaged hair cells (Forge, 1985; McDowell et al., 1989). From this study, damaged hair cell bodies also degenerated inside the vestibular sensory epithelia (Li at al., 1995) and the cell apical surfaces looked normal. The sensory epithelium remained intact as the cuticular plate of the degenerated hair cell was closely connected by the tight junctions with the supporting cells. As the degenerated cuticular plate was lost, the apical parts of the supporting cells expanded to seal the lumenal surface so that an undisrupted barrier was maintained. When the degenerated hair cell body was fragmented and disappeared, the long narrow supporting

cell body expanded to occupy the space of the lost hair cells. These characteristics suggest that there is an internally controlled process of repair in the inner ear vestibular sensory epithelia, as in the organ of Corti where outer hair cell loss is closely coupled to supporting cell expansion after gentamicin treatment (Forge, 1985). From thin sections of the vestibular specimens of gentamicin treated animals, tight junctions have been found intact at the region of hair cell loss and they form a new sealing between supporting cells without the involvement of hair cells, similar to previous work on the organ of Corti (McDowell et al., 1989; Raphael and Altschuler, 1991a; 1991b).

There are two typical cellular degenerating processes in tissues, apoptosis and necrosis. Apoptosis is associated with programmed cell death, and it is considered as a common process to remove unwanted or damaged cells in a wide range of tissues without disturbing tissue integrity or inducing inflammation (Alison and Sarraf, 1992; Kerr et al., 1972). Apoptosis is an active regulatory response by inducible cells to a specific inducing stimulus and endonucleolysis is considered as the key biological event of apoptosis, resulting in cleavage of nuclear DNA into fragments. In general, cells undergoing apoptosis display some characteristic structural changes in the nucleus and cytoplasm. The morphological features of apoptosis are that cells die individually, their cytoplasm shrinks with intact organelles, their nuclear chromatin condenses and fragments, and "apoptotic bodies" are formed and are phagocytosed by adjacent normal cells or macrophages. Also, apoptotic cell death occurs without stimulating an inflammatory response in the tissues (Kerr et al., 1972). In this study, hair cell degeneration inside the vestibular sensory epithelium shows these morphological characteristics of apoptosis. There was no evidence of oedema or disturbance of the vestibular sensory epithelium architecture when hair cell

loss occurred indicating that hair cell loss does not stimulate an inflammatory response in the vestibular sensory epithelia. These results suggest that apoptosis may be the predominant mode of hair cell death following gentamicin injury. In thin sections of control tissues, a small number of darkly stained apoptotic like hair cells could also be found at a very low level indicating hair cell apoptosis may occur naturally to remove unwanted, damaged or aged cells in the inner ear tissues.

To further confirm this hypothesis that gentamicin trigger apoptosis, in vitro studies were performed. This allowed direct comparison between different mammalian species to examine the generality of the phenomenon. The same responses to gentamicin treatment were found in the guinea pig and gerbil vestibular cultures and the morphological features characteristic of those described for apoptosis in vivo appeared in thin sections of the utricular cultures from both species examined. The presence of the hair cells protruding to the lumenal space above the epithelium in vitro, similar to the other mode of hair cell loss in vivo, was also observed, but less commonly.

6.2.3 Apoptosis Labelling

Morphological description of hair cell death through apoptosis had also been examined in the cultured utricles using propidium iodide and in situ end labelling methods. The nuclei of apoptotic cells stained by propidium iodide are brighter showing condensation of chromatin and fragmentation which has been used to identify apoptotic nuclei in other cell types (Jacobson et al., 1993). Propidium iodide staining also provides a means of assessing cell numbers by evaluation of the nuclear numbers. In gentamicin treated cultures, hair cell nuclei brightly labelled by propidium iodide were mainly found in the striolar (central)

regions and the size of most stained nuclei was small, suggesting condensation. Some hair bundles stained by phalloidin still remained at the apical surfaces of those hair cells with brightly labelled cell nuclei. This result supports the findings from thin sections where hair cell nuclei appeared degenerated but the cuticular plate with the hair bundle was present. When the hair cell nuclei became fragmented or disappeared, the associated hair bundles disappeared as well and scars were present at the sites. The fluorescence staining result corresponds to the morphological features of apoptosis in thin sections examined by TEM and is in agreement with the apoptotic nuclear changes of other cell types by fluorescence microscopy (Jacobson et al., 1993; Didenko and Hornsby, 1996).

In situ end labelling (ISEL) using ApopTag kit has been reported to detect apoptotic cells only (Didenko and Hornsby, 1996; Hungerford et al., 1996). This ISEL method utilizes terminal deoxyribonucleotidyl transferase (TdT) to detect the free 3'-OH ends of double or single stranded DNA generated during apoptosis that are not present in normal or proliferative nuclei. Visualization of focal in situ labelling inside intact apoptotic nuclei exactly correlates with the morphological characteristics of apoptosis observed from thin sections and propidium iodide labelling. It is difficult to determine hair cell types from whole mount surface preparation using ISEL method or propidium iodide to detect apoptosis. Further experiments on sections are planned to investigate whether both types of vestibular hair cells degenerate through apoptosis. Apoptotic hair cell death has also been found in the organ of Corti of guinea pigs exposed to noise (Niedermeyer et al., 1997) and in the cochleae and saccular maculae of ageing mice (Usami et al., 1996). These studies are consistent with the finding in this study that hair cell death in the mammalian inner ear is through apoptosis.

6.2.4 Roles of Supporting Cells after Gentamicin Treatment

In this work, the degenerating changes were mainly found in hair cells, and supporting cells appeared to be more resistant to gentamicin induced apoptosis than hair cells in the vestibular sensory epithelia both in vivo and in vitro. As hair cells degenerated, the supporting cells in the vestibular sensory epithelia become active to expand themselves to form scars. Neither modes of hair cell loss and replacement by the adjacent supporting cells caused an inflammatory response inside the sensory epithelium and there were no obvious lesions formed at the apical surface. Supporting cells could be observed to expand at their apical surface, re-organize the apical intercellular junctions during hair cell degeneration, increase the volume of the cell body, and their nuclei to migrate towards the lumenal surface of the epithelium. When degenerated hair cells disappeared, the supporting cells completely occupied the space inside the vestibular sensory epithelium. Using fluorescent phalloidin labelling to detect actin in the vestibular tissues, the hair bundles and the cuticular plates disappeared after gentamicin treatment. The outlines of the supporting cell actin ring associated with the intercellular junctions remained and became wider, and their apical surfaces were enlarged. The process of scar formation in the vestibular sensory epithelia both in vitro and in vivo could be seen immediately after gentamicin treatment in the site of hair cell loss and all supporting cells that surrounded a degenerating hair cell contributed to scar formation, similar to the results from the organ of Corti and the vestibular epithelia (Raphael and Altschuler, 1991a; 1991b; Meiteles and Raphael, 1994b; Wersäll et al., 1973). It suggests that supporting cells are actively involved in reparative processes during hair cell degeneration inside the vestibular sensory epithelia.

It should be emphasized here that aminoglycosides are a specific killer of hair cells in the acousticolateralis organs. Hair cells from the mammalian organ of Corti (Forge, 1985; McDowell, 1982; Raphael and Altschuler, 1991a; 1991b), the vestibular cristae and otolithic macular organs of mammals, birds, amphibians and fish (Baird et al., 1993; Lindeman, 1969a; Watanuki et al., 1972; Wersäll et al., 1973; Meiteles and Raphael, 1994b; Weisleder and Rubel, 1993; Rubel et al., 1995), the avian basilar papilla (Cruz et al., 1987; Hashino et al., 1991; Janas et al., 1995), and the neuromast of the lateral line system (Song et al., 1995) are primary targets for the drug action. In this series of animal experiments, using thin sections for TEM, phalloidin labelling of fluorescence microscopy and in situ end labelling, supporting cells showed no sign of apoptotic degeneration. However, apoptotic bodies from fragmented hair cells were seen inside supporting cells in both in vivo and in vitro studies suggesting that supporting cells may be able to eliminate degenerated hair cells by phagocytosis.

The general process of structural repair by supporting cells appears to be the same in all hair cell containing sensory epithelia. In lower vertebrates, supporting cells participate in scar formation to effect repair during hair cell degeneration and produce new hair cells for regeneration. Therefore, it is important that undamaged supporting cells in the sensory epithelia could control the repair of lesions effectively in the process leading to hair cell regeneration. Supporting cells also appear to be very important for subsequent hair cell recovery and regeneration processes in the mammalian vestibular sensory epithelia.

6.3 HAIR CELL RECOVERY IN THE VESTIBULAR EPITHELIA

One important finding in this work is evidence that hair cell recovery occurs in the vestibular sensory epithelia of the mature mammalian inner ear after hair cell loss induced by gentamicin treatment. Redevelopment of immature hair cells in the mammalian vestibular sensory epithelia (Forge et al., 1993) indicates that hair cell regeneration may not be restricted only to birds, amphibians and fish. The morphological reappearance of immature hair cells and labelling of vestibular supporting cell nuclei with proliferation marker, as found in this study, have subsequently been reported in vestibular organs of guinea pigs, humans and other mammalian animals (Warchol et al., 1993; Lambert, 1994; Rubel et al., 1995; Yamashita and Oesterle, 1995; Yamane et al., 1995). Taken together, these results suggest that the post-embryonic production of mammalian vestibular hair cells may be possible.

6.3.1 Morphological Evidences

Recovery of hair cell numbers after loss, and hair cell development are the main anatomical indications of repair and regeneration processes in the inner ear. Following gentamicin treatment, hair cell loss occurred and subsequently small and short, immature like stereociliary bundles appeared in the vestibular sensory epithelia in mature guinea pigs. Counts of the total number of hair cells and of the immature like hair bundles in the vestibular sensory epithelia, strongly suggest that hair cell recovery occurs in the mammalian vestibular organs. After an initial hair cell loss at early time post-treatment, hair cell numbers recovered significantly between one and three months and this phenomenon was found in all work from several separate experiments carried out at different times. In general, the hair cell numbers (Table 5.5) at three key time points were

all significantly different from each other: 1) the hair cell number fell to nearly 40% of normal hair cell numbers in the striolar region of the utricle at 1-2 weeks post treatment; 2) an increase of hair bundles to about 60% of the control number occurred as immature hair bundles emerged in the hair cell loss area at 4 weeks post-treatment; 3) at 12 weeks (three months) post-treatment, hair cell numbers in the treated group showed a further increase to nearly 70% of normal control hair cell number. The result (Table 5.7) from topical application with gentamicin to guinea pigs also showed a similar extent of hair cell recovery. Hair cell loss and recovery in the saccular maculae after topical gentamicin treatment was similar to the course in the utricular maculae. Statistical analysis supports the trend of continuously increasing hair cell numbers. However, the hair cell number at 12 weeks, the highest number found in the whole series of experiments and nearly double the hair cell number of the early group, is significantly lower than the mean number of hair cells in the control group, indicating that hair cell recovery is incomplete. Partial recovery has been found in birds after noise exposure. Up to 15 days survival, the basilar papilla returned to a nearly normal appearance by scanning electron microscopy, but the number of new hair cells was approximately 22%, of the hair cells number lost (32%) within the lesion immediately following 48 hours exposure (Marsh et al., 1990).

Hair cell recovery in the guinea pig vestibular sensory epithelia appears to be derived from immature hair cell development. In control tissues immature hair bundles were very rare (Table 5.3). In contrast, about 30% of hair bundles with the easily recognizable and distinctive morphology of the most immature forms were found in the striolar region of the utricles from treated animals. This large difference confirms that immature hair bundles appear in the sensory epithelia after gentamicin treatment. As survival time increased, the

number of immature hair bundles declined, but the number of short and mature hair bundles increased, indicating that the immature hair bundles continued to develop towards maturity. From thin sections, the hair cells with immature or short hair bundles resembled type II hair cells without an afferent nerve calyx.

From the Tables 5.10 and 5.11 (data from Dr Forge), there are 40% more type I hair cells than the type II hair cells in thin sections of the striolar region of the control utricular maculae. In gentamicin treated utricular maculae, the number of type I hair cells in the striolar region decreased significantly and it did not significantly increase during the recovery period, in agreement with other reports that type I hair cells are more sensitive to aminoglycosides (Lindeman, 1969a; Wersäll et al., 1973; Watanuki et al., 1972). Although type II hair cells were also found to degenerate, the number of type II hair cells did not significantly decrease at one week post-treatment. The reason may be related to the slow process of hair cell degeneration after gentamicin treatment and some hair cells still remained inside the sensory epithelia. The SEM results also showed that the area of hair cell loss continued to extend towards the periphery at two to four weeks post-treatment and in the meantime, immature hair bundles appeared. Another possibility is that immature hair cells with the characteristics of type II like hair cells emerged in the area soon after hair cell loss. More type II hair cells were present in the sensory epithelia by four weeks post-treatment and the number of hair cells gradually recovered. During the period of observation up to 33 weeks post-treatment, the trend of hair cell number decrease followed by an increase as seen in thin sections, was consistent with the SEM results. It provides further evidence that damaged hair cells disappear from the sensory epithelia after gentamicin treatment, and replaced by a new population of hair cells, morphologically type II hair cells. Development of type II like hair cells is triggered by hair cell loss. However, the hair cell numbers from both TEM and SEM fail to show any further increase between 12 weeks and 33 weeks and are lower than the control number.

6.3.2 Functional Evidence

The immature hair cells could restore the hair cell numbers and further development of these cells could also provide a basis for functional recovery. From this morphological study, at least some of the new hair cells became innervated by 12 weeks and 33 weeks after gentamicin treatment, making it likely that they may contribute to a recovery of sensory function. Can immature hair cells fully develop towards maturity and function? There was no physiological assessment of the animals showing hair cell recovery in this study. The incomplete hair cell recovery in the vestibular sensory epithelia of mammalian animals may make the functional recovery test related to hair cell regeneration more difficult. However, it has been reported that degeneration of vestibular hair cells with disappearance of post-rotatory nystagmus responses after streptomycin treatment of guinea pigs was followed by recovery of the functional responses, but whether this is related to vestibular hair cell regeneration cannot be stated (Meza et al., 1992).

6.3.3 Proliferation of Inner Ear Sensory Epithelia

The re-development of immature hair cells has been extensively examined in this work by BrdU labelling and other microscopic methods. The BrdU immunolabelling results presented in this in vivo study confirm the reports that cellular proliferation is also stimulated in the mature mammalian vestibular sensory epithelium following hair cell loss induced by aminoglycosides (Warchol et al., 1993; Goldstein et al., 1994; Rubel et al., 1995). A small number of BrdU positive labelled nuclei were found in wax sections of the gentamicin affected utricles at the supporting cell level and more lumenal, at the hair cell level after two weeks of continuous infusion of BrdU. One utricle (two weeks after systemic gentamicin treatment) also showed positive BrdU labelling in the whole mount

preparation of the utricular sensory epithelium. The number of proliferating cells observed in the vestibular sensory epithelium both by systemic intraperitoneal BrdU injection or by implantation of the osmotic pump studies was, however, considerably less than the immature hair cells seen by SEM.

It is possible to assume that the procedures used here may underestimate the number of cells which entered S-phase of the cell cycle in the vestibular sensory epithelium. Based on morphological evidence obtained in vivo and the data of hair cell number recovery, it appears that there is a considerable delay in hair cell redevelopment. Hair cell number does not increase significantly in damaged guinea pig utricular maculae until about four weeks post-treatment (Forge et al., 1993; Table 5.6). Subsequently, hair cell numbers increase further to reach the highest number by 12 weeks. Thus, a two week period of BrdU infusion may not be long enough to "capture" proliferative events. Hair cell degeneration was still a predominant feature at two weeks time after gentamicin treatment (Tables 5.2, 5.7), although SEM shows that immature hair bundles were present at two weeks after systemic gentamicin treatment. It is difficult to judge the origin of BrdU labelled nuclei at the hair cell level. They might be immature hair cells, but it is more likely that they were to be the nuclei of supporting cells that had migrated towards the lumenal surface, as was seen to occur in TEM thin sections. This result is in agreement with an in vivo study in which osmotic pumps were used to deliver tritiated thymidine to gentamicin topically treated animals (Rubel et al., 1995) and proliferation marker was found only in the nuclei of supporting cells. Longer term continuous delivery of BrdU has not been performed in this present work, but Rubel and colleagues reported that only a few labelled supporting cells could be detected in the gentamicin affected vestibular epithelia after six weeks

continuous delivery of tritiated thymidine into the ear (Rubel et al., 1995). Their results might be influenced by the extensive hair cell loss on the treated utricles and it appears that far more damage of hair cells may affect the proliferative regeneration. However, another in vivo study showed that one or two months after 30 consecutive days of systemic gentamicin treatment, BrdU labelling appeared in the lower and the lumenal portions of the crista sensory epithelia of guinea pigs (Yamane et al., 1995).

In comparison with the use of tritiated thymidine autoradiography, BrdU is not radioactive and can be identified and examined rapidly by immunohistochemical methods. The principle underlying assessment of cell proliferation by immunohistochemical methods is that there are enough cellular proteins and other molecules that could be recognised as antigens. The antigen may exist in the cells as a consequence of exogenous administration, like BrdU incorporated into DNA during S phase (Gratzner, 1982). Immunohistochemical detection and quantification of BrdU in the inner ear tissues depend upon several factors. First is the distribution of the BrdU and subsequent incorporation into the inner ear cells. The affinity and specificity of the monoclonal antibody, cellular fixation method, DNA denaturation, because anti-BrdU antibody binding requires BrdU exposure in single stranded DNA, and the stoichiometry of the immunohistochemical staining reaction are key steps for this reaction (Dolbeare, 1995). Any non-optimal procedure could impede the results of immunolabelling.

Great efforts were made during this work to optimise BrdU labelling procedures and many factors might have influenced the present results. However, different routes of BrdU delivery to animals, different specimen preparations (whole mount preparation and wax

sections), different HCl concentrations, treatment times and temperatures for DNA denaturation, different antibody concentrations and incubation conditions, DAB development of peroxidase and haemotoxylin counterstain, were all tested. Furthermore, there was extensive positive BrdU labelling in both the small intestine, where continuous cell proliferation normally occurs, and in the liver where cell proliferation also normally occurs at a relatively low rate. In addition, BrdU labelling was found in the nuclei of the connective tissues underlying the inner ear sensory epithelium with different methods of BrdU administration and different preparation techniques used. These BrdU labelling control results indicate that BrdU entered into different tissues in the body and the immunohistochemical labelling procedure used in this work was adequate.

Recovery of hair cell numbers after hair cell loss occurred in the inner ear vestibular sensory tissues, but BrdU labelling showed inefficient proliferative regeneration. It is possible that BrdU enters the inner ear sensory epithelium less readily than into other tissues because there is no direct blood supply inside the sensory epithelial layer, or that mammalian inner ear hair cells and supporting cells incorporate BrdU more slowly or less than other cell types. BrdU was also directly applied to the cultured mammalian vestibular utricles after gentamicin treatment in vitro and mitotic activity was noted mainly in the nuclei of marginal tissues showing BrdU positive labelling in both guinea pig and gerbil cultured utricles. No convincing positive BrdU labelling was found inside the sensory epithelium, although some morphological evidence demonstrated immature like hair cells in the cultured vestibular tissues (Li and Forge, 1995). This result contrasts with the initial work on cultured guinea pig and human utricles (Warchol et al., 1993) and cultured mouse utricles and cristae (Lambert, 1994), both of which reported that proliferation of

supporting cells appeared in the cultured tissues. The reasons for the difference may be related to the extent of hair cell loss, or that supporting cell proliferation had not been stimulated in the sensory epithelia, or the timing of study. There was another technical consideration that only a few thin sections from each gentamicin affected utricles were processed for immunoelectron microscopy, so that the result did not take account of the whole tissue. It is possible that more extensive thin sections for TEM (which would be very time consuming and expensive) might have revealed positive labelled nuclei in the vestibular sensory epithelia.

Nevertheless, the result reported here, at least would suggest that proliferation is involved in the hair cell recovery process but the extent is insufficient to account for all the immature hair cells which were seen by SEM and TEM. This result raises the possibility that mechanisms other than proliferative regeneration may also be invoked after drug induced hair cell damage. Proliferation might not be the only process involved in the appearance of new hair cells in the vestibular sensory epithelia of the mammalian inner ear.

6.3.4 Mechanism of Hair Cell Regeneration

Structural re-organization in the mammalian vestibular sensory epithelia following aminoglycoside induced hair cell damage includes supporting cell expansion to replace the lost hair cells (Meiteles and Raphael, 1994b; Li et al., 1995) and development of immature hair cells (Forge et al., 1993; Rubel et al., 1995). In addition to proliferative regeneration, there are several other sources which could also be considered as theoretical mechanisms accounting for the appearance of the immature hair bundles: 1) stereocilia bundles are damaged but hair cells still remain alive, followed by repair of the apical surface; 2) cell

migration from other regions; or 3) derived from a process of cellular transformation in which some non-sensory cells inside the epithelium convert to hair cells.

It is possible that some immature hair cells are injured hair cells but non-lethally killed and then can repair themselves. If this occurs, then the damaged hair cells must become de-differentiated to appear like non-sensory cells then differentiated as hair cells. There is no evidence consistent with hair cell dedifferentiation followed by differentiation in this study and no such report has been published on mature inner ear sensory epithelia. Repair of the apical surfaces of immature cochlear hair cells after the mechanical injury has been observed in cultured explants (Sobkowicz et al., 1996). But in this case, only the cell apices were injured and the cells retained their differentiated morphology. This present work has demonstrated that gentamicin induced hair cell loss in the mature vestibular sensory epithelia is predominant through apoptosis, which is different from the mechanical damage to the hair bundles.

No evidence has been reported that other types of cells migrate inside the sensory epithelium in mammalian inner ear, although it could happen in lower vertebrates (Girod et al., 1989). In this work, the immature hair bundles were found in the striolar/central regions (where hair cell loss occurred) of the sensory epithelia, not in the periphery. Morphological study could not find other cell types inside the vestibular sensory epithelium. The non-sensory cells within the avian inner ear tissues and in the mammalian vestibular epithelia are usually classified as a single population of supporting cells. Thus, the most likely explanation for the appearance of immature hair cells is the supporting cells in the sensory epithelia. Hair cell loss can trigger supporting cell proliferation and

differentiation both in vitro and in vivo (Corwin and Cotanche, 1988; Corwin and Warchol, 1991; Ryals and Rubel, 1988; Girod et al., 1989; Hashino and Salvi, 1993; Raphael, 1992; Raphael et al., 1994b; Raphael et al., 1996; Roberson et al., 1992; Stone and Cotanche, 1992; Weisleder and Rubel, 1992; Tsue et al., 1994b; Warchol et al., 1993; Lambert, 1994; Rubel et al., 1995; Yamashita and Oesterle, 1995). In general, the extent of proliferative regeneration is low. It is possible that some supporting cells may not need to go through mitosis, but may directly transdifferentiate into hair cells without divisions (Raphael et al., 1994; Adler and Rapheal, 1996; Roberson et al., 1996; Baird et al., 1996). Quantitative analysis of the hair cell number labelled with tritiated thymidine suggested that not all immature hair cells arise from supporting cell mitotic proliferation and direct transdifferentiation of supporting cells gives rise to about one third of new hair cells in the regenerating gentamicin affected avian basilar papilla (Roberson et al., 1996). Such cell conversion has been suggested as a mechanism for hair cell production in the embryonic organ of Corti of mice after laser ablation (Kelley et al., 1995). It is also thought to be the predominant mechanism for the production of hair cells in the amphibian vestibular sensory epithelia after gentamicin induced hair cell loss (Baird et al., 1993; 1996).

Considering the low level of proliferation and significant hair cell recovery observed in this study, the most likely interpretation of some of those cells showing immature hair bundles is that they arise through supporting cell conversion. In continuing this work, some morphological evidence has been obtained (Forge et el., in press) suggesting the possibility that supporting cells may directly convert into hair cells. Some cells have been found in the gentamicin affected utricles showing the features of both hair cells and supporting cells, being innervated and having immature stereocilia but still in contact with or apparently just

detaching from the basement membrane. Further examination by applying markers specific for hair cells or supporting cells to identify cells expressing some of both sets of markers would provide a further means to confirm the cellular transformation (Stone et al., 1996; Baird et al., 1996).

There must be an intercellular signal system to control cell proliferation, cell differentiation and cell survival throughout life in normal and in traumatized inner ear sensory epithelia. The mammalian vestibular sensory epithelia may have a similar process to the avian inner ear in that hair cell loss stimulates undamaged supporting cells in the epithelium to proliferate with subsequent differentiation to hair cells or they may directly convert to hair cells without intervening mitosis. This trigger mechanism is acting during hair cell regeneration and persists until recovery is nearly complete. One hypothesis is that the loss of hair cells may reverse an inhibitory influence on progenitor cells locally, allowing them to re-enter the cell cycle (Corwin et al., 1991) or re-differentiate as hair cells. When the adhesive intercellular junctions among the hair cell and the surrounding supporting cells are broken, the receptor sites of the supporting cells become open, which could provide a potential signal that no hair cell is in contact with those supporting cells and that the epithelium requires restoration (Corwin and Warchol, 1991). In this study, immature hair cells were found mainly in the area of hair cell loss and significantly increased at two weeks after systemic gentamicin treatment, suggesting that hair cell loss triggers the supporting cell repair and regeneration processes.

An unexpected finding of the BrdU labelling study was up-regulation of cell proliferation in the connective tissue underlying the gentamicin affected vestibular sensory epithelia. The

cell types labelled with BrdU in the connective tissue were not identified but the number of positive labelled nuclei was greater than the number in the connective tissue underlying the sensory epithelia from undamaged control ears. Stimulation of cell division in the spiral ligament, spiral limbus and stria vascularis outside the sensory epithelium has been found in the gerbil cochlea after acoustic trauma (Roberson and Rubel, 1994). These observations suggest that mitogenic factors may be released locally from the damaged sensory epithelia that influence mitotically competent cells elsewhere to proliferate, though the connective tissue might be damaged by gentamicin and produced the mitogenic factors itself or secreted from macrophages which have been activated and attracted to the damaged epithelium (Jones and Corwin, 1996). The existence of soluble mitogenic substances in the damaged avian inner ear epithelium, which can diffuse to reach and stimulate mitosis in progenitor cells has been proposed (Tsue et al., 1994a; 1994b; Warchol and Corwin, 1996). Culturing streptomycin damaged utricles with undamaged utricles, led to an increase of proliferating cell numbers in the undamaged tissue and culturing of several undamaged utricles together led to a decrease in the proliferating cell numbers. This study supports the hypothesis that soluble mitogenic control factors and soluble inhibitory factors produced by the sensory epithelium may be able to act at a distance to stimulate or suppress cell proliferation in normal tissue.

6.4 ORGANOTYPIC CULTURE SYSTEM

It is important to establish an in vitro system to study vestibular hair cell degeneration and recovery processes, in which the conditions can be carefully controlled. This study has shown that explanted mature mammalian vestibular tissues can survive up to two weeks in culture. This provides a means for direct examination of the vestibular sensory epithelia

under controlled and easily manipulated conditions. From thin sections, hair cells in the cultured vestibular sensory epithelium lost innervation and were surrounded by supporting cells without neural elements at 2-3 days in vitro, the earliest time examined. This is similar to the tissue cultures of avian inner ear sensory epithelia, where no innervation was present at two days in vitro (Oesterle et al., 1993). These results indicate that loss of hair cell innervation is rapid in vitro, and the vestibular sensory epithelia can survive in vitro without factors produced by their synapsing neurons. The vestibular hair cells including hair bundles and supporting cells remain intact in the tissue cultures of mature vestibular sensory epithelia despite loss of innervation.

The cultured mature macular sensory epithelia were directly affected by gentamicin in vitro and progressive loss of hair cells began in the striolar region and extended towards the periphery, similar to the pattern of hair cell loss in vivo when gentamicin was given to animals systemically or locally. The explants of vestibular sensory tissues were cultured at the defined time under the same incubation conditions, but hair cells in different locations showed the differential sensitivity to gentamicin treatment. The differential sensitivity to aminoglycosides found from hair cells in vitro appeared to be related to inherent properties of hair cells in the sensory epithelia rather than pharmacokinetic features of aminoglycosides. The differential uptake of drugs by hair cells in the different sensory epithelium may be associated with the different activities in hair cells (Aran et al., 1995). Studies on neonatal mouse cochleae incubated with aminoglycosides for short time demonstrated a similar result to this work: that the outer hair cells in the basal turn were damaged while those in the apical turn were not affected by aminoglycosides in vitro (Richardson and Russell, 1991; Kotecha and Richardson, 1994). The culture system is

useful to compare the effects of the direct application of drugs in vitro in an effort to identify the initial effects of aminoglycosides upon the hair cells and other factors that might influence the development of the ototoxic response.

The morphological and immunolabelling evidence obtained in this in vitro work support the idea that degeneration and loss of hair cell induced by gentamicin is mainly through apoptosis. Apoptosis, the morphological manifestation of programmed cell death, commonly occurs in the developing embryo, in normal healthy adult tissues and in many pathological conditions. Apoptotic degeneration is triggered at the end of a cascade of biochemical reactions which may occur very quickly and DNA fragmentation in apoptotic cells is followed by cell death and removal from the tissue within a short time (Bursch et al., 1990; Alison and Sarraf, 1992). Once cells are triggered to undergo apoptosis, the process is thought to be irreversible. Only a small number of labelled apoptotic hair cell nuclei were found in the control cultured utricles providing biologically significant data. The direct experimental manipulation, tissue environment and nutrient medium may be varied from in vivo conditions and that may in some extent affect the hair cells of the cultured vestibular tissues. The percentage of positively labelled hair cell nuclei in gentamicin treated cultures reached up to 20% of the total number of hair cell nuclei (Table 5.13) counted per field compared with under 5% in control tissues indicating that gentamicin is an apoptosis trigger and that it triggers hair cell death. In apoptotic labelling, apoptotic hair cell nuclei were labelled immediately after completion of the required gentamicin treatment. Up to 5 days in vitro after treatment, more hair cell nuclei disappeared. From thin sections of gerbil cultured utricles, apoptotic hair cells could be identified after 6 hours gentamicin treatment and most hair cells disappeared from the

sensory epithelium examined at 13 days post-treatment. Apoptotic hair cell nuclei were positively labelled more centrally rather than peripherally in the same cultured utricle indicating a similar pattern of hair cell loss to that occurring in vivo. Supporting cells of the cultured vestibular sensory epithelia appeared to be resistant to gentamicin and showed no sign of degeneration up to two weeks in vitro. Their responses to hair cell loss induced by gentamicin is precisely similar to that which occurs in vivo.

In addition, the culture system may also help in understanding regeneration for investigating hair cell recovery in the mammalian vestibular tissues. Immature hair cells were found in the cultured utricles at two weeks after direct gentamicin incubation (Li and Forge, 1995). This time course of the appearance of immature hair bundles was similar to that in vivo, although in both cases the individual immature hair bundle might be found earlier. The morphology of the immature hair bundles in vitro also resembled the immature form seen in vivo, with thin and short stereocilia arising from a small apical surface. The appearance of immature like hair cells spontaneously after gentamicin treatment in vestibular cultures was limited, but it does suggest that hair cells can develop in vitro following gentamicin treatment. Therefore, this organotypic culture system could provide a means for examination of the regeneration process in the damaged mature mammalian vestibular tissues to allow comparison with similar tissues from other vertebrates which are currently in use to study the process of hair cell regeneration (Warchol and Corwin, 1993; Oesterle et al., 1993; Tsue et al.,1994a; Baird et al., 1996).

Cultures of neonatal mouse cochleae have been extensively used to study ear development (Van De Water and Ruben, 1971; Sobkowicz et al, 1993) but little work has been done on

the mature mammalian inner ear cultures. Recently, the use of mammalian vestibular tissues maintained in the organotypic cultures has been described in connection with hair cell loss and regeneration to complement morphological observation in vivo (Warchol et al., 1993; Lambert, 1994; Yamashita and Oesterle, 1995). Increasing attempts have been made to identify factors involved in hair cell regeneration and the proliferative activity of the inner ear sensory epithelia using organotypic cultures. Growth factors and other factors that stimulate hair cell regeneration have been tested systematically in vitro. It has been shown that proliferative activity in neomycin damaged cultured mouse utricles and cristae can be up-regulated by adding transforming growth factor alpha (TGF-α) to the culture medium (Lambert, 1994). Epidermal growth factor (EGF) and TGF-α, in combination with insulin appear to induce proliferative activity in both the supporting cells and the hair cells of the cultured mouse utricles, which could be labelled by DNA synthesis markers (Yamashita and Oesterle, 1995). The organotypic culture system has also been used to detect the apparent stimulation of hair cell production after drug induced damage in the cultures of immature mammalian organ of Corti (Lefebvre et al., 1993; Chardin and Romand, 1995). TGF-α might be a factor involved in the production of hair cells in the neonatal mammalian organ of Corti after aminoglycoside injury (Staecker et al., 1995).

In further experiments, this culture system could be used as a model to study apoptotic process and relevant factors promoting cell survival or preventing cell death. Several of the biochemical events that contribute to apoptotic cell death have recently been elucidated. The key pro-apoptotic gene, ced-3, was found to be homologous to the gene for mammalian interleukin-1β-converting enzyme (ICE), which encodes a protease that is

necessary for apoptosis. Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and Boc-Aspartyl-fluoromethylketone (BAF), the cell permeable peptides which inhibit the ICE proteases, have been shown to effectively block apoptotic cell death in cultures (Deshmukh et al., 1996; Jacobson et al., 1996). If it is the case that aminoglycoside antibiotics cause hair cell death through apoptosis, it might be possible to inhibit apoptosis and then prevent hair cell loss in the inner ear. Preliminary work on applying one of the ICE inhibitors, BAF, to vestibular tissue cultures has shown attenuation of the toxic effect of gentamicin.

6.5 HAIR CELL LOSS AND REGENERATION IN THE COCHLEA

There was no morphological sign of hair cell recovery in the mammalian auditory sensory epithelium, the organ of Corti, after gentamicin induced hair cell loss. To date, there is no report of cell proliferation in the mature mammalian cochlear sensory epithelium. But some evidence for regeneration of hair cells in the immature organ of Corti of the neonatal rats has been reported (Lefebvre et al., 1993). Nearly 99% of the auditory hair cells were lost following exposure to neomycin and after further incubation in the presence of retinoic acid and foetal bovine serum the replacement of nearly all the hair cells was said to occur in the damaged cochlear cultures. This regeneration of hair cells was assessed by fluorescence microscopy using phalloidin labelling and irregularly arranged stereociliary bundles were seen in the recovered organ of Corti. However, this work has not yet been replicated and no evidence for proliferation has been presented. Another intensive study of this system failed to find reappearance of hair cells in vitro (Chardin and Romand, 1995). Behavioural evidence for recovery of auditory function in guinea pigs has been reported and the recovery appeared to take long time following kanamycin administration without

morphological evidence of hair cell regeneration (Nicol et al., 1992). Although there is no sign for hair cell regeneration in the organ of Corti in this work, the extent of cochlear hair cell loss may serve as a measure of gentamicin treatment effectiveness and also an internal control for vestibular hair cell regeneration in vivo. The recovery of vestibular hair cells at different survival periods after gentamicin treatment suggests the possibility that hair cell regeneration exists in the vestibular sensory epithelia of the mammalian inner ear.

The clinical literature does contain several reports of partial and complete hearing and balance recoveries in patients after aminoglycoside ototoxicity. Fully hearing recovery was found in four patients among 10 patients suffered gentamicin induced hearing loss (Winkel et al., 1978). A study reported that from one week to nine months after aminoglycoside treatment, 55% patients with hearing loss and 53% patients with vestibular depression showed improvement and recovery in auditory and vestibular functions (Fee, 1980). Glasscock and colleagues reported that a patient with bilateral Ménière's disease recurred vertigo and redeveloped the vestibular caloric response after streptomycin had abolished vestibular responses (Glasscock et al., 1989). Although these cases are isolated and circumstantial, the recovery pattern for the vestibular and auditory functions in patients is consistent with the data of hair cell regeneration from the avian model. The results of hair cell regeneration in the mammalian vestibular sensory epithelia from this work may offer an explanation for the reports on the recovery of vestibular functions.

6.6 CLINICAL IMPLICATIONS

Therapeutic uses of aminoglycosides in Ménière's disease started in late 1940's and control of the drug side effects is critical and difficult (Monsell et al., 1993). In this animal work, the vestibular and cochlear hair cells were affected by gentamicin treatment in vivo.

This may be related to the high dosage of gentamicin used in animal experiments or the animals may have different responses to aminoglycosides due to biological and anatomical differences. Gentamicin has been reported to be more toxic to the vestibular system than the cochlea in humans. In medical practice, some successes of "pharmacological labyrinthectomy" were reported in the treatment of Ménière's patients with vertigo using aminoglycosides. The aims of this procedure are to cure the vestibular symptoms, avoid major surgery, whilst causing minimal damage to the cochlea and preserving residual hearing. Gentamicin has been used locally to the middle ear to treat vertigo patients associated with Ménière's disease (Bagger-Sjöbäck et al., 1990; Beck and Schmidt 1978; Jahnke, 1988; Lange, 1989; Ödkvist et al., 1984; Pfaltz, 1988; Möller et al., 1988) as a clinical procedure for relieving the debilitating vertigo. The major advantages of this procedure have been reported to be over 90% of the vertigo control rate (Lange, 1989; Hellström and Ödkvist, 1994; Watanabe et al., 1995) and that hearing could be preserved or improved after local application with gentamicin (Beck and Schmidt 1978; Lange, 1989; Pfaltz, 1988; Hellström and Ödkvist, 1994). In these animal experiments, based on the morphological examination of the organ of Corti, hair cell loss appeared extensive after topical application with gentamicin. This result does not support the reported clinical advantages of "pharmacological labyrinthectomy".

6.7 CONCLUSION

The major side effects of gentamicin on hair cells of the sensory epithelia of the mammalian inner ear has been documented. This study has shown that there are two modes of hair cell loss in the vestibular sensory epithelia of guinea pigs and gerbils after gentamicin treatment and the results have been substantiated in a series of studies both in

vivo and in vitro. Gentamicin triggers programmed cell death in hair cells. The loss of most hair cells in response to gentamicin treatment can occur via apoptosis, that is degeneration of hair cells within the sensory epithelia, and is similar to the mode of outer hair cell loss in the mammalian organ of Corti after ototoxic treatment. Loss of hair cells by extrusion from the apical surface was also found in the vestibular sensory epithelia. This is similar to the process of hair cell loss in birds and other lower vertebrates, where hair cell regeneration also occurs. Extrusion has not been reported in the mammalian organ of Corti, this may be because the structural organization of the mammalian cochlea is highly differentiated, therefore, limitation of the repair and regeneration processes that can occur in the organ of Corti.

Supporting cells are more resistant to gentamicin than hair cells and they repair the lesions caused by hair cell loss. They become active expanding into the site of the lost hair cell to form scars. These steps maintain intact tissue architecture and prevent the formation of obvious lesions after gentamicin induced hair cell loss in the vestibular sensory epithelia. Apoptotic bodies from fragmented hair cells were observed inside supporting cells suggesting that supporting cells eliminate dying hair cells by phagocytosis. These processes may be important for subsequent hair cell recovery and regeneration mechanisms to operate in the mammalian vestibular system.

The first morphological evidence that all of the vestibular sensory epithelia, the cristae, utricles and saccules, of guinea pigs have the potential to restore hair cells after hair cell loss induced by gentamicin has been presented in this work. Immature hair cells have been identified in the mature vestibular sensory epithelia from the gentamicin treated animals

and the hair cell number increased significantly at four and 12 weeks post-treatment. BrdU labelling the inner ear tissues demonstrated that regenerative proliferation occurred in the vestibular sensory epithelia of guinea pigs in vivo after hair cell loss. This confirms the conclusion of other studies in which a proliferation phenomenon occurs in damaged mammalian vestibular sensory epithelia. However the extent is insufficient to account for all the immature hair cells seen by SEM.

This study also provides evidence that the processes of hair cell loss and recovery in the vestibular sensory epithelia that occur in vivo can be replicated in the organotypic cultures. However, the hair cell recovery was limited at the time of study. The process of supporting cell expansion and repair of the sensory epithelia in vitro is similar to that process in vivo. Therefore, this organotypic culture system can be used to examine hair cell loss, repair and recovery phenomenon under controlled, easily manipulated conditions.

The ultimate objective for studying the processes of hair cell loss and regeneration is to learn what is needed to stimulate and control the underlying repair processes in the human ear. The potential for the development of pharmacological / therapeutically strategies to protect inner ears from apoptotic death of hair cells or up-regulate hair cell regeneration in the mammalian inner ear may exist. Further understanding of the process and factors that influence the mammalian hair cell regeneration may lead to direct stimulation of hair cell production and recovery in the damaged human inner ear. The knowledge may be useful to help patients suffering sensorineural hearing loss and/or peripheral vestibular disorders.

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