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DISSERTATION

Gene and Protein Expression Patterns in the Rat Inner Ear during Ototoxicity and Otoprotection

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1 Introduction

1.1 Inner ear anatomy

The inner ear is located deep in the petrous portion of temporal bone. It consists of two functional parts: the vestibular system (consisting of the vestibule and semicircular canals) which serves as the balance organ, and the cochlea, which serves as the microphone, converting sound pressure impulses from the outer ear to electrical impulses which are passed to the brain *via* the auditory nerve [Roland et al., 1997].

1.1.1 Cochlea

The cochlea is a snail-shell like structure that is coiled two and three-quarter turns (in human) around the bony modiolus. The modiolus contains the cochlear vessel and spiral ganglion. The central processes of spiral ganglion neurons form the cochlear nerve and exit the temporal bone in the internal acoustic meatus, and the dendrites run through the osseous to make synaptic contacts with hair cells [Roland et al., 1997].

The cochlea contains three fluid-filled compartments. The scala vestibuli and scala tympani are filled with perilymph fluid, communicating at the apex of the cochlea, the helicotrema. Between the vestibular and tympanic scalae lies scala media (cochlear duct) containing endolymph fluid [Roland et al., 1997]. In a cross section, as shown in Fig. 1, the base of scala media is constituted by the osseous lamina (which winds around the modiolus) and the basilar membrane, which separate the cochlear duct from scala tympani. Human basilar membrane is approximately 35 mm in length [Krstic, 1991] and in rat it reaches about 22 mm [Ding et al., 2001a]. Situated on the basilar membrane is the auditory receptor organ, called organ of Corti (OC). OC contains hair cells, supporting cells and tectorial membrane. The lateral wall of cochlear duct is formed by stria vascularis, which plays an important role in producing endolymph. The fibrous spiral ligament lies between stria vascularis and the bony lateral wall of the cochlear duct. The spiral limbus, a thicken endosteum of the osseous spiral lamina, is located on the margin of the osseous spiral lamina and extends as two lips (tympanic lip and vestibular lip) to delimit the inner spiral sulcus. The upper wall of cochlear duct is formed by vestibular membrane (Reissner's membrane), which stretches between the inner margin of the spiral limbus and the upper edge of the stria vascularis [Krstic, 1991; Roland et al., 1997].

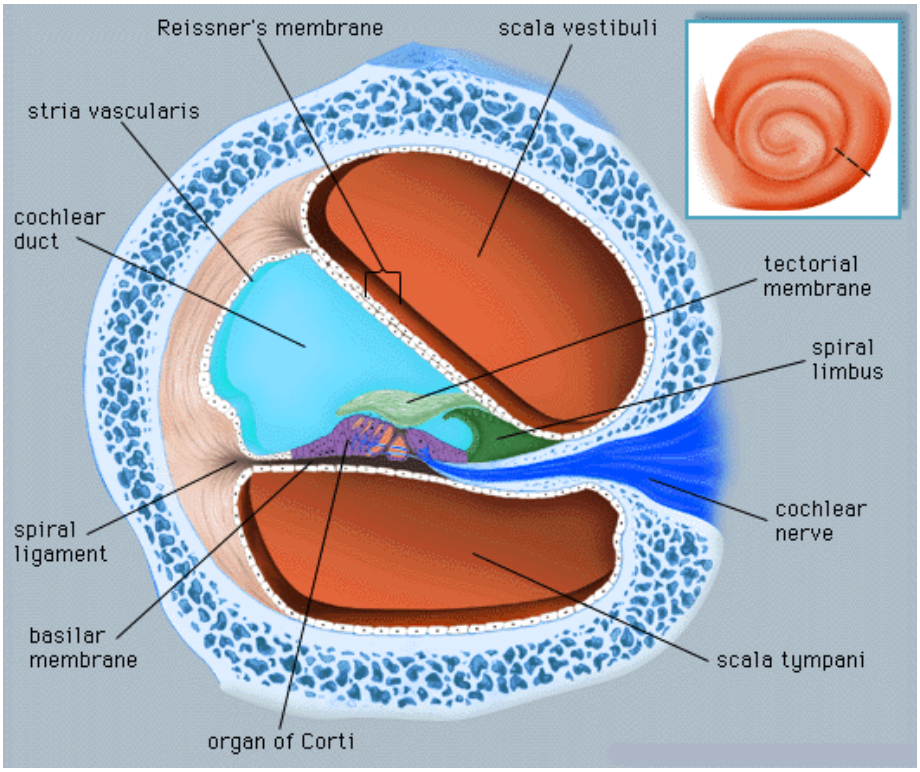


Fig. 1 A cross section through one of the turns of the cochlea showing the scala tympani, scala vestibuli and the cochlear duct.

[taken from: Human ear, Encyclopædia Britannica, Inc. (http://www.britannica.com/EBchecked/topic-art/123552/534/A-cross-section-through-one-of-the-turns-of-the)]

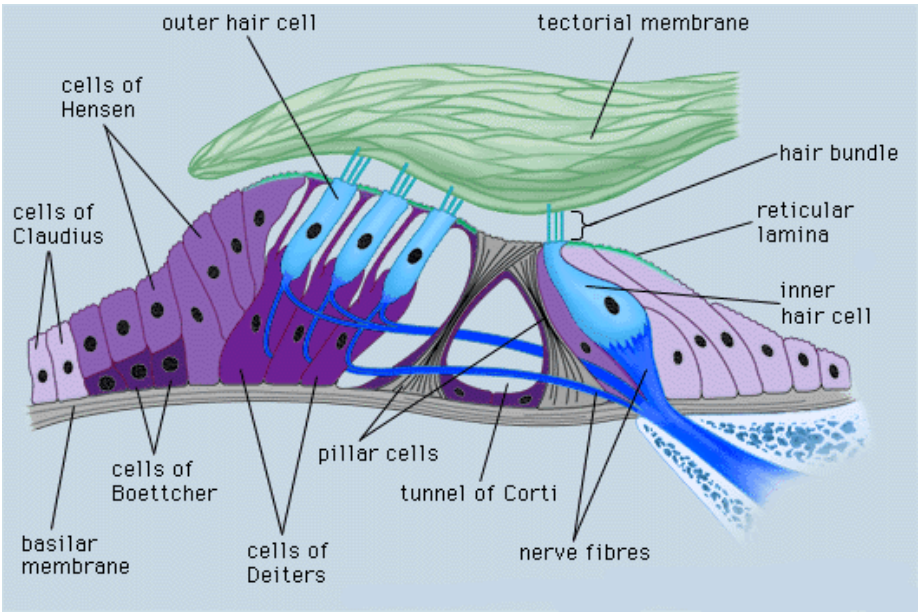


Fig. 2 Structure of organ of Corti

[taken from: Human ear, Encyclopædia Britannica, Inc. (http://www.britannica.com/EBchecked/topic-art/123552/534/A-cross-section-through-one-of-the-turns-of-the)]

1.1.2 Organ of Corti

The sensory epithelium of the inner ear is called organ of Corti. It is composed of inner and outer hair cells and their supporting cells. The supporting cells consist of: inner and outer pillar cells, inner and outer phalangeal cells, Hensen's cells, Boettcher's cells, and Claudius' cells. In cross section, inner pillar cells and outer pillar cells delimit the tunnel of Corti, which separates inner hair cells (IHCs) from outer hair cells (OHCs). Numerous nerve fibers run helically and radially through the tunnel of Corti. IHCs are supported by the inner phalangeal cells and OHCs are supported by outer phalangeal cells (Deiter' cells). On the inner side of IHCs and inner phalangeal cells is the inner spiral sulcus. The outer spiral sulcus is a groove situated at the level of Claudius' cells between the OC and the spiral ligament. The tectorial membrane extends from the vestibular lip of spiral limbus to overlie the hair cells [Roland et al., 1997] (Fig. 2).

1.1.3 Hair cells

Hair cells are the sensory receptors of the auditory system. There are two types of hair cells in OC: inner hair cells and outer hair cells. They differ in their shape, the pattern of stereocilia and the innervation.

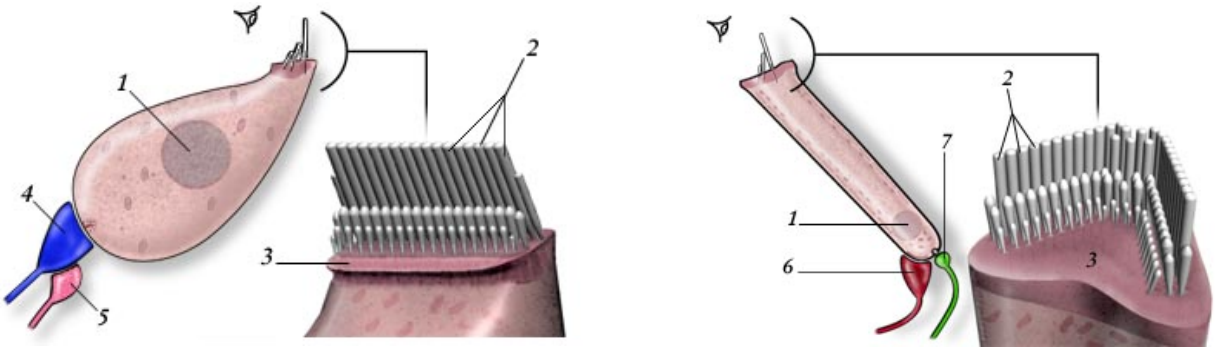
1.1.3.1 Inner hair cells

IHCs are located on the medial side of the tunnel of Corti. They are large, pear-shaped sensory cells, arranged in a single row of about 3,500 cells in humans [Krstic, 1991; Roland et al., 1997] (about 1,000 in rats[Ding et al., 2001a]. The nucleus is round and in a central position. At the apical pole, IHCs bear 40 to 60 stereocilia arranged in two or three rows [Krstic, 1991], which emerged from a thick terminal web, the cuticular plate. The cuticular plate and stereocilia consist of actin filament. IHCs are much more uniform in size than OHCs. On average, one IHC is innervated by ten of synaptic complexes, as shown in Fig. 3, including a radical afferent bouton from type I spiral ganglion neurons and a lateral efferent ending from small neurons in the ipsilateral lateral superior olivary complex in each complex. About 95% of afferent fibers of the cochlear nerve end on the IHCs [Pujol and Lenoir, 1986].

1.1.3.2 Outer hair cells

OHCs are situated on the lateral side of the tunnel of Corti. They are smaller than IHCs and cylindrical in shape, arranged in three to five rows. There are approximately 12,000 OHCs in the organ of Corti in humans [Krstic, 1991] (about 3,650 in rats [Ding et al., 2001a]). The nucleus is located in the basal part of the cell. At the apical pole, 100 to 200 stereocilia are anchored into

the cuticular plate forming a “V” or “W” pattern and the longest stereocilia are embedded in the tectorial membrane [Krstic, 1991]. Across the basilar membrane, OHCs vary in the length of the stereocilia bundles and in that of their bodies. The OHCs forms a synapse with a small afferent type II spiral ganglion neurons and large medial efferent fibers from the medial superior olivary complex. About 5% spiral ganglion neurons terminate on OHCs, and a single type II neuron innervates approximately 10 OHCs generally in the same row [Roland et al., 1997]. OHCs change their somatic length when the voltage across their membrane is altered. This electromotility is belived to provide the local mechanical amplification in response to sound. In recent years, a motor protein (prestin) which resides in the lateral membrane of OHCs has been identified to underlie somatic electromotility in OHCs [Zheng et al., 2002].



(A) (B)

Fig. 3 Inner hair cell (A) and outer hair cell (B)

1. Nucleus
2. Stereocilia
3. Cuticular plate
4. Radial afferent ending (type I neuron)
5. Lateral efferent ending
6. Medial efferent ending
7. Spiral afferent ending (type II neuron)

[taken from: Promenade ‘round the Cochlea, Rémy Pujol. (http://www.cochlea.org/)]

The final number of hair cells is reached very early in embryonic development and from this stage, hair cells are unable to proliferate in mammals. When hair cells degenerate or disappear as a result of aging, disease or injury, their places are covered by the adjacent supporting cells, which form a scar [Raphael, 2002].

1.2 Auditory physiology of inner ear

The process of hearing involves the transmission of vibrations and the generation of nerve impulses. When sound waves enter the ear canal, vibrations travel down the external auditory canal and strike the tympanic membrane. The vibrations of the tympanic membrane are then

passed to three tiny bones in the middle ear called the ossicles. The ossicles amplify the sound and send the sound waves into the fluid-filled hearing organ (cochlea) through the action of the stapes footplate on the oval window. The mechanical vibration creates pressure waves in the perilymph of the scala vestibuli of the cochlea. These waves move into the scala tympani through the helicotrema. The wave motion is transmitted to the endolymph inside the cochlear duct. As a result, the basilar membrane vibrates, causing the organ of Corti to move against the tectorial membrane. The resultant shearing forces bend the stereocilia of hair cells [Krstic, 1991]. The mechanical movement of hair bundle generates depolarization, resulting in contracting of OHCs (electromotility), which is an active process and the release of neurotransmitters from the base of the IHCs, stimulating the nerve fibers at the base of hair cells, which lead to the spiral ganglion in the modiolus of the cochlea [Moller, 2006]. The impulses are carried by the cochlear nerve to the brain. Sounds are heard and interpreted in the auditory areas of the temporal lobes. Moreover, active electromotility of OHCs feeds energy back into the OC, amplifying the vibration of the basilar membrane, which enables the high sensitivity, wide dynamic range and sharp frequency selectivity of hearing [Krstic, 1991]. The active process of OHCs is assumed to account for approximately 40-50 decibels (dB) of hearing sensitivity and loss of function of OHCs causes hearing loss of approximately 40-50 dB [Moller, 2006].

As the basilar membrane decreases remarkably in stiffness and the OC increase in mass from base to apex, a certain degree of tuning is formed in the structure of cochlear duct and its contents. Thus, the basal part of OC with greater stiffness and less mass is more attuned to the sound of higher frequencies, while the apical part with less stiffness and greater mass is more responsive to lower frequencies [Roland et al., 1997]. The ability of outer hair cell to respond to particular frequencies depends not only on its position along the length of the basilar membrane but also on its resonance, which varies with the length of the bundle of stereocilia and cell body.

1.3 Auditory dysfunctions of inner ear

Auditory dysfunction commonly presents as hearing loss and tinnitus.

Hearing loss is a decrease in the ability to perceive or process auditory information [Schacht et al., 2008]. It can be categorized as conductive or sensorineural. Any disruption of the neural signal transmitted from the hair cells in the cochlea or VIII cranial nerve will lead to sensorineural hearing loss, which is further divided into cochlear and retrocochlear categories [Schacht et al., 2008]. Hair cell loss is the leading cause of hearing loss [Raphael, 2002]. Hearing loss often occurs with tinnitus.

Tinnitus is the sensation of sound in the absence of external acoustic stimuli and perceived only by the sufferer. Tinnitus can be divided into two types: objective and subjective. Subjective tinnitus, accounting for 95 percent [Schacht et al., 2008] of tinnitus cases, is reported to be present in about 80 percent of individuals with profound hearing loss [Schacht et al., 2008].

The inner ear is vulnerable to damage or destruction from a variety of sources. Improper function or loss of cochlear hair cells may result in a hearing loss because of the failure of these cells to transmit proper signals to auditory centers. Malfunction or destruction of inner ear (especially the hair cells in cochlea) mainly due to acoustic trauma, ototoxic drugs and aging may lead to hearing loss and/or tinnitus [Schacht et al., 2008].

A number of drugs such as aminoglycoside antibiotics (e.g. gentamicin), chemotherapeutic drugs (eg cisplatin), non-steroidal anti-inflammatory drugs (e.g. salicylate), loop diuretics, antimalarials have the potential to damage inner ear and cause hearing loss and tinnitus, called "ototoxicity" [Schacht et al., 2008]. The clinical signs of ototoxicity are various, but the most common symptoms include sensorineural hearing loss and/or tinnitus.

1.4 Salicylate and inner ear

Acetylsalicylic acid (aspirin) is one of the most commonly used drugs for its antipyretic, analgesic and anti-inflammatory properties. Salicylate is the active component of aspirin. Over one century ago, the auditory toxicity of salicylate has been associated with high doses employed in the treatment of chronic inflammatory diseases [Cazals, 2000]. Since then, many observations indicated that high dosage of salicylate induces auditory dysfunction including tinnitus and hearing loss. In contrast to platinum and aminoglycoside ototoxicity, that of salicylate is mostly reversible upon treatment cessation, which has been noted in people and demonstrated in animal models [Cazals, 2000]. In addition, vestibular manifestations are absent. Thus, salicylate intrigues many researchers with its unique pattern of ototoxicity. Because of this unique ototoxicity pattern, salicylate was used to produce animal model tinnitus in many experimental researches [Bauer et al., 1999; Cheng et al., 2005; Jastreboff and Sasaki, 1994; Ruttiger et al., 2003; Zheng et al., 2006].

Though the central nervous system may also be sensitive to salicylate, which was measured by activity of voltage-gated channel currents or post-synaptic currents [Wang et al., 2006a; Yang et al., 2007], and central auditory structures are necessarily involved in perceptual alterations, the inner ear seems to be the origin of auditory dysfunction. Recent morphological and electrophysiological studies showed that sodium salicylate modulates cochlear function,

especially that of OHCs [Cazals, 2000]. These changes were attributed to the reversible blockage of nonlinear capacitive current and electromotility by salicylate [Zheng et al., 2000]. In a few studies, salicylate has been demonstrated to have influences on gene expression in cochlea [Im et al., 2007; Yu et al., 2008; Zheng et al., 2006], which suggested a possible molecular mechanism of salicylate ototoxicity. However, the exact molecular mechanisms of generation of tinnitus induced by salicylate are still obscure.

Interestingly, in recent years salicylate has shown a beneficial effect on the inner ear, besides ototoxicity. It has been demonstrated that salicylate protects against auditory side effects of gentamicin and cisplatin [Jiang et al., 2005; Li et al., 2002; Sha and Schacht, 1999]. In those reports, salicylate significantly attenuated the gentamicin and cisplatin-induced threshold shift and dramatically reduced the loss of cochlear OHCs. The underlying mechanism of bidirectional effect of salicylate on inner ear is poorly understood.

1.5 Cisplatin ototoxicity

Cisplatin is a widely used chemotherapeutic agent for the treatment of various malignant neoplasms. It binds to DNA and forms intra- or inter-strand cross-links and protein-DNA cross-links that inhibit DNA replication and RNA transcription, which directly leads to apoptosis [Wang and Lippard, 2005]. However its use is limited by serious side effects such as nephrotoxicity, neurotoxicity and ototoxicity [Fram, 1992]. Cisplatin-induced ototoxicity is manifested as bilateral, dose-related progressive, irreversible sensorineural hearing loss, which begins in the high frequencies and then progresses to involve low frequencies [Rybak, 2007]. Cisplatin-induced damage is prominently present in auditory hair cells, especially in outer hair cells. The damage first occurs in the basal cochlea and progresses towards the apex. Spiral ganglion cells also degenerate, mostly in the basal turn, and even more profoundly than hair cells [Hinojosa et al., 1995; Hoistad et al., 1998]. Deleterious effects are also frequently present on stria vascularis [Meech et al., 1998]. In cochlear tissue, cisplatin produced reactive oxygen species, causing depletion in antioxidant enzymes, which eventually increase apoptosis [Rybak, 2007]. Recently, an *in vitro* study on HEI-OC1 cells (a cell line expressing markers specific for OHCs) demonstrated that inflammatory cytokines including IL-6, IL-1 and TNF-α may mediate cisplatin ototoxicity [So et al., 2007].

1.6 Gentamicin ototoxicity

Gentamicin is an aminoglycoside antibiotic, used to treat many types of bacterial infections, particularly those caused by Gram-negative bacteria. It is well known that gentamicin has two major side effects, nephrotoxicity and ototoxicity [Matz, 1993]. Gentamicin ototoxicity involves auditory system and/or vestibular system. Clinically, hearing loss caused by gentamicin , which is often irreversible, occurs initially at high frequencies, and then progressively at low frequencies [Matz, 1993]. The ototoxicity of gentamicin is attributed to the selective toxic effect on sensory hair cells in cochlea and vestibular organ. Nerve fibers and spiral ganglion cells may degenerate subsequent to hair cells loss [Schacht et al., 2008]. With respect to the cochlea, hair cell loss begins in the basal region and progresses towards the apex, which is consistent with the pattern of hearing loss. And the pathology is first evident in OHCs, while IHCs are more resistant than OHCs and they generally disappear only after OHCs in their immediate vicinity are lost [Schacht et al., 2008]. A number of studies have addressed the gentamicin-induced mechanism of hair cell damage, and found two main ones, 1. overproduction of reactive oxygen species (ROS) [Song and Schacht, 1996] and 2. overactivation of N-methyl-D-aspartate receptors resulting in excitotoxic damage [Forge and Schacht, 2000; Hong et al., 2006]. Previous studies on intracellular signaling mechanism indicated that gentamicin-induced hair cell damage occurs by apoptosis [Forge and Schacht, 2000; Ylikoski et al., 2002].

Although new generations of antibiotics have emerged in the last decades, aminoglycoside gentamicin are still used in a variety of medical conditions. Therefore, there is a considerable interests in finding ways to prevent their ototoxicity. Intervention strategies such as preventing formation of ROS with a variety of antioxidants [Song and Schacht, 1996] or blocking apoptotic signaling pathways [Corbacella et al., 2004; Ylikoski et al., 2002] have been demonstrated to be effective against gentamicin-induced ototoxicity.

1.7 Proinflammatory cytokines and inner ear

Pro-inflammatory cytokines are a group of cytokines which act as inflammation mediators and can accelerate and/or regulate inflammatory reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types [Dinarello, 2000]. Proinflammatory cytokines play critical roles in a variety of cellular processes including development, apoptosis and cellular stress response as well as acute phase response [Adams, 2002; Dinarello, 2000]. The major proinflammatory cytokines that are responsible for

early responses are interleukine-1alpha (IL-1α, interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). Other proinflammatory mediators include interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-8, 11, 12, 17, 18, (IL-8, IL-11, IL-12, IL-17, IL-18) and a variety of other chemokines that chemoattract inflammatory cells, and various neuromodulatory factors [Kuchroo et al., 2002].

Proinflammatory cytokines are produced predominantly by activated immune cells such as leukocytes and microglia, and they are also produced in various organs after tissue damage and various types of insults including infection, trauma, and ischemia by various cell types including neuron and glia in the central nervous system. In several recent studies, proinflammatory cytokines were shown to be induced in damaged cochlea. It has been indicated that proinflammatory cytokines (IL-1, IL-6 and TNF-α) were produced in noise-induced damaged cochlea [Fujioka et al., 2006]. A very recent study implicated that these cytokines acted as important mediators of cisplatin ototoxicity in auditory hair cells [Kuchroo et al., 2002; So et al., 2007]. In addition, it was observed that exposure to IL-1β, IL-6 and TNF-α caused morphological changes, including shortening and ballooning, in isolated cochlear OHCs [Park et al., 2004]. These results suggest a possible involvement of certain cytokines in the pathogenesis of inner ear disorders. Nevertheless, little is known about the exact roles of inflammatory cytokines in inner ear damage or dysfunction.

In another aspect, IL-6 presents a protective effect on other tissue or cells, such as neuronal cells [Kushima and Hatanaka, 1992]. IL-6 was shown to protect rat PC12 cells (pheochromocytoma cell line) from serum deprivation or chemotherapeutic reagents [Kunioku et al., 2001]. It was also found that IL-6 protected cerebellar granule neurons from NMDA-induced neurotoxicity [Wang et al., 2007]. Overall, IL-6 seems to be involved in the protection mechanism as well as injury in damaged or stressed tissues.

1.8 HSP70 and inner ear

1.8.1 HSP70 in inner ear

Heat shock proteins (HSPs) are a group of highly conserved proteins that exist in all living organisms and are involved in the response to various stresses. The 70 kilodalton heat shock proteins (HSP70) are a family of ubiquitously expressed heat shock proteins. Constitutively expressed HSP70 (70 kDa heat shock cognate protein, HSC70), functions under normal

physiological conditions as chaperone during protein synthesis, intracellular transport and degradation. Under stress conditions, such as hyperthermia, oxidative stress, inflammation, exercise, exposure to toxins and ultraviolet light, starvation, hypoxia, or water deprivation, inducible HSP70 (iHSP70, HSP72) provide cellular protection by repairing the damaged proteins in cytoplasm and by blocking apoptosis [Beere et al., 2000; Beere, 2004] (The term "HSP70" mentioned in the present study specifically refers to inducible HSP70). Consequently, the heat shock proteins are also referred to as stress proteins and their up-regulation is sometimes described more generally as part of the stress response.

In the cochlea, HSPs have been found in OHCs, spiral ganglion cells and stria vascularis in rat and guinea pig [Leonova et al., 2002]. Induction of HSP70 occurs in cochlea in response to a variety of stimuli. It has been shown that HSP70 is up-regulated in the cochlea exposed to hyperthermia [Dechesne et al., 1992], transient ischemia [Myers et al., 1992], acoustic overstimulation [Lim et al., 1993] and cisplatin [Oh et al., 2000].

1.8.2 Extracellular HSP70

In addition to the intercellular stress response, HSPs can be released from cells in various physiological and pathological events including necrosis [Calderwood et al., 2007]. After release into extracellular fluid, HSP70 may interact with adjacent cells and lead to cytokines transcription and release, which has been shown in monocyte [Asea et al., 2000] and airway epithelium [Chase et al., 2007] in vitro. It implies that HSP70 may have a dual role as chaperone and cytokine. It has been shown that acetylsalicylic acid induced the release of HSP70 from mast cells, resulting in up-regulation of IL-6 and TNF-α [Mortaz et al., 2006].

1.8.3 Induction of HSP70 as endogenous protective molecules

The role of heat shock proteins as an endogenous protective mechanism is well established. A study has shown that heat shock protected cochlea from a subsequent noise exposure [Yoshida et al., 1999]. It was found that heat shock induced HSP70 expression and inhibited ototoxic drug-induced (aminoglycoside and cisplatin) hair cell death in utricle in vitro [Cunningham and Brandon, 2006]. Furthermore, HSP70 was indicated to be necessary for the protection effect of heat shock against aminoglycoside-induced hair cell loss in utricle [Taleb et al., 2008].

In addition to heat shock, some reagents functioning as HSP70 inducers have been shown to have a cyto-protective effect in experimental studies.

Resveratrol, a polyphenolic compound, which was identified in 1992 as an ingredient of grape skin and red wine, possesses multiple physiological properties including anti-oxidation,

anti-inflammation and anti-coagulation. It shows therapeutic potential *in vivo* against cancer, cardiovascular and inflammation diseases [Baur and Sinclair, 2006]. A recent study indicated that resveratrol induced HSP70 and protected human cells from severe heat stress *in vitro* [Putics et al., 2008]. And resveratrol has shown protective effect against acoustic trauma [Seidman et al., 2003].

Geldanamycin, a benzoquinone ansamycin isolated from *Streptomyces hygroscopicus*, binds to N-terminal ATP-binding site of HSP90, resulting in activation of HSF under non-stress conditions and prolongs the heat shock protein response [Ali et al., 1998]. Geldanamycin has been shown to activate HSP70 expression and to provide protection in a number of mammalian cell types including neurons, astrocytes, sensory epithelial cells [Kaarniranta et al., 2005; Ouyang et al., 2005; Shen et al., 2005; Xu et al., 2003]. However, till now, there is no report discussing otoprotective effect of geldanamycin.

1.9 Aims

Damage and death of the auditory sensory epithelium due to ototoxic conditions lead to acquired hearing loss and tinnitus. This work was dedicated to study selected ototoxic and otoprotective mechanisms and molecules.

This study had following main aims:

- To define the effect of salicylate on gene and protein expression in VOT-E36 cell line;
- To define the effect of salicylate on gene and protein expression in organotypic culture of the organ of Corti;
- To determine the influence of IL-6 on hair cell viability in organotypic culture of the organ of Corti under normal and ototoxic conditions;
- To establish a pharmacological or chemical way of induction of HSP70 using resveratrol and geldanamycin in organotypic culture of the organ of Corti ;
- To uncover otoprotective properties of resveratrol and geldanamycin in gentamicin-induced hair cell loss in organotypic culture of the organ of Corti .

2 Materials and Methods

2.1 Cell line culture and treatment

2.1.1 Culture of VOT-E36 cells

The VOT-E36 cell line was kindly provided by Dr. Matthew C. Holley (Sheffield, UK). According to the report by G. Lawoko-Kerali [Lawoko-Kerali et al., 2004], this conditionally immortal cell line is derived from a transgenic mouse, which carries a temperature-sensitive variant of large T-antigen from the SV40 virus under the control of an IFN-γ inducible promoter element. Cells were cultured in 12 cm^2 flasks (Becton Dickinson Labware, NJ, USA) containing 3 ml of minimum essential medium supplemented with Earle's salts, Glutamax I (Invitrogen), 50 U/ml IFN-γ and 10% fetal bovine serum (FBS) at 33 ºC (growth permissive conditions). When the cells grew to approximately 90% confluence, they were allowed to differentiate by placing them in a medium without IFN-γ and transferring them to 39 ºC incubator for 7 days.

2.1.2 Time course of salicylate treatment

After a 7-day-differentiation period, VOT-E36 cells were exposed to medium containing 2.5mM sodium salicylate for 1, 2, 3, 4, 5 or 24 h. At the end of the salicylate treatment period, cells were collected and lysed for RNA preparation. This experiment was repeated four times.

2.2 Organotypic culture of OC and treatment

2.2.1 Animals and dissection procedure

Postnatal day 3 to day 5 Wistar rats were used to prepare cochlear organotypic cultures. All studies were performed in accordance with the German Prevention of Cruelty to Animals Act and were approved by the Berlin Senate Office for Health (T0234/00).

The dissection procedure is similar to that described by Sobkowicz [Sobkowicz et al., 1993]. After decapitation, the head were cleaned with 70% ethanol and positioned with ventral surface down. The scalp was removed and the skull was transected along the mid-sagittal plane. The brain was scooped out to expose the posterior fossa. The temporal bones were freed from the posterior hemi-skulls and transferred into Petri dishes containing cold sterile buffered saline glucose solution (BSG) (glucose 11.4mM). Under a stereomicroscope (Stemi, SV6, Zeiss,

Germany), the tympanic membrane and annulus were laterally peeled away and the surrounding cartilages were removed exposing the cochlear capsule.
The cochlear capsules were biten off in small pieces from the oval window to the apex or shucked off from the base integrally. The stria vascularis and spiral ligament were stripped off as a single piece from the base to the apex, and the OC was stripped off from the modiolus.

2.2.2 Organotypic culture of OC

The OC explants were incubated in 4-well culture dishes (4× 1.9cm^2, Nunc, Wiesbaden, Germany) containing 500 µl medium. For different further use, OC explants were cultured in two different ways. For further reverse transcription polymerase chain reaction (RT-PCR) and Western blotting, whole OC free-floating tissue was incubated in the medium. For histological use, the OC was cut into three parts consisting of apical, medial and basal turns, and then the OC segments were positioned on the bottom of dishes as a flat surface preparation and held in place by the surface tension of the culture medium. The culture medium consisted of DMEM/F12 (1:1) (Gibco, Karlsruhe, Germany) supplemented with 10% FBS (Biochrom AG, Berlin, Germany), 0.6% glucose, 2 µl/ml insulin-transferrin-Na-Selenit-Mix (Roche Diagnostics GmbH, Mannheim, Germany), 24 ng /ml recombinant human insulin-like growth factor-I (rhIGF-I, R&D Systems, Wiesbaden-Nordenstadt, Germany), and 100 U/ml penicillin (Grünenthal GmbH, Aachen, Germany). The explants were placed in an incubator (SANYO MCO-16AIC, 37 ºC, 5% CO_2) for 24 hours to condition them before further treatments.

2.2.3 Culture treatments

2.2.3.1 Salicylate treatment

Sodium salicylate (Cat. # S3007, Sigma-Aldrich, Munich, Germany) was dissolved in Dulbecco's phosphate-buffered saline (PBS) (Cat. # H15-002, PAA Laboratories GmbH, Cölbe, Germany) to make 250 mM stock solution.

After initial 24 h in culture, 50% of OC explants obtained from right ears of experimental animals were incubated for 3 or 24 h in the culture medium containing 2.5 mM salicylate, and the other 50% of the OC explants obtained from left ears were untreated and served as the control. At the end of the treatment period all cell cultures and OC explants were washed with PBS and then lysed for extraction of RNA or protein.

2.2.3.2 Exogenous IL-6 and cisplatin treatment

BioSourceTM recombinant rat IL-6 (Cat. # PRC0064, Invitrogen, Hamburg, Germany) was dissolved in 100 mM acetic acid to prepare 1 mg/ml stock solution. Cisplatin (P4394, CAS # 15663-27-1, Sigma-Aldrich, Munich, Germany) was dissolved in culture medium to final concentration of 15 μM. After 24 h conditioning, the culture medium was removed and 500 μl medium containing specific concentration of IL-6 (0.3, 3, 30 or 90 ng/ml), cisplatin (15 μM), or the combination of two drugs was added to the culture wells. The OC explants were incubated for 24 or 48 h. At the end of culture, the explants were fixed for further staining manipulations.

2.2.3.3 Resveratrol or geldanamycin treatment

Geldanamycin (Cat. # Ant-g1, Invivogen, Toulouse, France) and resveratrol (Cat. # FR-104, Biomol, Hamburg, Germany) were dissolved in DMSO to make 1 mg/ml and 100 mg/ml stock solutions, respectively. After initial 24 h of culture, the medium was replaced with new medium containing a specific concentration of geldanamycin (0.5, 1 or 2 μM) or resveratrol (50, 100, 200 or 400 μM) and cultured for up to 24 h. Then OC explants were fixed for staining, or lysed for RNA isolation and protein extraction.

2.2.3.4 Geldanamycin and gentamicin treatment

Gentamicin sulfate (G1264, CAS # 1405-41-0, Sigma-Aldrich, Munich, Germany) was dissolved in distilled water to prepare 50 mM stock solution and diluted into culture medium at final concentration of 500 μM. After initial 24 h for conditioning, OC explants were incubated in culture medium containing 2 μM geldanamycin for 4 h and then exposed to 500 μM gentamicin for 24 h, or were treated simultaneously with geldanamycin and gentamicin for 24 h. At the end of culture, OC explants were fixed for histochemical analysis.

2.3 Total RNA extraction

Total RNA samples were isolated from VOT-E36 cells or organotypic cultures using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and on-column DNase digested with RNase-free DNase Set (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

For VOT-E36 cells, after aspiration of culture medium and wash with PBS, 350 μl buffer RLT supplemented with β-mercaptoethanol (β-ME, 10 μl β-ME per 1 ml buffer RLT) was added to the monolayer cell cultures. Cell lysates were then collected with cell scrapers and pipetted into QIAshredder homogenizers (Qiagen, Hilden, Germany) and centrifuged for 2 min at

maximum speed. Equal volumes of 70% ethanol were added to the homogenized lysate and mix by pipetting. Samples were then transferred to RNeasy spin columns placed in 2 ml collection tubes and centrifuged for 20 sec at 8000 rpm. Flow-through was discarded and 350 µl buffer RW1 was added to RNeasy columns and centrifuged for 20 sec at 10000 rpm to wash the column membrane. Flow-through was discarded and 80 µl DNase I incubation mix was added to RNeasy column membrane and incubated at room temperature for 15 min. 350 µl buffer RW1 was then added to RNeasy columns and centrifuged for 20 sec at 10000 rpm. Flow-through and collection tubes were discarded and RNeasy columns were placed in new 2 ml collection tubes. 500 µl buffer RPE was pipetted into RNeasy columns and centrifuged for 20 sec at 10000 rpm. Flow-through was discarded and 500 µl buffer RPE was added to RNeasy columns and centrifuged for 2 min at 10000 rpm to wash the column membrane. Then flow-through and collection tubes were discarded and RNeasy columns were transferred in new 2 ml collection tubes and centrifuged for 1 min at 14000 rpm. RNeasy columns were placed in new 1.5 ml collection tubes and 30 µl RNase-free water was pipetted to the column membranes and centrifuged for 1 min at 10000 rpm to elute the RNA. The resulting RNA samples were stored at - 80 ºC or used directly for concentration measurement.

For free-floating OC explants, after aspiration of culture medium and wash with PBS, OC explants were directly lysed in micro-centrifuge tubes containing 350 µl buffer RLT supplemented with β-ME and then homogenized with QIAshredder homogenizers. The following procedure of total RNA isolation was the same as that described above.

2.4 RNA concentration measurement

To quantify the isolated RNA samples from VOT-E36 cells, 3 µl of RNA was diluted with 70 µl RNase-free water in an UVette cuvette (Eppendorf, Hamburg, Germany) and the concentration was measured with Eppendorf Biophotometer (Eppendorf, Hamburg, Germany).

The RNA samples isolated from OC explants were quantified spectrofluorimetrically with Ribogreen® RNA Quantitation Reagent (Molecular Probes, Göttingen, Germany) according to the manufacturer's instruction. Briefly, Ribosomal RNA standard (Molecular Probes, Göttingen, Germany) was diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, PH 7.5) to prepare standard dilution series and RNA samples were diluted using TE buffer. Working solution of Ribogreen reagent (1: 2000 in TE buffer) was added to each diluted sample, and then the fluorescence was measured using SpectroMax M5 microplate reader (Molecular Device). The concentration of RNA samples was calculated with SoftMax Pro Software. All RNA samples were stored at -80°C until needed.

2.5 cDNA preparation

First-strand cDNA was synthesized from 100 ng of total RNA in a thermocycler (Perkin Elmer-applied biosystems thermal cycler 9600, Foster City, USA). The reaction mixture contained 0.5 mM dNTP Mix (Invitrogen GmbH, Karlsruhe, Germany), 3.8 µM Oligo(dT) (Biotez, Berlin, Germany), 26U RNasin (Promega Co., Madison, WI, USA) and 25U MMLV Reverse Transcriptase (Promega Co., Madison, WI, USA) in a final volume of 21 µl of MMLV Reaction Buffer (Promega Co., Madison, WI, USA). The template was denatured by heating at 70°C for 5 min and cooled at 4 °C. The RT reaction was run at 42 °C for 60 min followed by enzyme inactivation at 95 °C for 5 min and cooling at 4 °C. To exclude the possibility of cross-reaction of PCR primers with contaminating DNA in the following PCR experiment, negative RT controls containing all reverse transcription components, including RNA samples, were prepared by carrying out the reaction in the absence of MMLV Reverse Transcriptase. In addition, negative RNA controls were set up containing nuclease-free water in place of total RNA.

2.6 Real-time quantitative PCR and gene expression level calculation

2.6.1 Real-time quantitative PCR

The Master Mix (containing FastStart Taq DNA Polymerase, reaction buffer, SYBR Green I dye and $MgCl_2$) for real-time quantitative PCR (qPCR) was prepared from solution 1a (Enzyme) and solution 1b (Reaction Mix) (LightCycler FastStart DNA Master^{plus} SYBR Green I, Roche Dianostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. The PCR mixture contained 2 µl of single strand cDNA, 10 pmol reverse and forward specific primers (BioTez, Berlin, Germany), 4 µl Master Mix and PCR degree water in a final volume of 20 µl. A control PCR reaction, which involved PCR master mix and the primers, but no cDNA template, was used as a blank PCR reaction. Housekeeping genes, encoding glyceraldehyde-OHCs-phosphate dehydrogenase (GAPDH) or ribosomal protein S16 (rS16) were used as internal control. PCR was initiated with preincubation at 95 °C for 10 min, followed by 35-45 cycles consisting of denaturation at 95 °C for 10 sec, annealing at 65-70 °C for 10 sec and extension at 72 °C for 10-25 sec, and melting at 60-95 °C with a heating rate of 0.1 °C /sec, and ended with cooling down to 4 °C in LightCycler System (Roche Diagnostics, Basel, Switzerland). Primers used for real-time qPCR are shown in Tab 1.

Table 1 PCR primers sequence and product sizes

Target gene	Primer sequence (5′ → 3′)	Product size (bp)
(r) Hsp70	F: ACC AGG ACA CTG TTG AGT TC	250
	R: ACT CAT CTC CGA GTT CAC AC	
(r) IL-1β	F: AAT GCC TCG TGC TGT CTG ACC	118
	R: TTG TCG TTG CTT GTC TCT CCT TG	
(r) IL-6	F: CAA GAG ACT TCC CAG CCA GTT GC	111
	R: TGT TGT GGG TGG TAT CCT CTG TG	
(r/m) IL-18	F: ACT GTA CAA CCG CAG TAA TAC	436
	R: AGT GAA CAT TAC AGA TTT ATC CC	
(r) TNF-α	F: TCT TCT GTC TAC TGA ACT TCG GGG	82
	R: ATG GAA CTG ATG AGA GGG AGC C	
(r) prestin	F: CAC AGA GTC CGA GCT ACA CAG TG	162
	R: TCA GTG CGC TGC TGT ACA AG	
(r) GAPDH	F: AGG TGA CCG CAT CTT CTT GT	225
	R: CTT GAC TGT GCC GTT GAA CT	
(r) rS16	F: GGG TCC GCT GCA GTC CGT TC	127
	R: CGT GCG CGG CTC GAT CAT CT	
(m) Hsp70	F: CAA GAT CAC CAT CAC CAA CG	238
	R: GAT HAC CTC CTG GCA CTT GT	
(m) IL-1β	F: GAT CCA CAC TCT CCA GCT GCA	152
	R: CAA CCA ACA AGT GAT ATT CTC CAT G	
(m) IL-6	F: GTT CTC TGG GAA ATC GTG GA	339
	R: GGA AAT TGG GGT AGG AAG GA	
(m) TNF-α	F: TGG GAG TAG ACA AGG TAG TAC AAC CC	175
	R: CAT CTT CTC AAA ATT CGA GTG ACA A	
(m) MyoVIIa	F: AAC TGC TGT GGC TGT GTA CG	231
	R: TGT CAT CGG GGA AGT AGA CC	
(m) prestin	F: GTC TCG AAG CCT TGT TCA GG	251
	R: ACA GGG AGG ACA CAA AGG TG	
(m) GAPDH	F: AAC TTT GGC ATT GTG GAA GG	223
	R: ACA CAT TGG GGG TAG GAA CA	

(r): rat; (m): mouse

2.6.2 Agarose gel electrophoresis

2 µl of each PCR product was separated by electrophoresis on a 2% or 3% agarose gel (Gibco BRL, Life Technologies, Paisley, Scotland) stained by GelStar® Nuleic Acid Gel Stain (Cambrex Bio Science, Rockland, USA), bands were visualized under ultraviolet light using a Syngene-Gene Genius imaging system (Synoptics Inc., USA), and the product size was confirmed by comparison with DNA ladder Marker V (Roche Diagnostics, Mannheim, Germany).

2.6.3 Gene expression level calculation

Threshold cycle (*Ct*) values acquired in real-time qPCR were normalized to GAPDH or rS16 that served as an endogenous reference and calibrated to the control. Relative expression level (fold change) of the target genes in each experimental sample was calculated using $2^{-\Delta\Delta Ct}$ method (Livak, KJ, 2001) [Livak and Schmittgen, 2001], where ΔCt = *C*t (target gene) – *C*t (reference gene) and $\Delta\Delta Ct = \Delta Ct$ (treated) – ΔCt (control).

2.7 Western blotting

For VOT-E36 cells, after aspiration of culture medium and wash with PBS, 200 µl ice cold modified RIPA buffer (50mM Tris-HCl, 150mM NaCl, 0.5% Na-Deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), and 1% non-ionic detergent P-40 (NP-40)) containing 67 µg/ml phenylmethylsulfonyl fluoride (PMSF) (Carl Roth, Germany) and 5 µg/ml aprotinin (Carl Roth, Germany) was added to the monolayer cell cultures in flasks. Cell lysates were collected with cell scrapers and transferred into microcentrifuge tubes and then sonicated for 5 sec and incubated on ice for 15 min. The lysates were then clarified by centrifuge at 14000 rpm for 15 min at 4 °C. The supernatants were aliquoted and stored at - 80 °C.

For free-floating OC explants, after aspiration of culture medium and wash with PBS, 6 explants were pooled into micro-centrifuge tubes containing 140 µl modified RIPA buffer for lysis. The following procedures were the same as that described above.

The protein concentration was determined using DC Protein Assay kit (Bio-Rad, Munich, Germany) based on Lowry assay. After denaturation at 95 °C for 5 min, 10 µg of total protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking and 12.5% separating gel) at 130 V for 18 min and 150 V for 60 min, with recombinant rat HSP70 protein (SPP-758, Streegen, Bioreagents, Ann Arboor, USA) and recombinant rat IL-6 (Cat. # PRC0064, Biosource, Solingen, Germany) as standards, and then electrotransferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, Freiburg,

Germany). The blots were blocked with blocking buffer containing 5% non-fat dry milk and 1% Tween in PBS at room temperature for 60 min and then incubated at room temperature for 120 min with monoclonal antibodies anti-GAPDH (Cat. # 5G4, Hy test Turku, Finland) or mouse Anti-β-Actin (Cat. #A5441, Sigma-Aldrich, Munich, Germany) (1: 100,000 dilution), mouse Anti-Hsp70 (Cat. # SPA-810Stressgen Bioreagents, Ann Arboor, USA) (1: 1000 dilution), and goat Anti-Rat IL-6 (Cat. # AF506, R&D System, Wiesbaden-Nordenstadt, Germany) (1: 200 dilution). After washing with blocking buffer, the membranes were incubated overnight at 4 °C with HRP-conjugated goat Anti-Mouse IgG (W402B, Promega, Mannheim, Germany) (1:5000 dilution) and HRP-conjugated donkey Anti-Goat IgG (sc-2033, Santa Cruz, Heidelberg, Germany) (1:2000 dilution). Immunodetection was visualized with Amersham ECL™ (Enhanced chemiluminescence) Western Blotting Detection Reagents (RPN2109, Amersham Bioscience, Freiburg, Germany) on Hyperfilm ECL autoradiography (RPN3103K, Amersham Bioscience, Freiburg, Germany). The bands were quantified by densitometric analysis software (Gelscan Standard V5.01) and the expression level was calculated by normalization to GAPDH or β-actin in the same sample.

2.8 ELISA

2.8.1 Measurement of the secretion of HSP70 and proinflammatory cytokines by OC cultures

At the end of culture, supernatants of control or OC cultures treated with salicylate for 24 h were collected and used for measurement of HSP70, IL-6 and TNF-α.

2.8.1.1 HSP70 measurement

The enzyme-linked immunosorbent assay (ELISA) kit, Human/Mouse/Rat Total HSP70 DuoSet IC (R&D Systems, Wiesbaden-Nordenstadt, Germany), was used to measure HSP70 concentration in OC culture supernatants. The 96-well microplate was coated with 100 µl per well of 2 µg/ml total HSP70 capture antibody (Part 841680) and incubated overnight at room temperature. After three washes in wash buffer (0.05% Tween 20 in PBS, PH 7.2-7.4), block buffer (1% BSA, 0.05 NaN_3 in PBS, PH 7.2-7.4) was added into each well and incubated at room temperature for 1 h. After three washes, 100 µl per well of total HSP70 standard (Part 841682) diluted series in IC Diluent # 4 (1 mM EDTA, 0.5% Triton X-100 in PBS, PH 7.2-7.4) and supernatant samples in IC Diluent # 4 were added and incubate at room temperature for 2 h. The plate was washed three times and 100 µl per well of 100 ng/ml detection antibody (Part

841681) in IC Diluent # 1 (1% BSA in PBS, PH 7.2-7.4) was added and incubated at room temperature for 2 h. The plate was washed three times and incubated with 100 µl per well of streptavidin-HRP (Part 890803) (1: 200 dilution in IC Diluent # 1) at room temperature for 20 min. After three washes, the plate was incubated with 100 µl per well of substrate solution (1: 1 mixture of H_2O_2 and tetramethylbenzidine (TMB)) at room temperature for 20 min, and then 50 µl per well of stop solution (2 M H_2SO4) was added. The optical density was determined using SpectroMax M5 microplate reader (Molecular Device) at 450 nm (reference 540 nm). The total HSP70 concentration was calculated from the standard curve with SoftMax Pro Software and then normalized to total protein concentration.

2.8.1.2 IL-6 measurement

The ELISA kits, Rat IL-6 CytoSet™ (Cat. # CRC0063, Biosource, Invitrogen, Hamburg, Germany) and CytoSet Buffer Set (Cat. # CNB0011, Biosource, Invitrogen, Hamburg, Germany) were used to measure IL-6 in supernatants. The 96-well microplate was coated overnight at 4°C with 100 µl per well of 1 µg/ml anti-rat IL-6 in coating buffer B (Cat. # CB01100, Biosource). After one wash-off with wash buffer (Cat. # WB01, Biosource), the plate was blocked by 300 µl per well of assay buffer (Cat. # DS98200, Biosource) and incubated for 1 h at room temperature. After removal of assay buffer, 100 µl standard and sample dilutions in assay buffer were added into designated wells whereafter 50 µl of biotin-conjugated anti-rat IL-6 (0.1 µg/ml in assay buffer) was added to each well and incubated for 3 h at room temperature. After five washes, 100 µl of streptavadin-HRP (1: 2000 in Assay Buffer) was added to each well and incubated at room temperature for 30 min. The plate was washed five times and 100 µl TMB substrate (Cat. # SB01, Biosource) was added to each well and incubated for 30 min. Then 100 µl of Stop Solution (Cat. # SS01100, Biosource) was added to each well and the absorbance was read at 450 nm using SpectroMax M5 microplate reader (Molecular Device) at 450 nm (reference absorbance 650 nm).

2.8.1.3 TNF-α measurement

Rat TNF-α ELISA kit (Cat. # KRC-3012, Biosource, Invitrogen, Hamburg, Germany) was used to measure TNF-α concentration in supernatants. 100 µl of prepared standard series, specimens (1: 2 dilution in Standard Dilution Buffer) and controls were added to appropriate wells pre-coated with monoclonal antibody specific for rat TNF-α and incubated at room temperature for 2 h. After four washes in wash buffer, 100 µl of biotinylated anti-TNF-α solution was added to each well except the chromogen blanks and incubated for 1.5 h at room

temperature. Wells were washed four times, 100 µl of streptavidin-HRP working solution was added to each well except the chromogen blanks and incubated for 45 min at room temperature. After four washes, 100 µl of stabilized chromogen (TMB) was added to each well and incubated at room temperature for 30 min in the dark. Then 100 µl of stop solution was added to each well and the absorbance was read at 450 nm (reference absorbance 650 nm) using SpectroMax M5 microplate reader (Molecular Device). The TNF-α concentration in specimens was read from the standard curve with SoftMax Pro Software.

2.8.1.4 IL-1β measurement

The ELISA kit, Rat IL-1β CytoSet™ (Cat. # KRC-0012, Biosource, Invitrogen, Hamburg, Germany), was used to measure IL-1β concentration in supernatants. 100 µl of prepared standard series, specimens (1: 2 dilution in Standard Dilution Buffer) and controls were added to appropriate wells pre-coated with monoclonal antibody specific for rat IL-1β and incubated at room temperature for 3 h. After four washes in wash buffer, 100 µl of biotin-conjugated anti-IL-1β solution was added to each well except the chromogen blanks and incubated for 1.5 h at room temperature. Wells were washed four times, 100 µl of streptavidin-HRP working solution was added to each well except the chromogen blanks and incubated for 30 min at room temperature. After four washes, 100 µl of stabilized chromogen (TMB) was added to each well and incubated at room temperature for 30 min in the dark. Then 100 µl of stop solution was added to each well and the absorbance was read at 450 nm (reference absorbance 650 nm) using SpectroMax M5 microplate reader (Molecular Device). The IL-1β concentration in specimens was read from the standard curve with SoftMax Pro Software.

2.8.2 Quantification of HSP70 protein in OC lysates

The ELISA kit, Human/Mouse/Rat Total HSP70 DuoSet® IC (Cat. # DYC1663-5, R&D Systems, Wiesbaden-Nordenstadt, Germany), was used to quantify HSP70 protein expression in OC explants. Free-floating OC explants were washed two times with PBS and lysed in 150 µl lysis buffer #12 containing 1 mM EDTA, 0.5% Triton X-100 and Protease Inhibitor Cocktail Set III (Cat. # 539134, Calbiochem, Darmstadt, Germany). The OC lysates were then vortexed briefly, homogenized with ultrasound wave for 5 sec, and incubated on ice for 45 min. The lysates were centrifuged at 4200 rpm for 5 min at 4 °C and the supernatants were aliquoted and stored at - 80 °C. The total protein concentration was determined using DC Protein Assay kit (Bio-Rad, Munich, Germany) based on Lowry assay. The following procedures were the same as HSP70 measurement in supernatants described above.

HSP70 concentration in OC lysates acquired from ELISA were normalized to total protein concentration and calibrated to the control. Relative expression level of HSP70 in each experimental sample was calculated as: Fold change = $\frac{\text{HSP70 / total protein (sample)}}{\text{HSP70 / total protein (control)}}$.

2.9 Histochemistry

2.9.1 HSP70 immunohistochemistry

At the end of culture, OC explants were fixed in 4% paraformaldehyde in 0.1 M PBS at room temperature for 30 min. Then the fragments were washed two times with PBS and permeabilized with 0.2% Triton X-100 in PBS for 30 min. After two washes in PBS, the fragments were incubated in blocking solution (0.8% goat serum, 0.4% Triton and 2% BSA in PBS) at room temperature for 3 h and then incubated overnight at 4 °C with mouse anti-Hsp70 monoclonal antibody (Cat. # SPA-810, Stressgen Bioreagents, Ann Arboor, USA) (1: 200 dilution in blocking solution, 5 µg/ml) and Alexa Fluor® 594 phalloidin (A12381; Molecular Probes (Invitrogen), Karlsruhe, Germany) (33 nM in blocking solution). For negative control samples, mouse IgG1 Ab-1 (Dianova, Hamburg, Germany) (1: 40 dilution in blocking, 5 µg/ml) was substituted for mouse anti-Hsp70 monoclonal antibody. The fragments were washed three times in PBS, incubated at room temperature with fluorescence isothiocyanate (FITC) goat anti-mouse IgG (Dianova, Hamburg, Germany) (1: 200 dilution in blocking solution, 7.5 µg/ml) for 3 h, and washed three times in PBS. The fragments were mounted on glass slides as surface preparation in Prolong Gold® antifade reagent (P36930, Molecular Probes (Invitrogen), Karlsruhe, Germany) and examined on a confocal microscope (Leica TCS SPE, Wetzlar, Germany).

2.9.2 Hair cell quantification

At the end of culture, OC explants were fixed for 30 min in 4% paraformaldehyde in 0.1 M PBS at room temperature. Then the fragments were washed two times with PBS and permeabilized with 0.2% Triton X-100 in PBS for 30 min. The fragments were washed two times with PBS and immersed in 5 µg/ml phalloidin-tetramethyl rhodamine isothiocyanate (TRITC) (P1951, Sigma-Aldrich, Munich, Germany) at room temperature for 30 min. After two times of washing with PBS, the fragments were mounted with mounting medium containing 1,4-diazabicyclo[2.2.2]octane (DABCO) (D2522, Sigma- Aldrich, Munich, Germany).

The OC fragments were examined under a fluorescence microscope (Leica DMIL, Wetzlar, Germany) with appropriate filters for TRITC (excitation: 544 nm, emission: 572 nm).

The hair cell numbers were counted over a 100 μm longitudinal distance in 5 separated regions of each cochlear part (magnification 400×). Cells were considered missing when there was a gap in the normal arrays and no stereocilia or cuticular plate was to be seen. A mean value was calculated for each explant and at least 4 explants were used for each experimental condition.

The fragments were photographed with a digital camera (Canon PowerShot S40). Images were stored in a personal computer, the contrast and brightness was adjusted by using Adobe Photoshop (version 9.0) software.

2.10 Statistical analyses

All data were processed with the SPSS 11 statistical package.

As some data from ELISA and most of data from real-time PCR did not meet criteria of normality, they were presented as "median (interquartile range, IQR)". The two-tailed Wilcoxon signed ranks test or Mann-Whitney test were used to compare gene expression and protein concentration between experimental samples and paired or unpaired control samples. A *p*-value of less than 0.05 was considered statistically significant.

Data from hair cell counting were expressed as mean ± standard error of mean (SEM). Comparison of numbers of surviving hair cells among groups was performed by paired or unpaired *t*-test or one-way ANOVA. A *p*-value of less than 0.05 was considered statistically significant.

3 Results

3.1 Effect of salicylate on the expression of target genes in VOT-E36 cells

3.1.1 Effect of salicylate on transcriptional expression of target genes in VOT-E36 cells

After 7 days under differentiation conditions, VOT-E36 cells were treated with 2.5 mM salicylate for 1, 2, 3, 4, 5 and 24 h and then the mRNA expression levels of myosin VIIa, prestin, HSP70, IL-6, IL-1β, IL-18 and TNF-α were detected by real-time RT-PCR.

3.1.1.1 Expression of myosin VIIa mRNA and prestin mRNA in VOT-E36 cells

Fig. 4 shows the time sequence of myosin VIIa expression in VOT-E36 cells following salicylate treatment. After 2 h and 3 h treatment, the relative expression level (presented as fold change: 1.12 (1.05-1.19) and 1.18 (1.04-1.21), respectively) showed statistically significant up-regulation, compared with 1-fold change (represents gene expression level in untreated controls) ($p < 0.05$, Wilcoxon signed ranks test). There was no significant change of myosin expression after 1, 4, 5 and 24 h of treatment.

After 7 days of differentiation, transcripts of prestin in VOT-E36 cells were not detectable with real-time RT-PCR. Even after 28 days of differentiation, prestin mRNA was barely detectable in those cells.

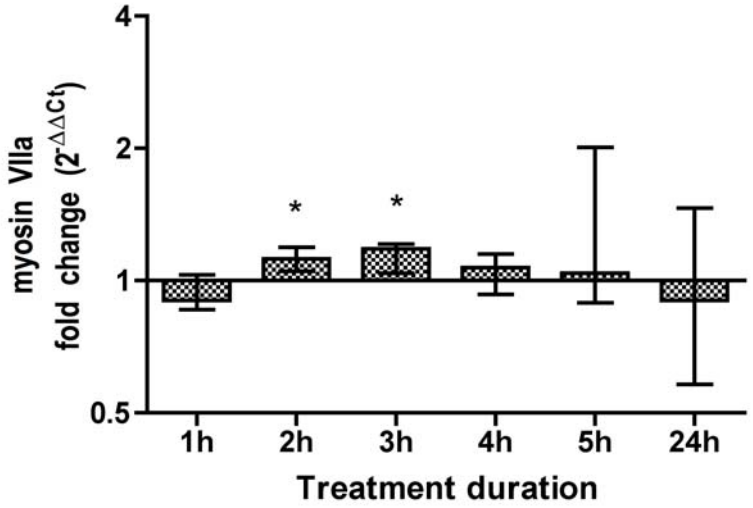


Fig. 4 Time course of Myosin VIIa mRNA expression during salicylate treatment in VOT-E36 cells. The bars represent median and the error bars represent IQR (each n = 7). * $p < 0.05$, significantly different from 1-fold change).

3.1.1.2 Expression of proinflammatory cytokines mRNA in VOT-E36 cells exposed to salicylate

3.1.1.2.1 Expression of IL-6 mRNA in VOT-E36 cells exposed to salicylate

The time sequence of IL-6 mRNA expression after salicylate treatment is presented in Fig. 5. The IL-6 expression increased significantly after 1 h of salicylate treatment to 1.56 (1.20-2.91) fold ($p < 0.05$, Wilcoxon signed ranks test). There was no significant regulation of IL-6 expression level after 2, 3, 4, 5 and 24 h of treatment ($p > 0.05$, Wilcoxon signed ranks test).

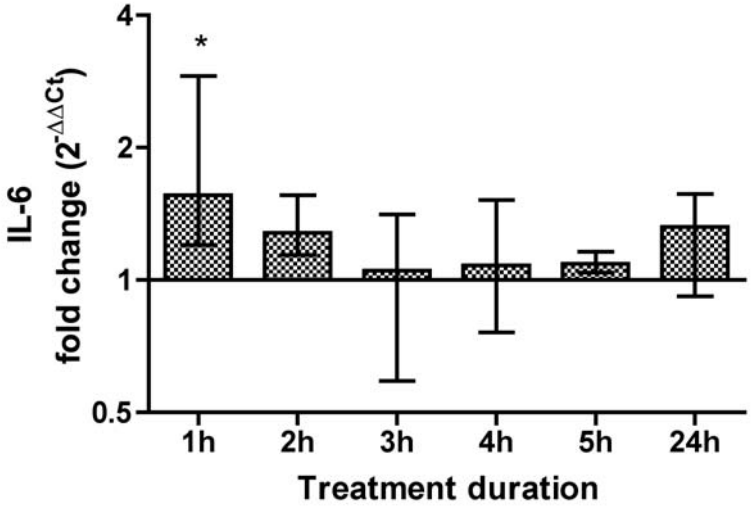


Fig. 5 Time course of IL-6 mRNA expression during salicylate treatment in VOT-E36 cells. Bars represent median and error bars represent IQR (each n = 7). * $p < 0.05$, significantly different from 1-fold change.

3.1.1.2.2 Expression of IL-18 mRNA in VOT-E36 cells exposed to salicylate

The time course of relative expression levels of IL-18 after salicylate treatment is displayed in Fig. 6. The expression level of IL-18 did not increase or decrease significantly after exposure to salicylate for 1, 2, 3, 4, 5 and 24 h ($p > 0.05$, Wilcoxon signed ranks test).

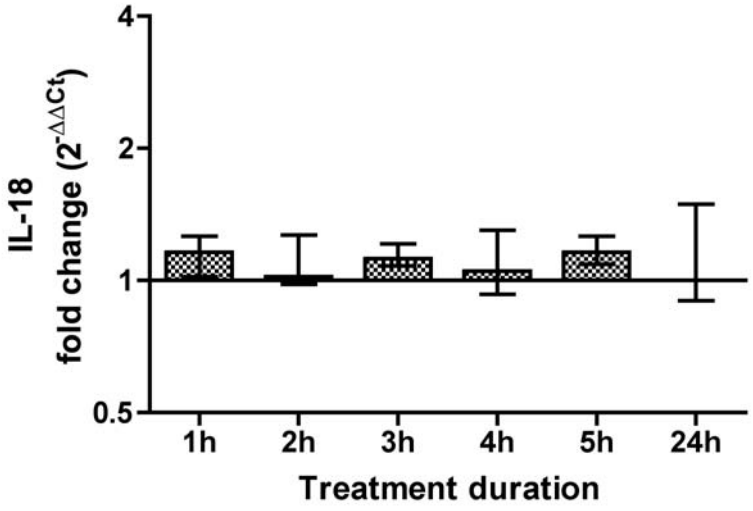


Fig. 6 Time course of IL-18 mRNA expression during salicylate treatment in VOT-E36 cells. Bars represent median and error bars represent IQR (each n = 7).

3.1.1.2.3 Expression of IL-1β mRNA and TNF-α mRNA in VOT-E36 cells

Transcripts of IL-1β in VOT-E36 cells either treated with salicylate or untreated were barely detectable with real-time RT-PCR. TNF-α transcripts were not detectable in VOT-E36 cells either.

3.1.1.3 Expression of HSP70 mRNA in VOT-E36 cells exposed to salicylate

Fig. 7 illustrates the time course of relative expression levels of HSP70 during salicylate treatment. Starting 2 h after salicylate treatment, HSP70 expression level increased. But there was statistical significance only at the time point of 2 h (1.37 (1.23-1.60) fold increase) and 5 h (1.11 (1.09-1.21) fold increase) ($p < 0.05$, Wilcoxon signed ranks test). After 24 h of exposure, HSP70 expression showed further increase to 2.38 (1.11-3.56) fold with statistical significance ($p < 0.05$, Wilcoxon signed ranks test).

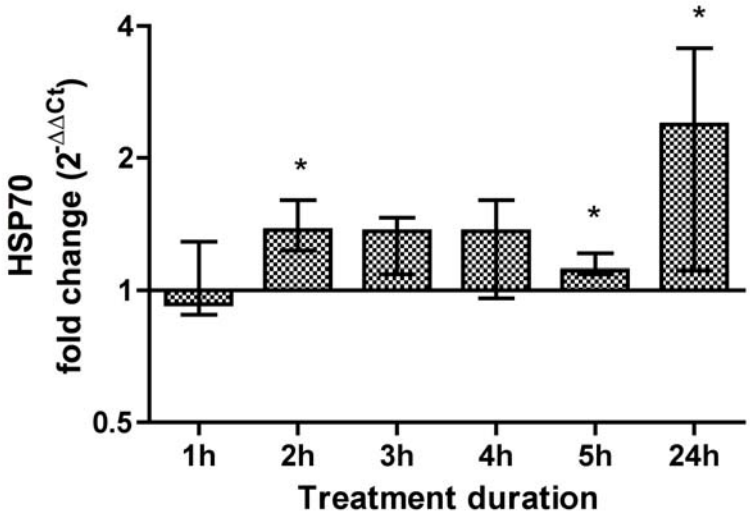
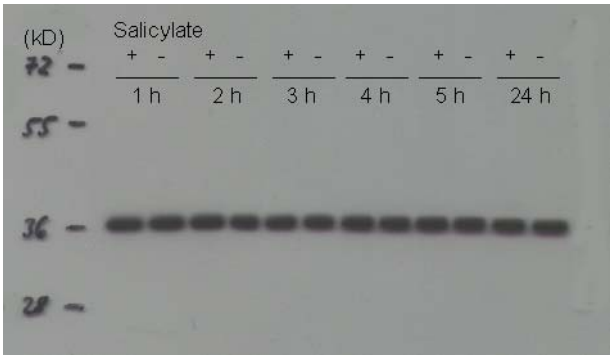
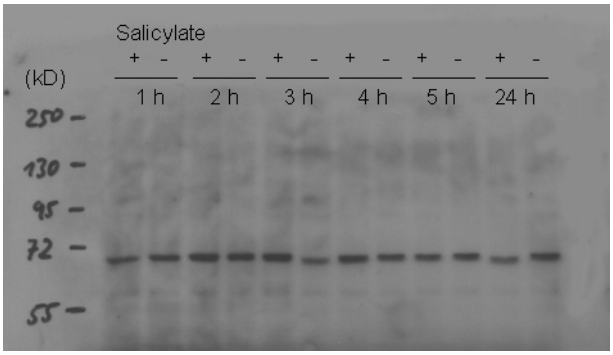


Fig. 7 Time course of HSP70 mRNA expression during salicylate treatment in VOT-E36 cells. Bars represent median and error bars represent IQR (each n = 7). * $p < 0.05$, significantly different from 1-fold change.

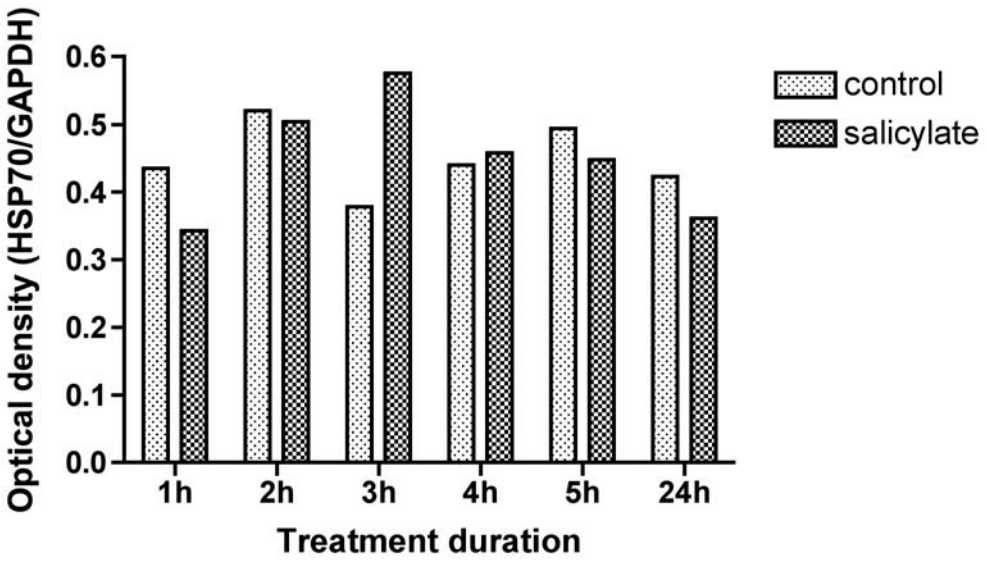
3.1.2 Expression of HSP70 protein in VOT-E36 cells exposed to salicylate

HSP70 protein levels in VOT-E36 cells treated or untreated with 2.5 mM salicylate for up to 24 h were analyzed with Western blot. Fig. 8 A shows the time course of HSP70 protein level in VOT-E36 cells after salicylate treatment. The 70 kDa protein was detected by immuno-blotting with monoclonal antibody against HSP70 in salicylate-treated and untreated samples. The optical density of HSP70 bands relative to those of GAPDH bands in salicylate-treated and untreated sample at each time point is shown in Fig 8 B. Compared with untreated samples, HSP70 protein levels were not modulated after salicylate treatment, even after 24 h when the induction HSP mRNA was maximal, except that at the time point of 3 h a probable higher level of HSP70 was seen.





(A)



(B)

Fig. 8 Time course of HSP70 protein expression in VOT-E36 cells treated with salicylate and untreated control. (A) Western blot analysis was performed using antibodies against GAPDH (upper) and HSP70 (lower). (B) The amount of HSP70 was quantified by densitometric analysis and normalized to GAPDH.

3.2 Effect of salicylate on the expression of target genes in OC cultures

3.2.1 Effect of salicylate on transcriptional expression of target genes in OC cultures

The OC explants were treated with 2.5 mM salicylate for 3 and 24 h and then the transcriptional expression levels of prestin, HSP70, IL-6, IL-1β, IL-18 and TNF-α were detected by real-time RT-PCR.

3.2.1.1 Expression of prestin mRNA in OC cultures exposed to salicylate

Fig. 9 shows prestin mRNA expression in OC cultures following salicylate treatment. After 3 h of treatment, the relative expression level (1.17 (0.83-1.43)) showed no significant change ($p > 0.05$, Wilcoxon signed ranks test). After 24 h of exposure to salicylate, prestin expression showed statistically significant up-regulation (1.66 (1.28-3.00)) compared with 1-fold change ($p < 0.001$, Wilcoxon signed ranks test).

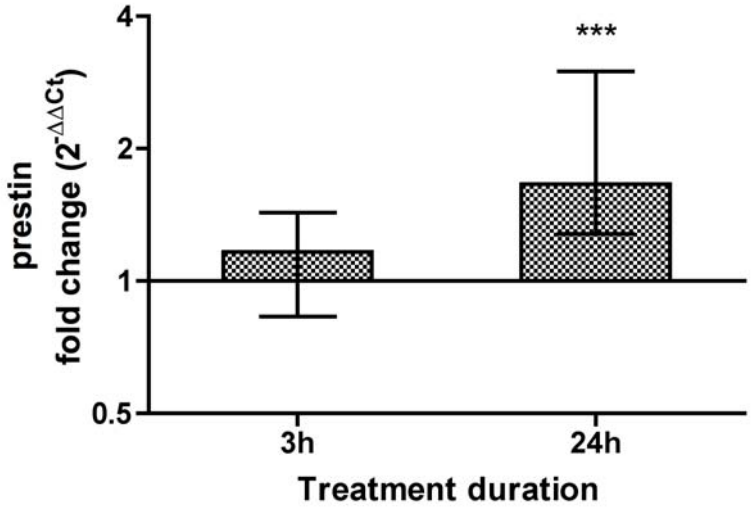


Fig. 9 Expression of prestin mRNA in OC cultures exposed to salicylate for 3 h and 24 h. Bars represent median and error bars represent IQR (each n = 20). *** $p < 0.001$, significantly different from 1-fold change.

3.2.1.2 Expression of proinflammatory cytokines mRNA in OC cultures exposed to salicylate

3.2.1.2.1 Expression of IL-6 mRNA and TNF-α mRNA in OC cultures exposed to salicylate

Figs. 10 and 11 show IL-6 and TNF-α mRNA expression in OC cultures following salicylate treatment, respectively. After 3 h of treatment, the relative expression levels of IL-6 and TNF-α showed statistically significant up-regulation (2.17 (0.93-5.43) fold and 1.23 (0.90-1.69) fold, respectively), compared with 1-fold change ($p < 0.01$ and $p < 0.05$, respectively, Wilcoxon signed ranks test). After 24 h exposure to salicylate, the expression levels showed no statistically significant up-regulation (0.94 (0.67-2.26) fold and 1.53 (0.81-1.76) fold, respectively) ($p > 0.05$, Wilcoxon signed ranks test).

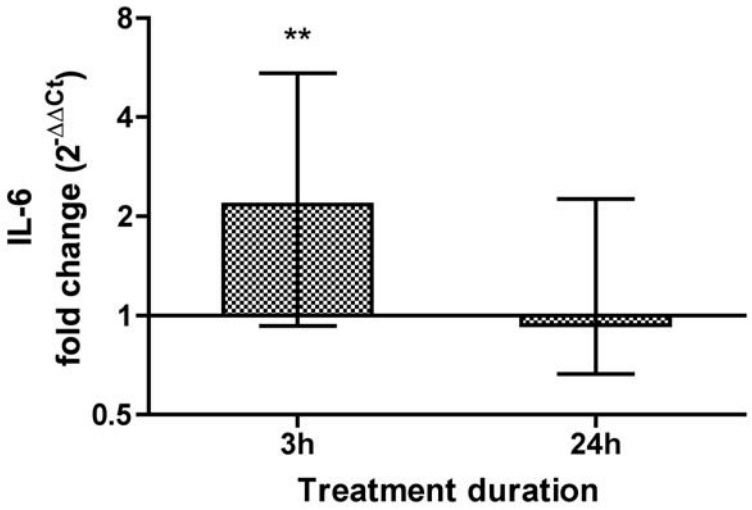


Fig. 10 Expression of IL-6 mRNA in OC cultures exposed to salicylate for 3h and 24h. Bars represent median and error bars represent IQR (each n = 20). ** $p < 0.01$, significantly different from 1-fold change.

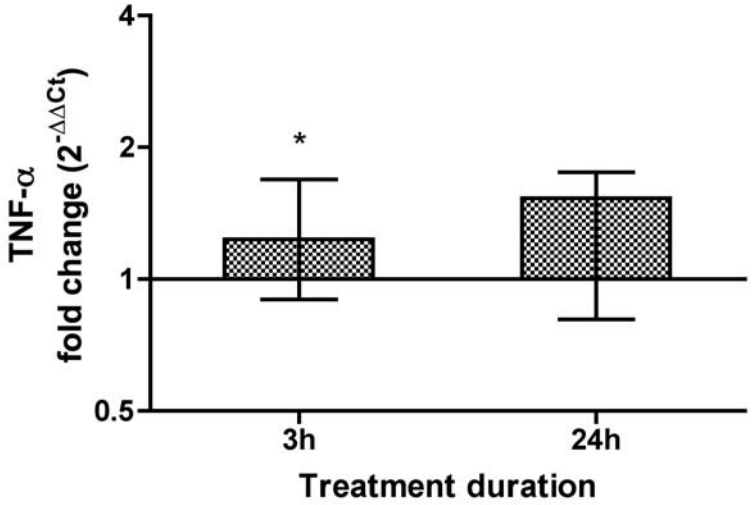


Fig. 11 Expression of TNF-α mRNA in OC cultures exposed to salicylate for 3h and 24h. Bars represent median and error bars represent IQR (each n = 20). * $p < 0.05$, significantly different from 1-fold change.

3.2.1.2.2 Expression of IL-1β mRNA and IL-18 mRNA in OC cultures exposed to salicylate

Figs. 12 and 13 display the relative expression levels of IL-1β and IL-18 in OC cultures after salicylate treatment, respectively. The expression level of IL-1β did not increase or decrease significantly after exposure to salicylate for 3 h or 24 h (1.18 (0.73-2.30) fold and 1.27 (0.72-2.45) fold, respectively) ($p > 0.05$, Wilcoxon signed ranks test). After 3 h of salicylate treatment, IL-18 mRNA expression was not altered (0.96 (0.57-1.35)); while after 24 h treatment

IL-18 expression decreased to 0.57 (0.32-1.20) fold but without statistical significance ($p > 0.05$, Wilcoxon signed ranks test).

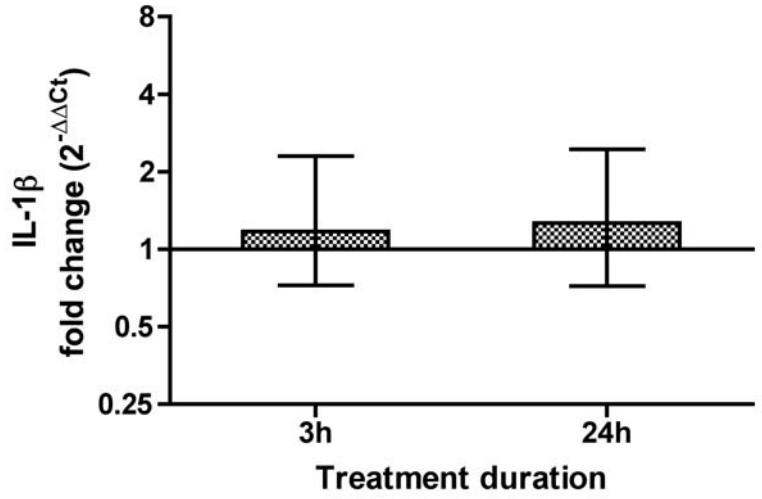


Fig. 12 Expression of IL-1β mRNA in OC cultures exposed to salicylate for 3h and 24h. Bars represent median and error bars represent IQR (each n = 20).

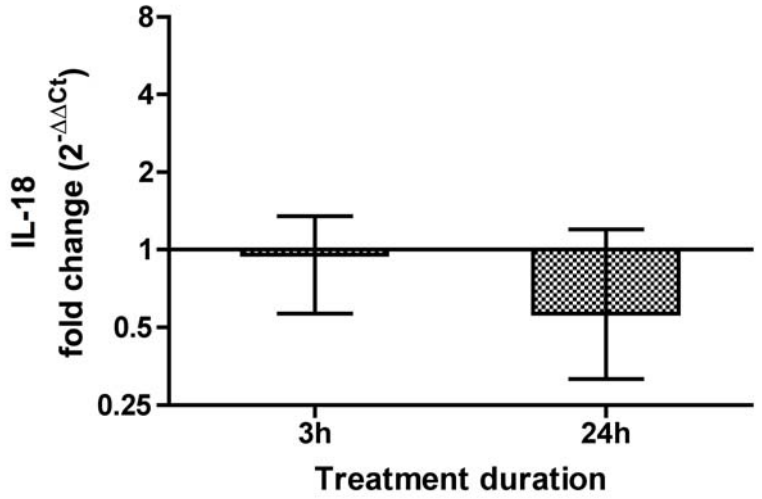


Fig. 13 Expression of IL-18 mRNA in OC cultures exposed to salicylate for 3h and 24h. Bars represent median and error bars represent IQR (each n = 20).

3.2.1.3 Expression of HSP70 mRNA in OC cultures exposed to salicylate

Following 3 h of exposure to salicylate, HSP70 mRNA level increased to 1.57 (1.06-4.05) fold, as compared with untreated samples. Exposure to salicylate for 24 h also augmented the expression of HSP70 mRNA level to 1.66 (0.94-3.61) fold. The HSP70 induction after 3 h and 24 h of exposure was statistically significant ($p < 0.01$ and $p < 0.05$, respectively, Wilcoxon

signed ranks test), and the increase in HSP70 expression level between 3 h and 24 h was of no statistical significance ($p < 0.05$, Mann-Whitney test) (Fig. 14).

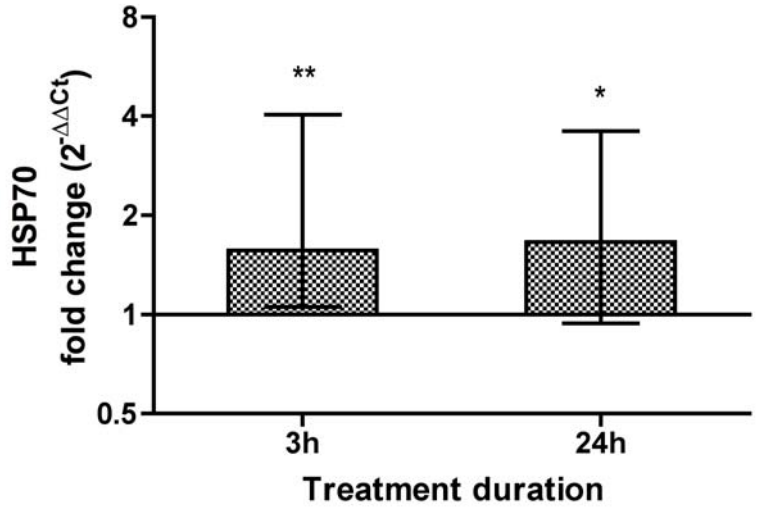
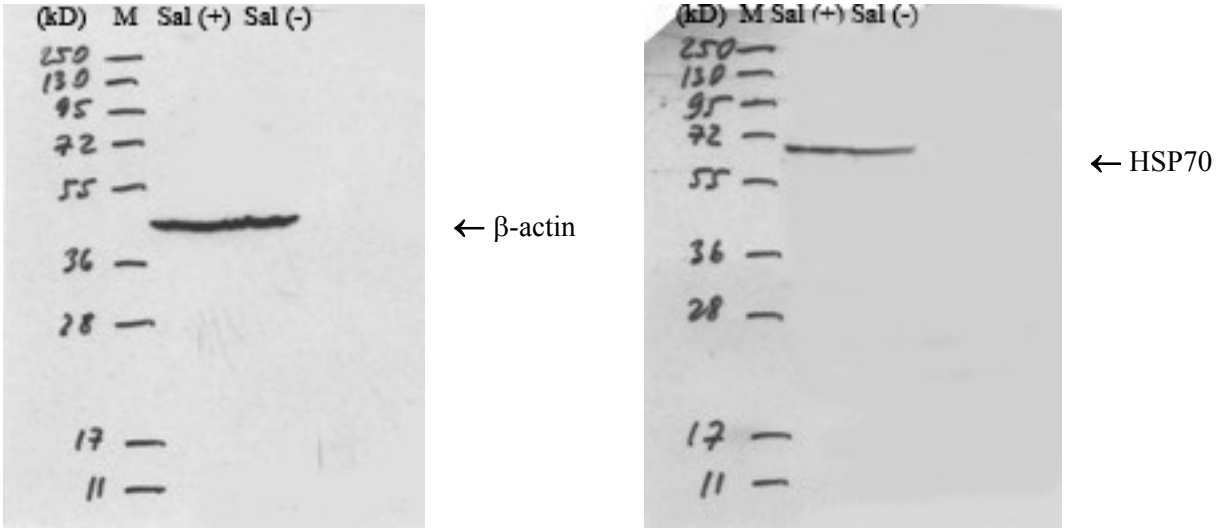


Fig. 14 Expression of HSP70 mRNA in OC cultures exposed to salicylate for 3h and 24h. Bars represent median and error bars represent IQR (each n = 20). ** $p < 0.01$ and * $p < 0.05$, significantly different from 1-fold change.

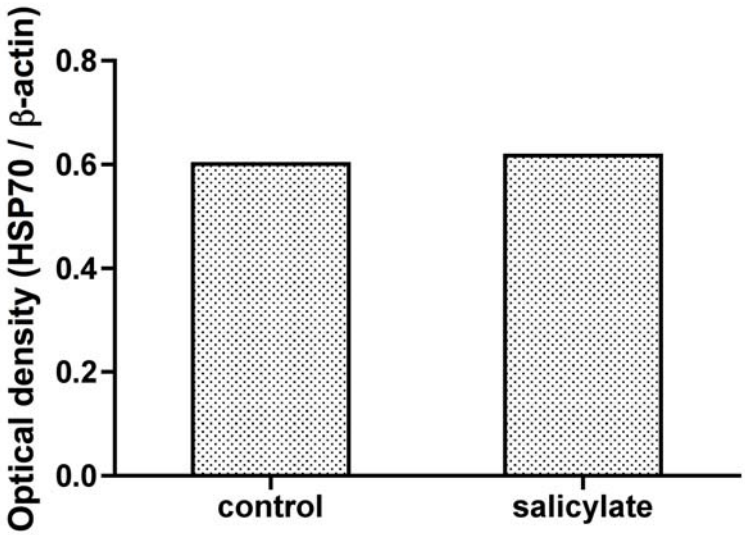
3.2.2 Expression of the target genes on protein level in OC cultures exposed to salicylate

3.2.2.1 Protein expression of HSP70 and IL-6 in OC cultures exposed to salicylate

The protein levels of HSP70 and IL-6 in OC cultures following 24 h of salicylate treatment were examined by Western blot. Bands were visible at about 70 kDa in salicylate-treated and untreated samples, corresponding to HSP70 (Fig. 15A). The optical density of HSP70 bands relative to β-actin was 0.617 and 0.601 in salicylate-treated and untreated sample (Fig. 15B). No noticeable difference of intensity could be detected in salicylate-treated and untreated OC where HSP70 was present. IL-6 bands (22 kDa) were detectable neither in salicylate-treated nor in untreated samples (Fig. 15A).



(A)



(B)

Fig. 15 HSP70 protein expression in OC explants treated with or without salicylate for 24 h. (A) Western blot analysis was performed using antibodies against β-actin (left) and HSP70 (right). (B) The amounts of HSP70 protein was quantified by densitometric analysis and normalized to β-actin.

ELISA was also used to measure HSP70 protein level in OC lysates. HSP70 protein expression level relative to total protein was 0.06 (0.05-0.09) ng/μg and 0.06 (0.04-0.10) ng/μg in salicylate-treated and untreated samples, respectively. There was no difference between salicylate-treated and untreated samples ($p > 0.05$, Wilcoxon signed ranks test) (Fig. 16).

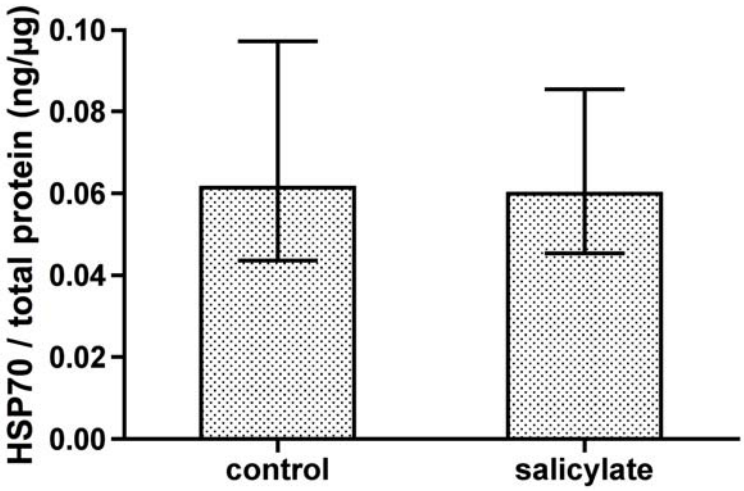


Fig. 16 Expression of HSP70 protein in OC lysates exposed to salicylate for 24h. Bars represent median and error bars represent IQR (each n = 6).

3.2.2.2 The protein secretion by OC cultures exposed to salicylate

3.2.2.2.1 Proinflammatory cytokines secretion by OC cultures exposed to salicylate

The protein levels of proinflammatory cytokines (TNF-α, IL-6 and IL-1β) in culture supernatant were quantified with ELISA.

TNF-α concentration in supernatants was 28.98 (25.85-41.04) pg/ml and 30.20 (28.00-37.16) pg/ml in salicylate-treated and untreated samples, respectively, and there was no significant difference between the two groups ($p > 0.05$, Wilcoxon signed ranks test) (Fig. 17).

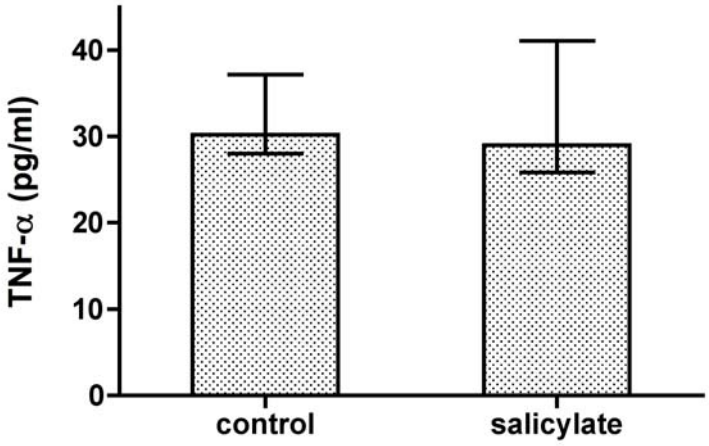


Fig. 17 Expression of TNF-α protein in supernatants of OC cultures exposed to salicylate for 24h. Bars represent median and error bars represent IQR (each n = 6).

The concentration of IL-6 and IL-1β in supernatants was too low to be quantified with existing standard curves (< 8 pg/ml and < 16 pg/ml, respectively).

3.2.2.2.2 HSP70 secretion by OC cultures exposed to salicylate

Extracellular HSP70 concentration in supernatants of OC culture after 24 h of salicylate treatment was measured by ELISA. In a salicylate-treated sample and an untreated control, the HSP70 concentration was 8.34 (7.42-8.93) ng/ml and 8.02 (7.58-9.25) ng/ml, respectively. There was no statistically significant difference of HSP70 concentration between salicylate-treated and untreated samples ($p > 0.05$, Wilcoxon signed ranks test) (Fig. 18).

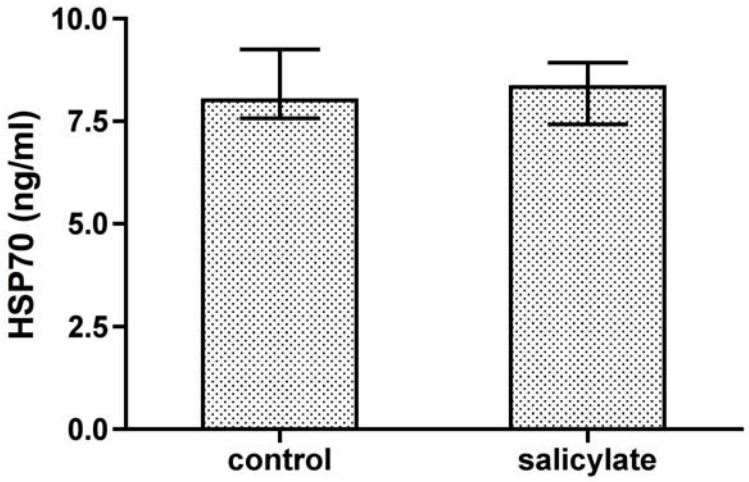
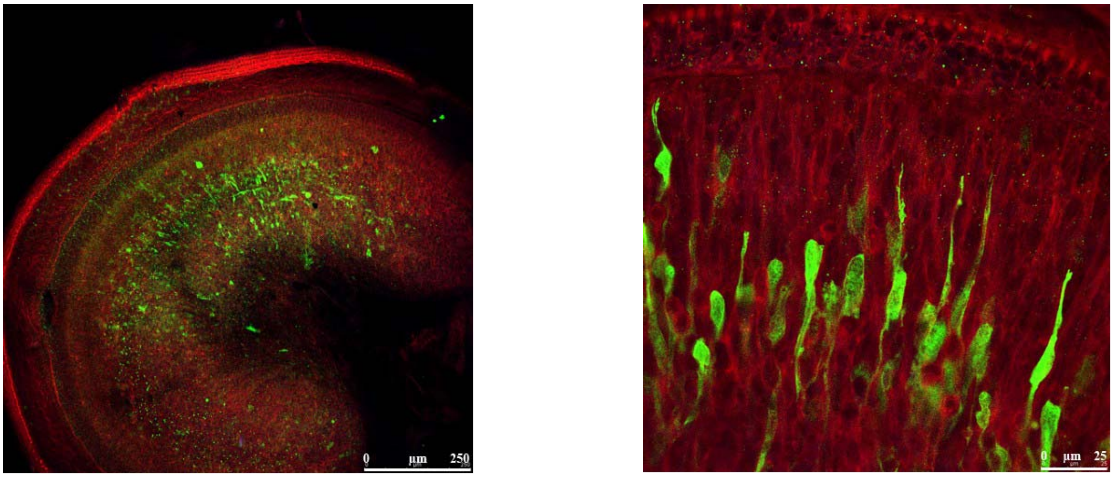


Fig. 18 Expression of HSP70 protein in supernatants of OC cultures exposed to salicylate for 24h. Bars represent median and error bars represent IQR (each n = 12).

3.2.2.3 Localization of HSP70 in OC cultures exposed to salicylate

To determine the localization of HSP70 protein in OC explants, immunohistochemistry was carried out using antibody against inducible HSP70. In OC explants exposed to salicylate for 24 h, HSP70 immunoreactivity was observed mainly in cells corresponding in shape, size and localization to spiral limbus fibrocytes (Fig. 19). Only diffuse weak immunoreactivity was detected in the OC. A similar localization pattern was seen in untreated cultures. No difference in HSP70 immunostaining could be observed between salicylate-treated and untreated OC explants.



(A) **(B)**

Fig. 19 Representative laser confocal images showing localization of HSP70 (green) in OC explant after 24 h salicylate exposure. The specimen was also stained with Alexa Fluor® 594 phalloidin (red).

3.3 Effect of exogenous IL-6 on the viability of hair cells in OC cultures under normal and cisplatin ototoxic condition

3.3.1 Effect of exogenous IL-6 on the viability of hair cells in OC cultures

To determine the possible damage effect of IL-6 on hair cells, OC explants were treated with recombinant rat IL-6 at varying concentration for 24 h and the viability of HCs was determined by hair cell counting.

Fig. 20 displays the survival of hair cells in OC cultures exposed to 0.3, 3, 30 and 90 ng/ml recombinant IL-6 and untreated controls.

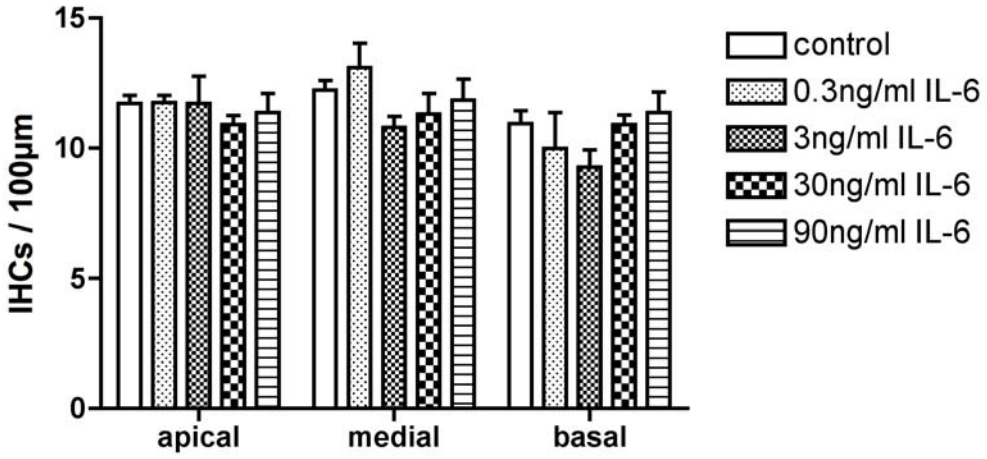
In the OC cultures exposed to 3 ng/ml IL-6, the number of IHCs in medial and basal parts decreased to 10.8 ± 0.41 (relative to 11.71 ± 0.31) and 9.27 ± 0.66 (relative to 10.94 ± 0.49), but without statistical significance. There is no decrease of surviving IHCs in other parts of OC explants treated with other concentrations of IL-6 ($p > 0.05$, one-way ANOVA including Duunnett's post test).

No significant loss of OHCs was observed in three parts of OC explants subjected to recombinant IL-6 at any certain concentrations ($p > 0.05$, one-way ANOVA including Duunnett's post test).

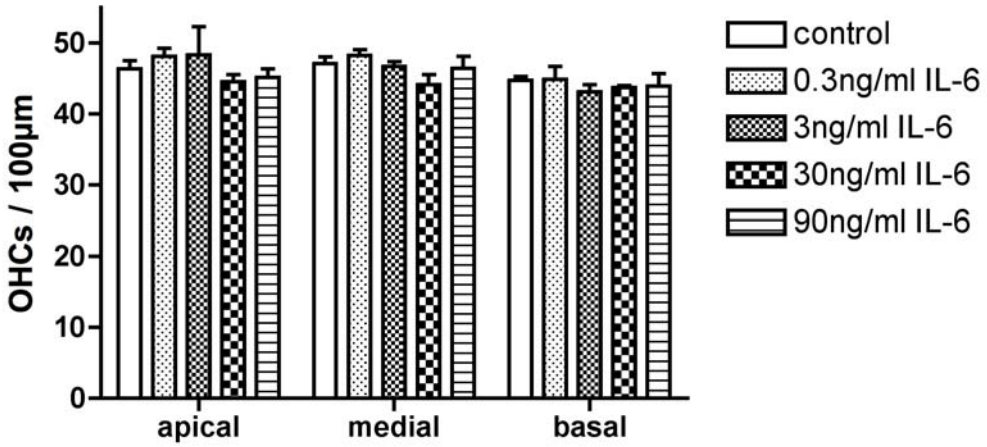
Fig. 21 shows OC cultures treated with 30 ng/ml IL-6 for 24 h and then stained with phalloidin-TRITC to label the actin filament, which is intensively expressed in the stereocilia

and cuticular plate of hair cells. After IL-6 treatment, the three rows of OHCs and single row of IHCs show a regular arrangement along the length of the cochlea.

These results indicate that exogenous IL-6 at concentration range from 0.3 to 90 ng/ml did not produce cytotoxic effect on hair cells in OC explants.

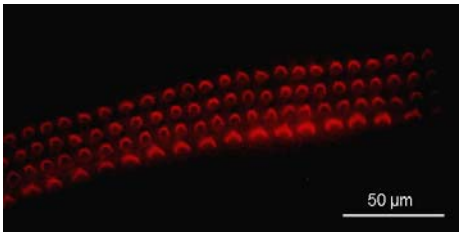


(A)

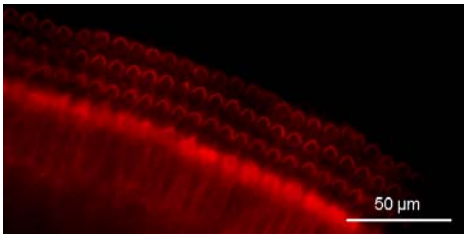


(B)

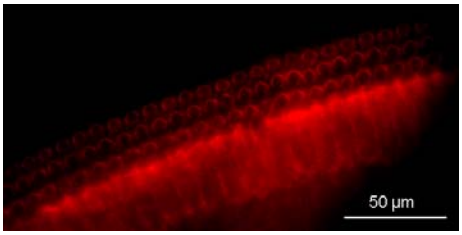
Fig. 20 Number of hair cells per 100µm length of the apical, medial and basal parts of the OC explants from controls and IL-6-treated samples. (A) IHCs; (B) OHCs. Bars represent mean and error bars represent SEM (control: n = 10; 0.3 and 3 ng/ml IL-6: n = 4; 30 and 90 ng/ml IL-6: n = 6).



(A)



(B)



(C)

Fig. 21 Representative fluorescence micrographs of phalloidin-TRITC-labeled hair cells in OC cultures treated with 30 ng/ml IL-6 for 24h. (A) apical part; (B) medial part; (C) basal part.

3.3.2 Effect of exogenous IL-6 on cisplatin-induced hair cell loss in OC cultures

To test the influence of exogenous IL-6 on cisplatin-induced hair cell damage, the OC cultures were pretreated with IL-6 (30 ng/ml) for 1 h and exposed for additional 48 h to cisplatin (15 μM) and IL-6 (30 ng/ml).

Fig. 22 showed the morphology of hair cells in OC cultures exposed to cisplatin only or combined with IL-6. It can be seen that exposure to cisplatin for 48 h led to a substantial OHC loss and partial IHC loss and irregular organization of hair cells in OC cultures. And more OHCs in the third row were missing than in the first and second row.

The number of IHCs in apical and medial parts treated with IL-6 in addition to cisplatin was 9.93 ± 0.14 and 10.23 ± 0.43, showing increase compared to those in cisplatin-treated cultures (relative to 7.11 ± 0.73 and 9.30 ± 0.31, respectively) (both $p < 0.05$, paired *t*-test), while no increase was observed in the basal part (Fig. 23 A).

Cisplatin-induced loss of OHCs was more evident than that of IHCs, and the loss of OHC was more evident in the basal part than apical and medial part. In the medial part of OC cultures exposed to cisplatin combined with IL-6, the surviving OHCs were quantified to be 31.47 ± 2.97,

showing significant promotion relative to cisplatin-treated cultures (28.30 ± 2.32) ($p < 0.05$, paired *t*-test). There is no difference in the number of OHCs in either apical or basal parts between cisplatin-treated cultures and cisplatin plus IL-6-treated cultures (Fig. 23 B).

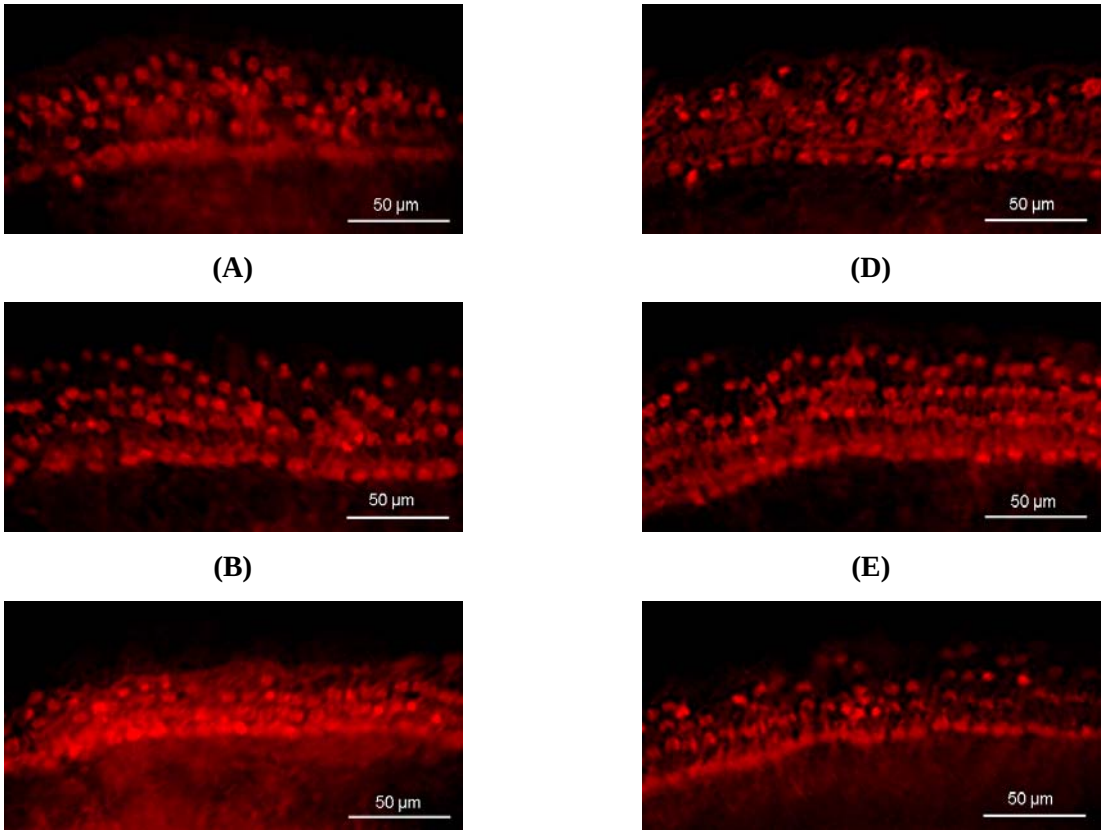
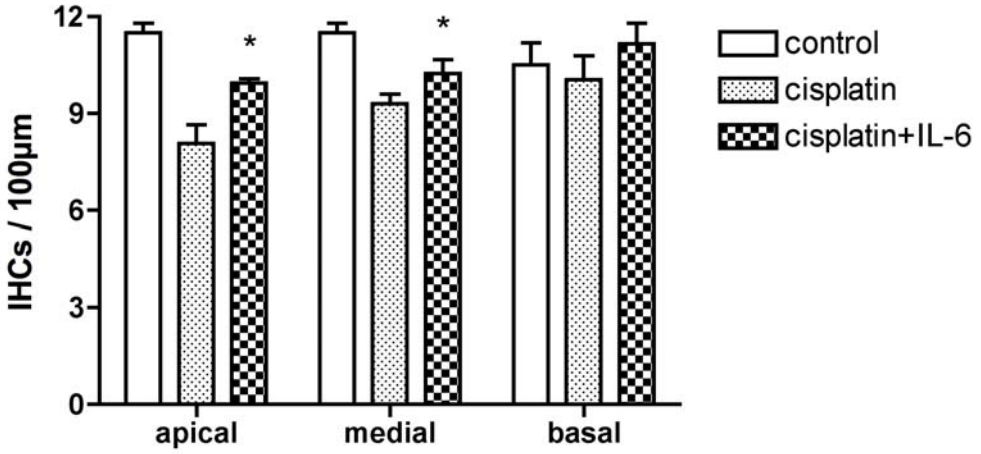
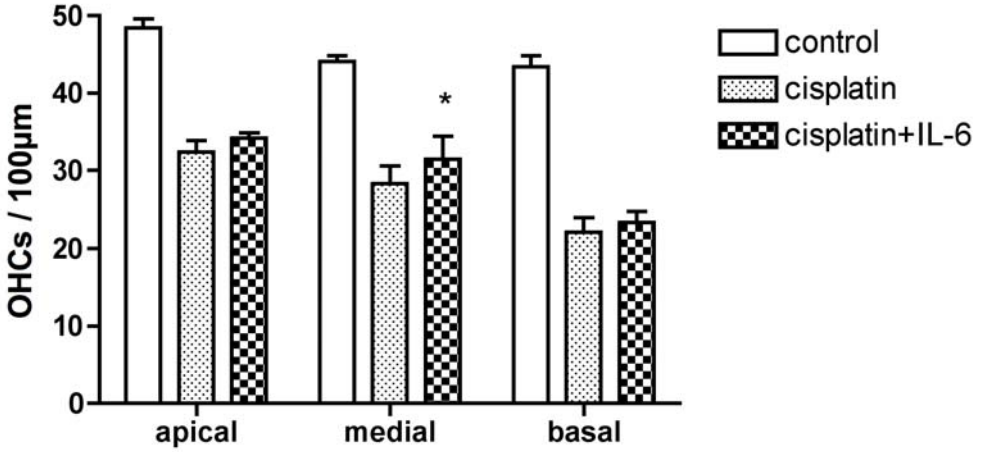


Fig. 22 Representative fluorescence micrographs of phalloidin-TRITC-labeled hair cells in OC cultures treated for 48h with cisplatin (15 μM) only or co-treated with IL-6 (30 ng/ml). (A) cisplatin (apical part); (B) cisplatin (medial part); (C) cisplatin (basal part); (D) cisplatin+IL-6 (apical part); (E) cisplatin+IL-6 (medial part); (F) cisplatin+IL-6 (basal part).



(A)



(B)

Fig. 23 Number of OHCs per 100 µm length of the apical, medial and basal parts of the OC explants from controls, cisplatin-treated samples, cisplatin- and IL-6-treated samples. (A) IHCs; (B) OHCs. Bars represent mean and error bars represent SEM (control, n = 4; cisplatin and cisplatin+IL-6, both n = 6). * $p < 0.05$, significantly different from the cisplatin-treated samples.

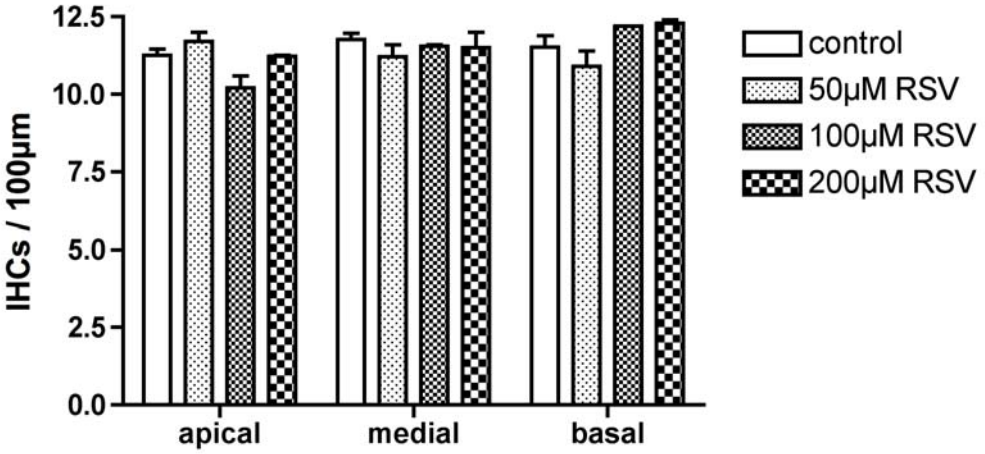
3.4 Effect of resveratrol and geldanamycin on HSP70 expression in OC cultures

3.4.1 Effect of resveratrol and geldanamycin on hair cell viability

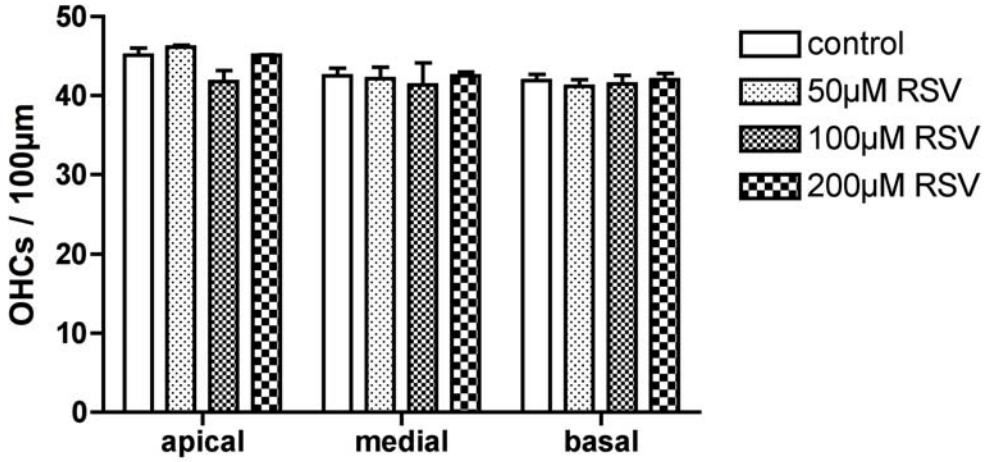
To investigate whether geldanamycin or resveratrol has cytotoxic effects on HCs, OC explants were exposed to geldanamycin or resveratrol at varying concentration for 24 h and HCs viability was determined by hair cell counting.

3.4.1.1 Effect of resveratrol on hair cell viability

Fig. 24 shows the numbers of IHCs and OHCs in OC cultures treated with 50, 100, 200 and 400 μM resveratrol and untreated. There was no significant decrease in numbers of hair cells in three parts of OC treated with resveratrol at the concentrations of 50, 100 and 200 μM, compared to the controls ($p > 0.05$, one-way ANOVA). Fig. 25 shows OC cultures treated with 200 μM RSV for 24h and then stained with phalloidin-TRITC. The morphology of OHCs and IHCs was not affected by resveratrol. It is indicated that resveratrol at the concentration no more than 200 μM has no significant cytotoxic effects on hair cells in OC cultures.

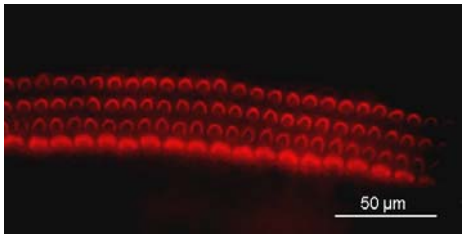


(A)

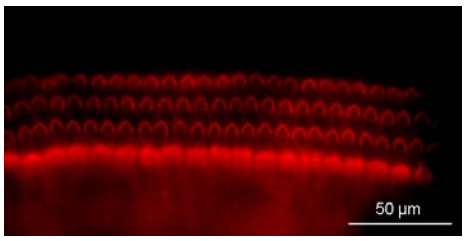


(B)

Fig. 24 Number of hair cells per 100μm length of the apical, medial and basal parts of the OC explants from controls and resveratrol-treated samples. (A) IHCs; (B) OHCs. Bars represent mean and error bars represent SEM (control: n = 6; 50, 100, 200 μM RSV: n = 2). RSV: resveratrol.



(A)



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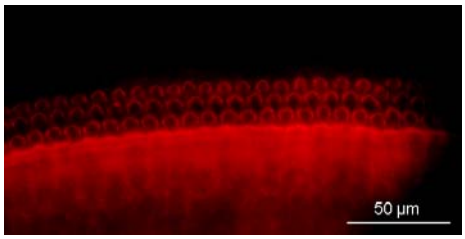
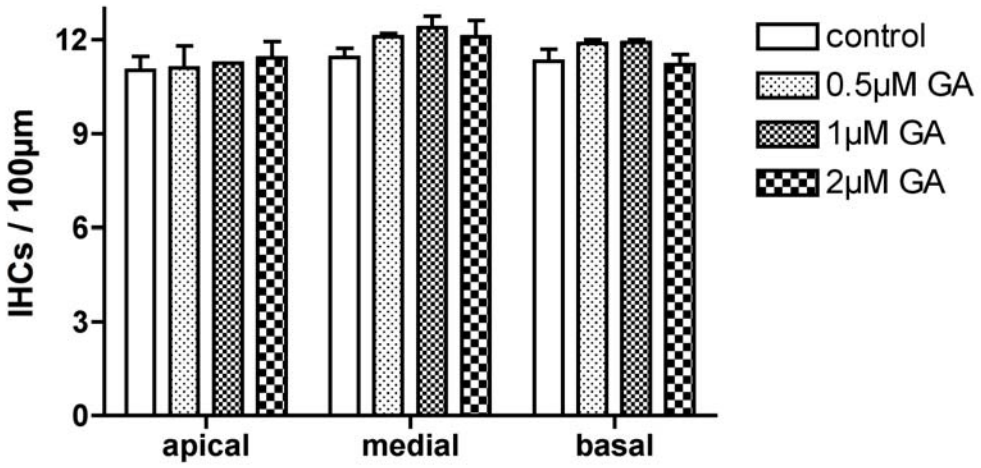


(C)

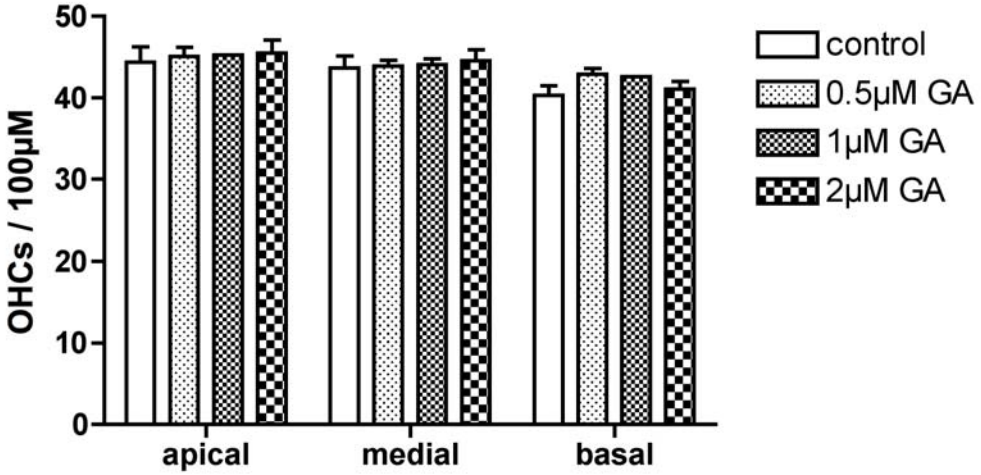
Fig. 25 Representative fluorescence micrographs of phalloidin-TRITC-labeled hair cells in OC cultures treated with 200 µM resveratrol for 24h. (A) apical part; (B) medial part; (C) basal part.

3.4.1.2 Effect of geldanamycin on hair cell viability

Fig. 26 shows the counts of IHCs and OHCs in OC explants treated with 0.5, 1 and 2 µM geldanamycin and untreated control explants. There was no significant decrease or increase in numbers of both OHCs and IHCs in apical, medial or basal part of OC treated with geldanamycin, compared to the controls ($p > 0.05$, one-way ANOVA). Fig. 27 shows OC cultures treated with 2 µM geldanamycin for 24 h and then stained with phalloidin-TRITC to label the actin. Three orderly rows of OHCs and one row of IHCs there were seen. These results suggest that geldanamycin at a concentration of no more than 2 µM has no significant cytotoxic effects on hair cells in OC cultures.

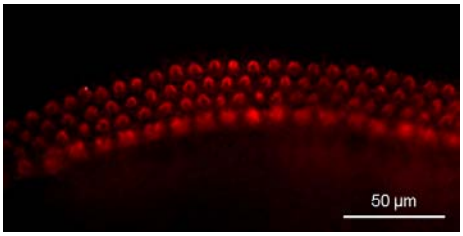


(A)

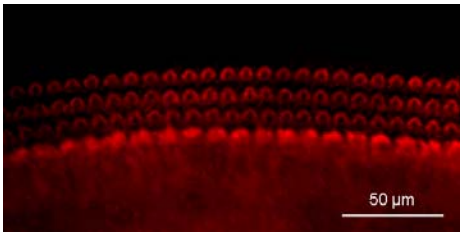


(B)

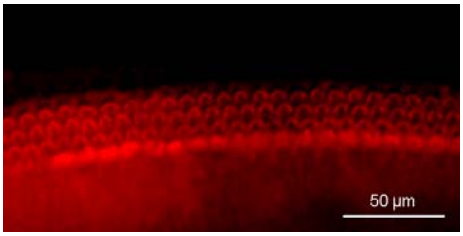
Fig. 26 Number of hair cells per 100μm length of the apical, medial and basal parts of the OC explants from controls and geldanamycin-treated samples. (A) IHCs; (B) OHCs. Bars represent mean and error bars represent SEM (control and 2μM GA: n = 6; 0.5 and 1μM GA: n = 2). GA: geldanamycin.



(A)



(B)



(C)

Fig. 27 Representative fluorescence micrographs of phalloidin-TRITC-labeled hair cells in OC cultures treated with 2 µM geldanamycin for 24h. (A) apical part; (B) medial part; (C) basal part.

3.4.2 Expression of HSP70 in OC cultures treated with resveratrol

To detect whether resveratrol induces the expression of HSP70, OC explants were exposed to 50, 100 and 200 µM resveratrol for 24 h and HSP70 mRNA level was examined by RT-PCR. Fig. 28 shows the HSP70 mRNA expression in OC explants induced by resveratrol. HSP70 mRNA was not induced in OC explants treated with resveratrol at the concentration of 50 and 100 µM (fold change: 0.96 (0.60-2.18) and 1.01 (0.43-1.45), respectively). At the concentration of 200 µM, resveratrol increased the expression of HSP70 mRNA (1.68 (1.03-2.74)), but without statistical significance ($p > 0.05$, Wilcoxon signed ranks test). Based on the result that the treatment with resveratrol at varying concentrations up to 24 hours failed to induce HSP70 expression in OC explants, the planning further time course experiment was not carried out.

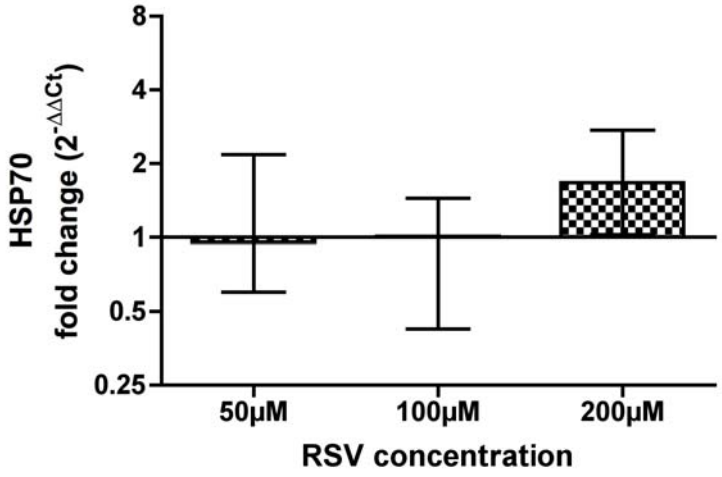


Fig. 28 Expression of HSP70 mRNA in OC explants exposed to resveratrol for 24h at the concentration of 50, 100 and 200 µM. Bars represent median and error bars represent IQR (50 µM, n = 4; 100 and 200 µM, both n = 6). RSV: resveratrol.

3.4.3 Induction of HSP70 by geldanamycin in OC cultures

3.4.3.1 Time course of HSP70 induction by geldanamycin in OC cultures

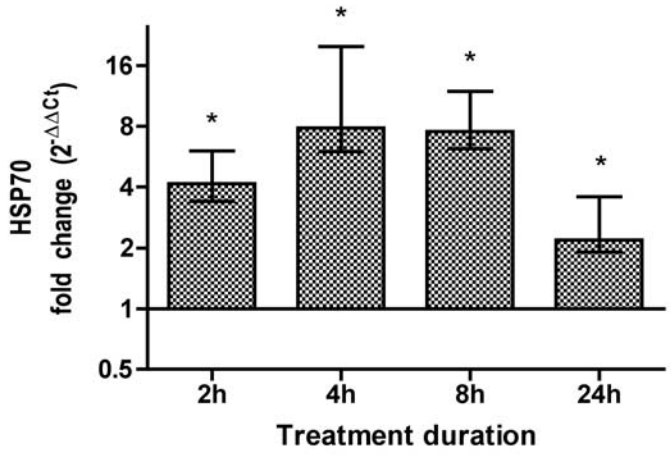
To determine whether geldanamycin induces HSP70 expression in OC, OC explants were treated with 2 µM geldanamycin for up to 24 h and HSP70 expression at mRNA and protein level was quantified by RT-PCR and ELISA of tissue lysates, respectively.

Fig. 29 A shows the time course of HSP70 mRNA expression in OC explants induced by geldanamycin. The HSP70 mRNA levels were significantly increased within 24 h of geldanamycin treatment ($p < 0.05$, Wilcoxon signed ranks test). The induction of HSP70 mRNA (fold change: 4.14 (3.39-6.02)) was evident after 2 h of geldanamycin treatment, was maximal after 4 h and 8 h of treatment (7.84 (5.98-19.84) and 7.52 (6.19-11.96)), and was maintained for at least 24 h in the presence of geldanamycin (2.19 (1.91-3.58)).

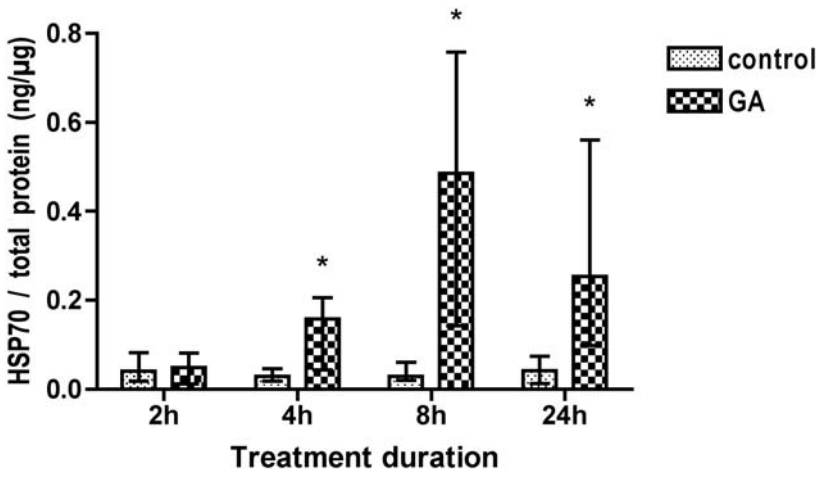
Fig. 29 B illustrates the time course of HSP70 protein expression in OC explants induced by geldanamycin. Compared to the untreated control (0.04 (0.02-0.07) ng/µg), the HSP70 protein level relative to total protein was not changed within 2 h of geldanamycin treatment (0.05 (0.01-0.08) ng/µg). HSP70 protein concentration increased significantly after 4 h of treatment (0.16 (0.05-0.19) ng/µg), compared to the control (0.03 (0.02-0.04) ng/µg) ($p < 0.05$, Wilcoxon signed ranks test). HSP70 protein concentration was further increased after 8 h of treatment (0.49 (0.15-0.74) ng/µg) relative to the control (0.03 (0.02-0.06) ng/µg) ($p < 0.05$, Wilcoxon signed

ranks test). HSP70 protein induction was maintained till 24 h of treatment (0.25 (0.11-0.44) ng/µg) compared with the control (0.042 (0.02-0.07)) ($p < 0.05$, Wilcoxon signed ranks test).

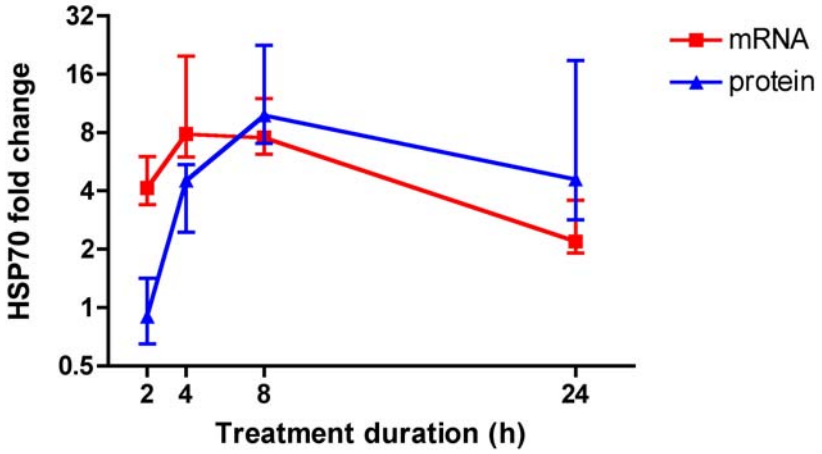
The HSP70 protein concentration relative to total protein in geldanamycin treated OC explants was normalized to the corresponding control and presented as fold change. The time course of HSP70 induction at mRNA level and protein level was compared and shown in Fig. 29 C. HSP70 induction at mRNA level started within 2 h of geldanamycin treatment, was kept at relative high level within 8 h, and declined within 24 h. At protein level, the induction was seen after 4 h of treatment, later than that at mRNA level, and was accumulated at relatively high level within 24 h.



(A)



(B)



(C)

Fig. 29 Time course of HSP70 mRNA and protein expression in OC explants during 2 μM geldanamycin treatment. (A) Time course of HSP70 mRNA relative expression (each n = 7). * $p < 0.05$, significantly different from 1-fold change. (B) Time course of HSP70 protein expression (relative to total protein) (each n = 6). * $p < 0.05$, significantly different from the control. (C) Comparison of time course of HSP70 mRNA and protein expression. The bars represent median and the error bars represent IQR in A and B. The points represent median and the error bars represent IQR in C.

3.4.3.2 Localization of HSP70 induced by geldanamycin in OC cultures

To localize the HSP70 protein induced by geldanamycin in the OC explants, OC cultures were exposed to 2 μM geldanamycin for 4 h and 8 h, and immunohistochemical analysis was performed using anti-HSP70 antibody.

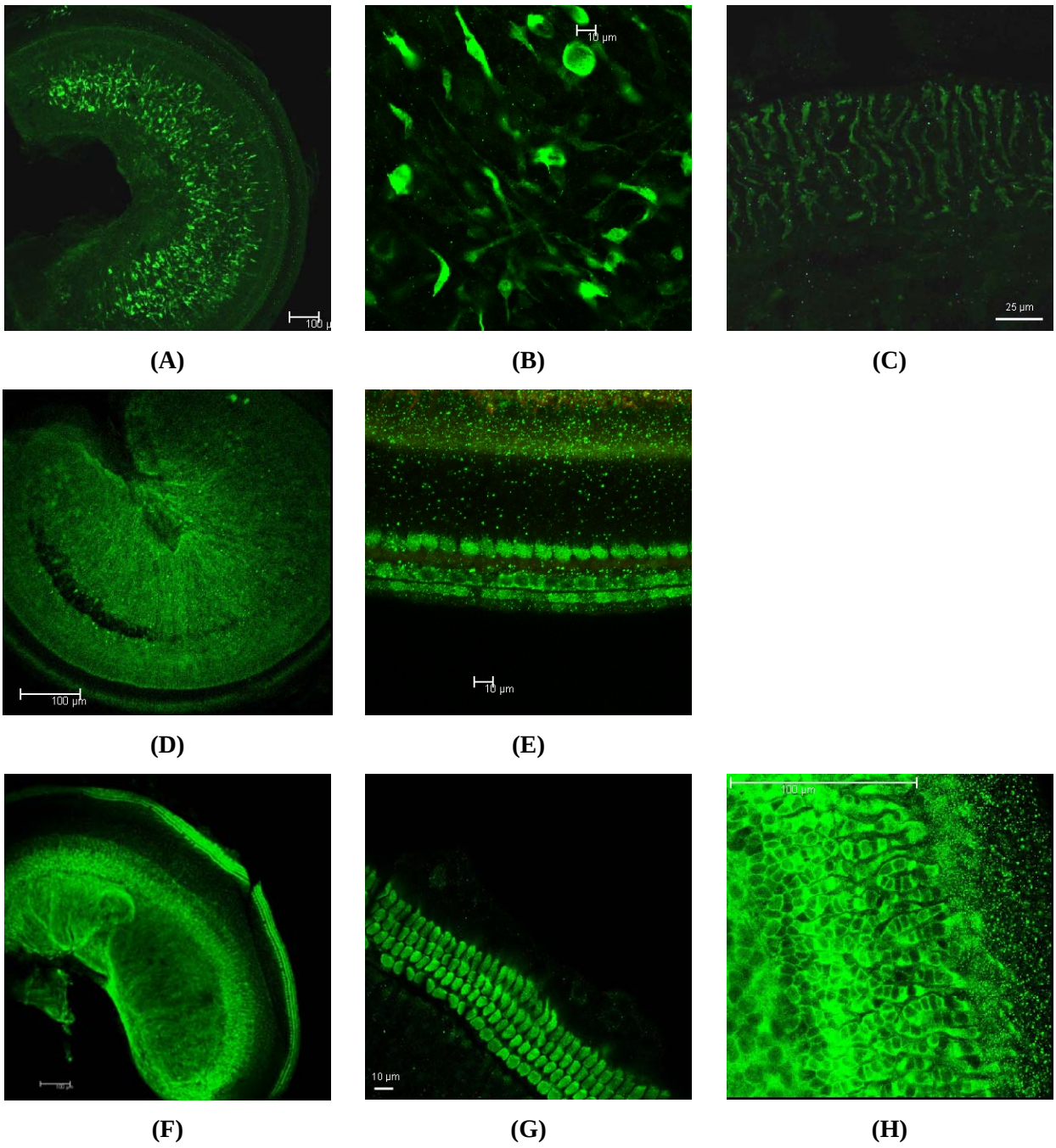
In untreated OC cultures, HSP70 immunoreactivity was mainly observed in spiral limbus (Fig. 30 A), where intensive immunoreactivity was located in irregularly shaped fibrocytes (Fig. 30 B) and less immunoreactivity was presented in interdental cells (Fig. 30 C). Very faint signals were noted over hair cells.

Figs. 30 D and E reveal that a robust HSP70 immunoreactivity in IHCs and all three rows of OHCs as well as spiral limbus after 4 h of geldanamycin treatment.

After 8 h of exposure to geldanamycin, positive immunoreactivity was more intensive in hair cells (Fig. 30 F and G) and interdental cells, which are lodged in groups in corresponding parallel slits formed by collagen fibers and have polygonal, plate-like processes [Krstic, 1991] (Fig. 30 F and H).

In either geldanamycin-treated OC or control fragment, no positive immunostaining reactivity was seen in the somata and peripheral neurites of spiral ganglion neurons. No HSP70

immunoreactivity was observed in the negative control OC fragments stained with the secondary FITC-labeled antibody only.



(A) **(B)** **(C)**

(D) **(E)**

(F) **(G)** **(H)**

Fig. 30 Representative laser confocal micrographs of geldanamycin-treated and untreated OC cultures labeled with FITC-conjugated secondary antibody against a primary antibody of HSP70. (A), (B) and (C): untreated control; (D) and (E): geldanamycin treatment for 4 h; (F), (G) and (H) geldanamycin treatment for 8 h.

3.5 Effect of geldanamycin on gentamicin-induced hair cell loss

To study the influence of geldanamycin on gentamicin ototoxicity, OC cultures were subjected to either treatment protocol: 4 h of geldanamycin (2 μM) pretreatment prior to 24 h of gentamicin treatment (500 μM), or simultaneous treatment with geldanamycin (2 μM) and gentamicin (500 μM) for 24 h, then stained with phalloidin-TRITC to display hair cells.

3.5.1 Effect of geldanamycin on gentamicin-induced outer hair cell loss

Exposure to gentamicin for 24 h led to a severe loss of OHC in the apical, medial and basal parts OC cultures. The stereocilia bundles were missing on most remaining OHCs. And more OHCs in the first row were missing than those in the second and third rows (Fig. 31).

OC samples pretreated with geldanamycin prior to gentamicin exposure presented significantly decreased OHC loss in all the three parts, compared to those treated with GM alone (Fig. 31). Hair cell quantification showed the number of surviving OHCs was 39.27 ± 0.80 (relative to 33.45 ± 1.33) in apical part, 31.48 ± 0.84 (relative to 25.14 ± 0.99) in medial part, and 22.64 ± 1.02 (relative to 19.13 ± 0.97) in basal part of OC explants pretreated with geldanamycin ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively, paired *t*-test) (Fig. 32 A).

Similar results were obtained from OC cultures treated with geldanamycin and gentamicin simultaneously. The surviving OHCs in OC cultures treated with geldanamycin and gentamicin were quantified to be 35.91 ± 1.70 (relative to 30.17 ± 1.42) in apical part, 28.17 ± 1.61 (relative to 20.73 ± 0.71) in medial part, and 22.10 ± 1.47 (relative to 17.48 ± 1.75) in basal part, showing significant promotion of OHC survival relative to gentamicin-treated OC cultures ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively, paired *t*-test) (Fig. 32 B).

These results suggested that geldanamycin treatment significantly inhibited gentamicin-induced OHC loss in OC explants.

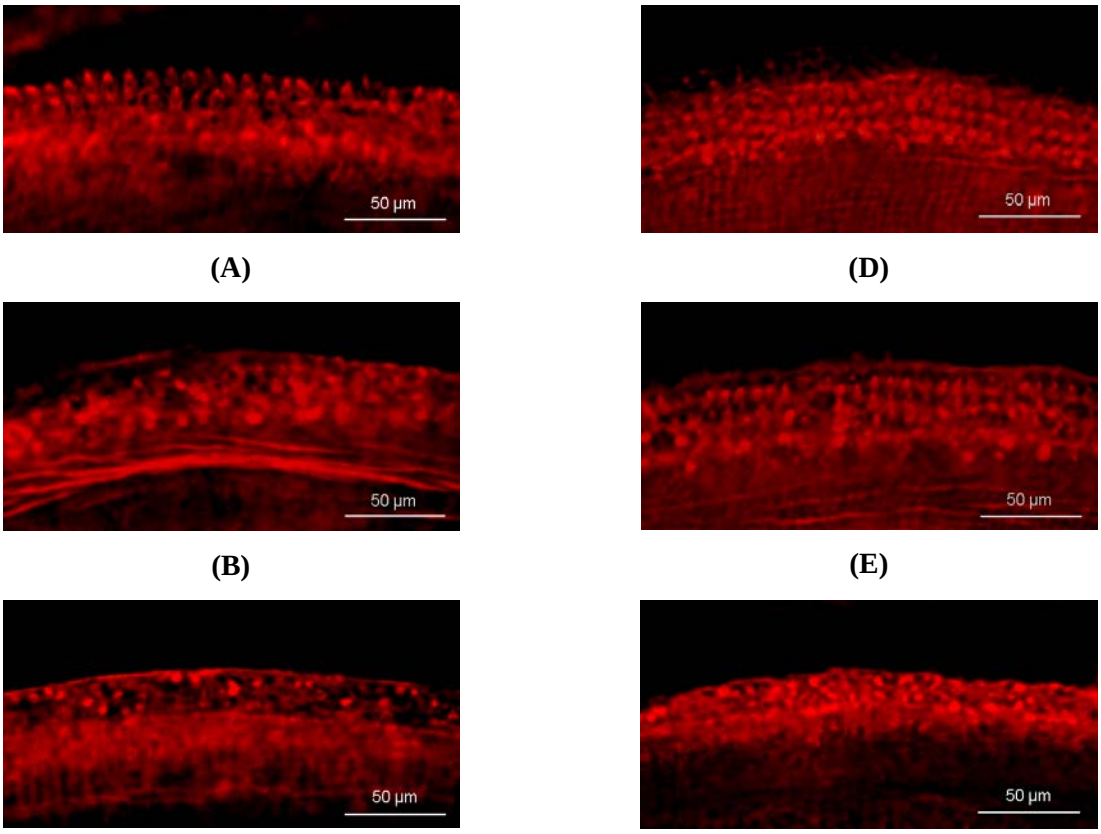
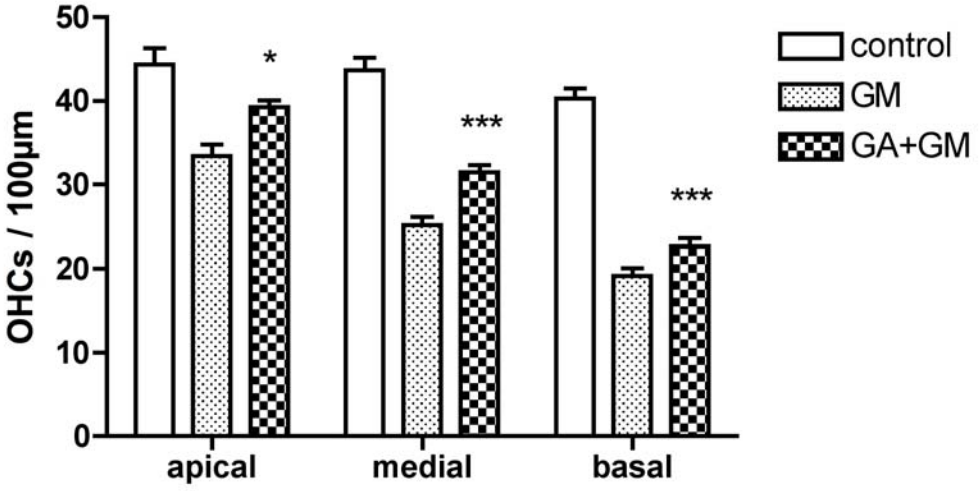
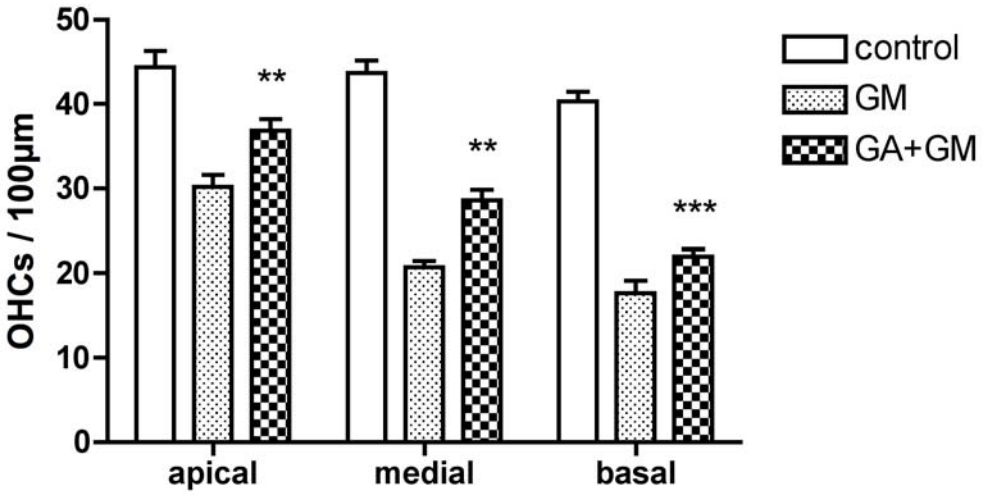


Fig. 31 Representative fluorescence micrographs of phalloidin-TRITC-labeled hair cells in OC cultures treated with gentamicin (500 μM) alone for 24 h or pretreated with geldanamycin for 4 h prior to 24 h of gentamicin exposure. (A) GM (apical part); (B) GM (medial part); (C) GM (basal part); (D) GA+GM (apical part); (E) GA+GM (medial part); (F) GA+GM (basal part). GM: gentamicin; GA: geldanamycin.



(A)

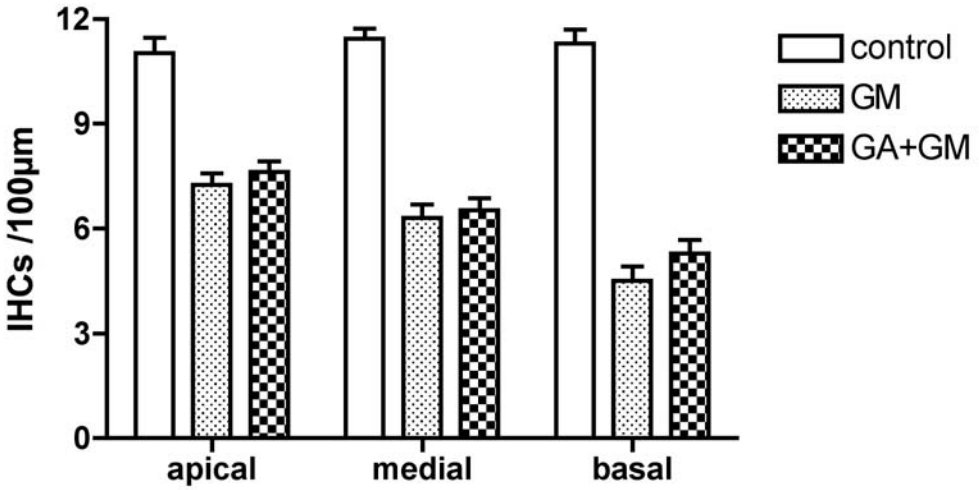


(B)

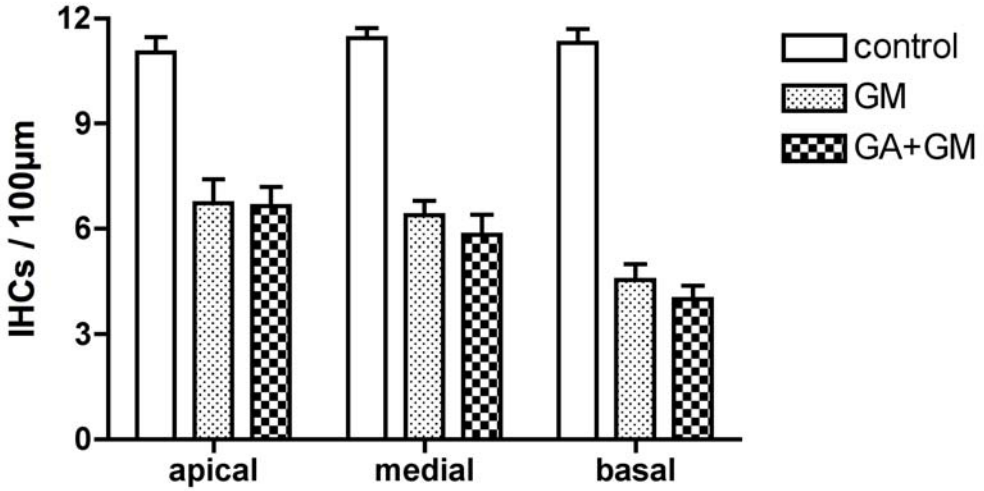
Fig. 32 Number of OHCs per 100 µm length of the apical, medial and basal parts of the OC explants from controls, gentamicin-treated samples, geldanamycin and gentamicin-treated samples. (A) 4 h of GA (2 µM) pretreatment prior to 24 h of GM treatment. (B) Concurrent treatment with GA (2 µM) and GM (500 µM) for 24 h. Bars represent mean and error bars represent SEM (control, n = 6; GM and GA+GM, both n = 8 in (A) and both n = 9 in (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from the GM-treated samples. GM: gentamicin; GA: geldanamycin.

3.5.2 Effect of geldanamycin on gentamicin-induced inner hair cell loss

The gentamicin treatment also caused a large loss of IHC as well as OHC. Fig. 33 displays the survival of IHC in all the three parts of OC explants exposed to gentamicin treatment and simultaneous treatment with geldanamycin and gentamicin. The number of surviving OHCs was 7.62 ± 0.30 (relative to 7.25 ± 0.33) in apical part, 6.53 ± 0.34 (relative to 6.31 ± 0.38) in medial part, and 5.30 ± 0.37 (relative to 4.52 ± 0.40) in basal part of OC explants pretreated with geldanamycin for 4 h (Fig. 33A). In OC explants exposed to geldanamycin and gentamicin simultaneously, the surviving IHCs were quantified to be 6.37 ± 0.66 (relative to 6.72 ± 0.68) in apical part, 5.60 ± 0.71 (relative to 6.37 ± 0.41) in medial part, and 3.98 ± 0.36 (relative to 4.20 ± 0.37) in basal part (Fig. 33 B). There is no difference of the number of surviving IHCs between samples treated with gentamicin alone and samples treated with geldanamycin and gentamicin ($p > 0.05$, paired *t*-test). This suggested that the loss of IHC induced by gentamicin was not attenuated by geldanamycin treatment either prior to or concurrent with gentamicin exposure.



(A)



(B)

Fig. 33 Number of IHCs per 100 µm length of the apical, medial and basal parts of the OC explants from controls, gentamicin-treated samples, geldanamycin plus gentamicin-treated samples. (A) 4 h-pretreatment with geldanamycin (2 µM) prior to 24-h treatment of gentamicin (500 µM). (B) concurrent geldanamycin treatment with 500 µM gentamicin treatment for 24 h. Bars represent mean and error bars represent SEM (control, n = 6; gentamicin and geldanamycin + gentamicin, both n = 8 in (A) and both n = 9 in (B).

4 Discussion

4.1 Cochlea and inflammation

Inflammation is a first step in the healing process. Inflammation involves a complex set of interactions among soluble factors and cells that can arise in any tissue in response to trauma, infection, ischemia, toxin or immune reaction [Nathan, 2002]. The inducers of inflammation can be classified into two groups: microbial inducers, which cause infectious inflammation, and non-microbial inducers such as allergens, physical injury, chemical irritants, toxic compounds and foreign bodies, which lead to sterile inflammation [Medzhitov, 2008]. Inflammation inducers trigger the production of a large number of mediators, which in turn alter the function of cells and tissues.

4.1.1 Inflammatory cytokines

A group of soluble inflammatory mediators, namely proinflammatory cytokines (including IL-1, IL-6, Il-18, TNF-α), are produced in a wide variety of body cells in response to various inflammation inducers and involved in the amplification of inflammatory reactions.

IL-1β (which belongs to IL-1 superfamily of cytokines) is a proinflammatory cytokine involved in immune regulation and various inflammatory processes. IL-1 induces the expression of many effector proteins such as other cytokines, chemokines and nitric oxide synthetase [Dinarello, 2002].

IL-6 is an endogenous pyrogen, one of the most important proinflammatory cytokines in the acute phase reaction and a stimulator of both B and T cell functions. Moreover, IL-6 activates endothelial cell production of a subset of chemokines and adhesion molecules, thus contributing indirectly to the recruitment of leukocytes at inflammatory sites [Feghali and Wright, 1997]. IL-6 is considered as an anti-inflammatory as well as a proinflammatory cytokine. In models of chronic inflammatory diseases, such as collagen-induced arthritis, murine colitis, or experimental autoimmune encephalomyelitis, IL-6 is proinflammatory, whereas in models of acute inflammation, IL-6 exhibits an anti-inflammatory profile [Gabay, 2006]. In the central neuronal system, IL-6 acts as a double-edged sword: it has been shown that blockage of IL-6 ameliorated functional recovery in experimental spinal cord injury [Okada et al., 2004], whereas in an experimental cerebral ischemic model it aggravated cerebral damage [Yamashita et al.,

2005]. It also plays important roles in anti-oxidative stress by upregulating anti-apoptotic genes [Lin et al., 2001]. Numerous studies have demonstrated that IL-6 exerts a protective effect on liver in ischemia/reperfusion injury [Tiberio et al., 2006], hyperoxic lung injury [Ward et al., 2000] and cardiomyocytes in ischemia/reperfusion injury [Smart et al., 2006].

IL-18, a recently described member of interleukin-1 superfamily, is important in both innate and acquired immune response. It stimulates neutrophil migration and activation as well as T helper 1 cell proliferation and interferon-γ secretion in a variety of cell types [Gracie et al., 2003].

TNF-α is a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF-α is regulation of immune cells. Depending on co-stimulatory molecules, TNF-α can induce apoptotic or necrotic cell death as well as inflammation [Locksley et al., 2001].

4.1.2 Intracellular and extracellular HSP70

HSP70 was first characterized as intracellular protein produced in response to heat shock. With time, several research groups have shown that there are various types of stress including environmental (ultraviolet radiation, heat shock, heavy metals and amino acids), pathological (viral, bacterial or parasitic infections, fever, inflammation, malignancy and autoimmunity) or physiological stress (growth factors, cell differentiation, hormonal stimulation or tissue development) that induce a marked increase in intracellular HSP70 synthesis, known as a stress response [Lindquist and Craig, 1988]. The intercellular HSP70 functions as chaperone maintaining the dynamic stability of protein folding and inhibiting protein aggregation [Bukau et al., 2006]. These functions serve to improve cell survival after cellular stress [Hartl, 1996]. Intracellular HSP70 was also demonstrated to be an endogenous anti-inflammatory molecule. In various experimental settings, HSP70 blocked neutrophil infiltration, inhibited the production of inflammatory cytokines, prevented responses to inflammatory cytokines and prevented reactive oxygen species-induced DNA strand breaks, thus protecting cells from the deleterious effects of inflammation [Ding et al., 2001b; Jacquier-Sarlin et al., 1994].

Recently, it has been found that HSP70 was expressed in extracellular microenvironment. HSP70 can be released from a variety of cell types including neuronal cells, epithelial cells, monocyte, rat embryo cells, B cells, and tumor cells [Giffard et al., 2008]. In a pathophysiological state, extracellular HSP70 exacerbates inflammatory diseases such as Alzheimer's disease and inflammatory bowel disease [Pockley, 2002]. Some reports

demonstrated that extracellular HSP70 induced the release of cytokines including IL-1, IL-6 and TNF-α from monocytes. This release was mediated through a toll-like receptor 2 (TLR2), TLR4 and involved in the activation of nuclear factor kappa B (NFκB), a transcription factor that regulates the inducible expression of a wide range of proinflammatory cytokines [Asea et al., 2002]. However, other reports suggested that this effect was actually, at least partly, due to contamination with lipopolysaccharide (LPS) [Gao and Tsan, 2003]. In opposite direction, extracellular HSP70 can also be anti-inflammatory. Administration of HSP70 *in vivo* can prevent or arrest inflammatory damage in experimental disease models and in patients with chronic inflammatory disease in initial clinical trials, and promote the production of anti-inflammatory cytokines [Van Eden et al., 2005].

4.1.3 Cochlea and inflammation

Inflammation and proinflammatory cytokines have been implicated to be involved in cochlear pathophysiology. The mRNA encoding NFκB, IL-1, TNF-α was found in cochlear tissue, and NFκB protein was evidently localized in type I fibrocytes and root cells within spiral ligament, interdental cells of spiral limbus and non-sensory cells within OC, indicating that those cochlear cells may be specialized in expressing inflammatory cytokines [Adams, 2002]. The effect of cytokines may be autocrine or paracrine, therefore, cochlear reactivity to cytokines implies the presence of corresponding surface receptors on cochlear cells, which could make cells vulnerable to damage by cytokines and related compounds.

In experimental animal model of cochlear sterile inflammation, an increase in IL-1β and IL-6 expression was observed in cochlear fibrocytes in response to local inflammation [Satoh et al., 2002]. It was demonstrated that proinflammatory cytokines were produced in noise-damaged cochlea [Cheng et al., 2005]. In another study, mechanical stimulus such as transcranial vibration during temporal bone drilling produced cochlear shear stress that is connected with up-regulation of TNF-α and its receptors [Zou et al., 2005]. Hypoxia-induced cochlear dysfunction is also mediated by inflammatory cytokines [Tabuchi et al., 1999]. The expression of IL-6 was induced in HEI-OC1 cell line under hypoxia mimic condition [Jeong et al., 2005]. Additionally, it was demonstrated that ototoxicity induced by cisplatin was mediated by proinflammatory cytokines [So et al., 2007].

Interestingly, corticosteroids, widely used anti-inflammatory medications, can improve hearing in immune-mediated hearing loss [McCabe, 1989] and sudden hearing loss [Rauch, 2008]. Non-steroidal anti-inflammatory drugs also display protective effect on the functional

recovery of the cochlea after acoustic injury [Hoshino et al., 2008]. In an experimental animal model for sensorineural hearing loss caused by sterile inflammation, cochlear microperfusion, which facilitates removal of cytotoxic products of inflammation during the acute phase of inflammation, produced significant elevation of hearing threshold [Barkdull et al., 2005].

This evidence suggest that inflammation and inflammatory cytokines are involved and in some case are critical in pathogenesis of many types of cochlear disorders including ototoxicity.

4.2 Effect of salicylate on gene and protein expression in cochlea and VOT-E36 cells

It has long been known that a number of drugs and chemicals have a damaging effect on the auditory and vestibular compartments of the inner ear. This effect is often referred to as "ototoxicity". Salicylate, a well-known ototoxic agent, presents a distinctive pattern of toxicity, that is, for the most part, completely reversible tinnitus and reversible hearing loss. The mechanism of salicylate auditory toxicity seems to be multilevel. Physiological data have revealed modifications of cochlear blood flow, OHCs and afferent cochlear nerve fiber activity in salicylate ototoxicity [Cazals, 2000]. Anatomical examination indicated significant alteration only at OHC lateral membrane. Dieler et al. observed dilation, vesiculation and modification of the parallel arrangement of lateral membrane surface cisternae, and these alterations were reversible [Dieler et al., 1991]. *In vitro* isolated OHCs showed reduction of their fast motile response, which are thought to result in loss of absolute acoustic sensitivity in humans and animals [Lue and Brownell, 1999]. Great efforts have been made in electrophysiological studies on auditory system and interference with mechanical and motile properties of OHCs induced by salicylate. Salicylate has been demonstrated to act as a competitive antagonist at the anion-binding site of prestin, the motor protein of OHCs [Oliver et al., 2001], resulting in inhibition of OHCs electromotility. It has been indicated that salicylate produced clear alteration of otoacoustic emission (OAE) including spontaneous OAE [Stewart and Hudspeth, 2000] and distortion product otoacoustic emission (DPOAE) [Kujawa et al., 1994].

Salicylate has been observed to interfere with normal transcription processes in animal cells. It has been found that salicylate inhibits NF-κB activity by preventing its translocation to the nucleus [Kopp and Ghosh, 1994]. In contrast to its effect on NF-κB, presence of salicylate activates heat shock factor 1 (HSF1), the transcription factor activating heat shock genes transcription in a variety of cell types and species [Housby et al., 1999; Jurivich et al., 1992]. However, there is very limited evidence showing the effect of salicylate on gene expression in

cochlea or OHCs. A recent study using oligonucleotide microarray revealed differential gene expression induced by salicylate ototoxicity. Authors demonstrated that approximately 200 genes involved in various biological processes were significantly up- or down-regulated [Im et al., 2007]. The property of salicylate altering gene expression can be useful for understanding how salicylate exerts its ototoxic effect in the auditory system on the molecular level.

In the present study, we analyzed the effect of salicylate on expression of major proinflammatory cytokines (IL-1β, IL-6, IL-18 and TNF-α) and HSP70 in two distinct *in vitro* models, VOT-E36 cells and OC explants.

4.2.1 Expression of hair cell markers in cochlea and in VOT-E36 cells

As an experimental model, VOT-E36 cell line was derived from the ventral otocyst of the immortomouse at embryonic day 10.5 and expresses a number of sensory epithelia specific markers, including hair cell cytoskeleton protein myosin VIIa. Thus, VOT-E36 cells can be used as a tool for the *in vitro* study of the influence and the mechanism of ototoxic agent on auditory cells.

The expression of myosin VIIA has been observed in the otocyst of mouse at embryonic day 9 [Sahly et al., 1997]. At embryonic day 15, myosin VIIA begins to be detected in IHCs in the basal region of the cochlea, and at embryonic day 16, myosin VIIA is clearly expressed in developing inner hair cells, and some of the outer hair cells in the basal end of the cochlea [Bermingham-McDonogh et al., 2006]. In VOT-E36 cells, myosin VIIa protein was not detected when the cells were growing under proliferation conditions, whereas under differentiation conditions myosin VIIa was detected at low levels after 14 days [Helyer et al., 2007]. The present study shows the expression of *myosin VIIa* at transcriptional level already after 7 days of differentiation. It is possible that the expression of transcript encoding *myosin VIIa* on 7th day of differentiation is connected with a low-level protein expression.

Prestin, a molecular marker of cochlear OHCs, was also examined on transcriptional level in VOT-E36 cells. During development of rat cochlea, prestin is initially expressed throughout the basolateral membrane of OHCs and subsequently redistributed only to lateral membrane between postnatal day 7 and day 12 [Weber et al., 2002]. In a recent study, Abe et al. [Abe et al., 2007] demonstrated that prestin mRNA in mouse OHCs peaked near postnatal day 10 and OHC motor activity reaches adult levels at postnatal day 18. VOT-E36 cells were derived from otocyst of immortal mouse at embryonic day 10.5. In this study, regardless of length of time used for differentiation of VOT-E36 cells, they never have expressed prestin. This suggests that *prestin* is a gene expressed late during outer hair cell maturation and VOT-E36 cells after differentiation

for up to 28 days still have an immature phenotype. Alternatively, it could be possible that VOT-E36 cells differentiate into IHC rather than OHC phenotype, as IHC does not express prestin.

4.2.2 Effect of salicylate on the expression of hair cell markers

In the inner ear, myosin VIIa has been localized along the length of the stereocilia of hair cells and the cuticular plate and pericuticular necklace, a vesicle-rich zone within hair cells [Hasson, 1997]. By binding to vezatin and harmonin, myosin VIIa links the actin core to the plasma membrane of the cilia [Kussel-Andermann et al., 2000; Verpy et al., 2000]. Myosin VIIa plays an important role in sustaining the normal morphology of hair bundles. Mice lacking myosin VIIa have disarrayed hair bundles, which causes deafness [Liu et al., 1999]. Additionally, myosin VIIa is involved in mechanotransduction in hair cells by fixing membrane-bound elements to the actin core of the hair bundles [Gillespie, 2002]. A recent study reported that administration of salicylate did not increase the myosin VIIa mRNA expression in adult guinea pigs and mice [Yu et al., 2008]. In the present study, *myosin VIIa* showed a relatively stable expression pattern in VOT-E36 cells after salicylate treatment.

Prestin is the motor protein of outer hair cells responsible for the OHC motile activity [Zheng et al., 2000]. Prestin is localized to the lateral plasma membrane of OHCs, the region where electromotility occurs, but not in the nonmotile inner hair cells [Belyantseva et al., 2000]. Prestin belongs to the anion transporter family, SLC26, which mediate the exchange of chloride and carbonate (two anions found to be essential for OHCs motility) across the plasma membrane [Lohi et al., 2000]. Salicylate, a competitive antagonist at the anion-binding site of prestin, significantly reduces OHC electromotility [Oliver et al., 2001]. Defective expression of prestin (e.g. mutation) may cause a loss of OHC electromotility. A targeted *prestin* gene deletion strategy resulted in loss of outer hair cell electromotility *in vitro* and a 40-60 dB loss of cochlear sensitivity *in vivo* [Liberman et al., 2002]. Hypoxia and ischemia were demonstrated to decrease prestin mRNA in OC cultures, parallel with OHC loss [Gross et al., 2005]. Chen showed that prestin expression in rat cochlea was significantly up-regulated after noise exposure [Chen, 2006]. A very recent study reported that long-term administration of salicylate increased prestin expression at mRNA and protein level. Consistent with that, the present study indicates that prestin gene expression is significantly up-regulated in OC explants in relatively late phase after 24 h exposure to salicylate. It is not known whether the increase of prestin mRNA affects the protein level. In addition, it is not clear if the increased transcription of *prestin* reflects an attempt to replace prestin "blocked" by salicylate with a functional new prestin or if the overexpression of prestin contributes to the dysfunction of OHCs electromotility. Further

experiments are needed to determine possible modulation of prestin on protein level. Nevertheless, it is tempting to speculate that overexpression of prestin could possibly be one of elements responsible for the hearing loss and tinnitus induced by salicylate.

4.2.3 Effect of salicylate on transcriptional expression of proinflammatory cytokines in cochlea and in VOT-E36 cells

The influence of salicylate on the expression of transcripts encoding major proinflammatory cytokines was examined in this study. NFκB is a major transcription factor regulating the expression of several proinflammatory cytokines. Salicylate has been reported to inhibit NFκB activation in a cell culture [Yin et al., 1998]. In addition, salicylate inhibited the expression of proinflammatory cytokines induced by LPS through NFκB-dependent mechanism in various cell types such as splenocytes [Wang et al., 2006b], monocytes [Housby et al., 1999], lung epithelial cells [Bitko et al., 1997] and tumor cells [Portis et al., 2001]. However, it was indicated that salicylate at pharmacological concentration (10^{-7} to 10^{-3} M) had no effect on NFκB transactivation induced by TNF-α in quiescent human fibroblasts, in contrast to the potent anti-NFκB effect exerted by salicylate at suprapharmacological concentration (>5 mM) [Saunders et al., 2001].

In this study, the transcriptional expression of IL-6 rose upon salicylate treatment in VOT-E36 cells and in the OC explant cultures. Similarly, expression of TNF-α in OC was also upregulated after salicylate treatment (TNF-α mRNA was not detected in VOT-E36 cells) in the early phase, which was similar to the results from the studies on noise-induced damaged cochlea [Fujioka et al., 2006] and cisplatin-induced damaged cochlear cells [So et al., 2007]. It was reported that aspirin enhanced the expression of proinflammatory cytokines (IL-1, IL-6 and TNF-α) in human intestinal myofibroblasts. This enhancement was regulated at the level of message stability *via* activation of p38 (mitogen-activated protein kinase, MAPK) [Mifflin et al., 2004]. It was indicated that hypoxia-induced IL-6 production is mainly associated with activation of p38 in the cochlear auditory cell line (HEI-OC1 cells) [Jeong et al., 2005]. Lee et al. (2004) demonstrated that in epithelial cells, IL-18 signal transduction is primarily *via* p38 MAPK pathway rather than NFκB [Jeong et al., 2005; Lee et al., 2006]. In addition, NFκB translocation and binding were found to be inhibited after kanamycin treatment, whereas cotreatment with salicylate promoted NFκB translocation into nuclei of OHCs and prevented the increase of IκB kinase α, the inhibitory binding protein of NFκB, induced by kanamycin [Jiang et al., 2005]. In the present study, unlike IL-6 and TNF-α, expression of transcripts encoding IL-

1β and IL-18 was not significantly induced after salicylate treatment of OC explants. This implies that the expression of IL-1β and IL-18 is mediated through a different signaling pathway than IL-6 and TNF-α. Thus, it is likely that the effects of salicylate may vary in different cells. Additionally, the mechanism of salicylate-induced IL-6 and TNF-α mRNA expression in OC observed in this study may still be NFκB-dependent

An *in vivo* loss of function analysis demonstrated that IL-1β expression was a general cochlear response to trauma and that TNF-α was a deteriorating factor in cochlear inflammation [Satoh et al., 2002]. It was also demonstrated that proinflammatory cytokines including TNF-α, IL-1β and IL-6 were produced in noise-stimulated cochlea and by cisplatin-treated HEI-OC1 cells, which was considered to initiate an inflammatory response in cochlear damage [Fujioka et al., 2006; So et al., 2007]. Therefore, although no direct evidence was obtained, we conclude that the up-regulation of IL-6 and TNF-α observed in presence of salicylate could be a possible part of the salicylate ototoxicity.

4.2.4 Effect of salicylate on transcriptional expression of HSP70 in cochlea and in VOT-E36 cells

In mammalian cells, the transcription of various heat shock protein genes is mediated by the conversion of a transcription factor heat shock factor (HSF) from an inactive to an active form and translocation to the nucleus [Morimoto et al., 1992]. Salicylate activates HSF in mammalian cells (HeLa cells), but the transcription of HSP genes may not be induced by a relatively low dose of sodium salicylate (20-30 mM) [Jurivich et al., 1992]. It has been demonstrated that in HeLa cells, at a concentration of 20 mM, salicylate activates binding of HSF to DNA but does not activate the transcriptional competency [Jurivich et al., 1992]. A recent study showed that a high dose of sodium salicylate (45-60 mM) induced the activation of HSP70 promoter activity and resulted in the accumulation of HSP70 protein in mouse cells (mouse fibroblast C3H10T1/2 cells and mouse embryonic F9 cells) and human cells (HeLa cells) [Ishihara et al., 2003]. These results suggested that a relative high concentration of salicylate is needed to induce a transcription of HSP genes. In contrast to above reports, the present study shows that sodium salicylate at a much lower concentration (2.5 mM) induces transcriptional expression of HSP70 in VOT-E36 cells and rat OC explant cultures.

In this study, it was found that the up-regulation of HSP70 transcription in OC cultures was initiated in an early stage (3 h) of exposure to salicylate. Moreover, this up-regulation event was also observed after 24h exposure. The induction of HSP70 in VOT-E36 cells displayed a similar

temporal expression pattern. As HSP70 plays important roles in cell resistance against stress, induction of HSP70 by salicylate may imply protective effects on salicylate.

4.2.5 Effect of salicylate on protein expression in cochlea and in VOT-E36 cells

In the present study, protein expression of proinflammatory cytokines and HSP70 were determined in VOT-E36 cells and OC explant cultures.

First, intracellular HSP70 protein level in VOT-E36 cells and OC explant cultures was examined by Western blot. HSP70 protein was detected in both salicylate-treated and untreated samples. However, the expression of HSP70 protein seemed not to be regulated by salicylate in both VOT-E36 cells and OC, which is inconsistent with the transcript level.

Western blot is not a method of choice for quantitative analysis, therefore ELISA was also used to determine the intracellular HSP70 protein concentration in the lysates of OC cultures. Despite its quantitative nature and high sensitivity, ELISA did not detect modulation in intracellular HSP70 concentration, as compared to salicylate-untreated controls.

As for extracellular (secreted) HSP70, no augmentation of protein expression was observed with ELISA in supernatant of OC cultures. Thus, it appears that HSP70 protein expression, both intracellular and extracellular, was not induced by salicylate in OC.

Dissimilar expression of HSP70 at mRNA and protein level has been observed in some previous studies. In a study of hyperthermia-stressed rodent cochlea, it was found that HSP70 mRNA was induced in rat OC after heat stress, but no HSP70 protein was produced; and in guinea pig, HSP70 was present in both unstressed and heat-stressed cochlea and no difference was revealed between them [Dechesne et al., 1992]. A similar situation was noted in another study on gerbil brain, which indicated that induction of HSP70 mRNA was elevated after ischemia, but HSP70 protein failed to accumulate in hippocampal CA1 neurons [Nowak, Jr., 1991].

It is possible that noticeable expression of HSP70 protein in VOT-E36 cells and OC explants occurred beyond the period during which protein expression was detected in this study. It is also likely that the inconsistency between the expression of protein and that of mRNA could be attributed to a relatively low level of protein translation. The HSP70 protein synthesis appears to be correlated with types of stress used to induce HSP70. It has been shown that HSP70 protein level increased in rat OHCs stressed by ischemia [Myers et al., 1992] or noise exposure [Lim et al., 1993], but not by hyperthermia [Dechesne et al., 1992].

It should not be overlooked that those previous results were obtained from *in vivo* experiment preformed on adult animals, while the subjects in the present study are a cell line

derived from embryo and OC explants of neonatal rat, which are relatively immature and may present a reaction different from that of mature animals under certain stress conditions.

In addition, the discrepancy between mRNA expression and protein expression was also observed in inflammatory cytokines. The induction of TNF-α at transcriptional level was evaluated by RT-PCR, but no increase of corresponding protein in supernatants was detected by ELISA. The underlying reasons might be that the release of inflammatory cytokines to extracellular medium was blocked or that the synthesis of inflammatory cytokines was suppressed by salicylate. In agreement with latter, it has been demonstrated that salicylate inhibits protein synthesis in human cells through activation of one of stress-activated kinases (PERK), which initiates phosphorylation of the translation initiation factor (eIF2α) [Silva et al., 2007]. Nevertheless, there is a necessity to verify these options in future studies.

4.2.6 Expression of HSP70 in OC during organotypic culture

In this study, HSP70 protein was detected in both salicylate-treated and untreated OC explant cultures by Western blot. In addition, the cellular localization of HSP70 protein in OC explants with use of immunohistochemistry revealed presence of HSP70 in spiral limbus fibrocytes. This localization remained unchanged between salicylate-treated and salicylate-untreated OC cultures.

Our previous studies indicated absence of HSP70 protein in freshly dissected OC. These results are in agreement with previous reports that no HSP70 was found in unstressed rat cochlea [Dechesne et al., 1992; Lim et al., 1993; Myers et al., 1992]. Our previous studies also showed a substantial increase of HSP70 mRNA and protein expression in OC during the first 24 h of organotypic culture, compared with fresh OC (unpublished results). A very similar finding was also reported by Sano and colleagues [Sano et al., 2007].

During tissue dissection, OC explants endure mechanical stress and temporary ischemia, suggesting that salicylate-untreated specimens were not “unstressed”. As a universal stress protein in all cells, HSP70 was produced in cultured skeletal muscle cells [Goto et al., 2003], fetal lung epithelial cells [Copland and Post, 2007] and chondrocytes [Sironen et al., 2002] under mechanical stress. Truettner et al. indicated that HSP70 mRNA and protein were strongly induced in the brain after traumatic injury [Truettner et al., 2007]. Moreover, ischemia stress has been proven to enhance HSP70 expression in cochlea [Myers et al., 1992].

In this study, because of tissue culture medium used (see 2.2.2), OC was exposed to a number of growth factors (IGF-1, insulin, transferrin, selenium and serum proteins in FBS) during the organotypic culture. It has been shown that HSP70 gene expression in human HeLa

and 293 cells was induced markedly by serum stimulation following serum deprivation [Wu and Morimoto, 1985]. Bhagat reported that both HSP70 mRNA and protein were relatively low in freshly prepared pancreas explants, but rose rapidly within 6 h after the start of culture and remained high for up to 24 h [Bhagat et al., 2000]. These results imply that *in vitro* incubation may represent a stress, which in turn induces HSP70 expression.

Thus, the induction of HSP70 in OC cultures may be caused by mechanical stress and temporary ischemia during tissue dissection and subsequent organotypic culture condition, which resulted in relatively high background of HSP70 expression in OC cultures and might have concealed possible induction of HSP70 by salicylate.

4.3 Influence of IL-6 on cisplatin-induced ototoxicity

Inflammation and proinflammatory cytokines have been shown to be involved in the pathophysiology of inner ear disorders and the role of proinflammatory cytokines in pathogenesis of cochlear damage has been discussed in some previous studies. Nevertheless, there are few reports regarding the ototoxicity induced by inflammation and proinflammatory cytokines, and there is little evidence showing the direct role of proinflammatory cytokines in pathophysiology of ototoxicity.

Among numerous ototoxic drugs, cisplatin was studied for its ototoxicity correlated with presence of inflammatory cytokines. In a previous study on HEI-OC1 cells, it was demonstrated that cisplatin increased the production of proinflammatory cytokines including IL-6 [So et al., 2007]. In addition, it was shown that the exogenous treatment with TNF-α alone has not significantly affected the viability of HEI-OC1 cells, while treatment of TNF-α combined with IL-6 (20 ng/ml) decreased cell viability, and lastly, IL-6 alone at higher concentration of 25 ng/ml decreased cell viability. These results suggest that IL-6 exerted a cytotoxic effect on HEI-OC1 cells [So et al., 2007]. In contrast, the present study clearly demonstrates that exogenous IL-6 treatment dose not cause significant loss and any noticeable morphological change of hair cells in OC cultures, even at relatively high concentrations (30 and 90 ng/ml), indicating that IL-6 is not cytotoxic to hair cells in OC cultures. The discrepancy between our results and those delivered by So et al. may be explained on at least two levels. First, we used OC explant culture whereas So used cell lines; second, the IL-6 we used was of tested low endotoxin, whereas the possible endotoxin contamination is not mentioned by So.

In the central nervous system, IL-6 was shown to exert neurotrophic and neuroprotective effects [Kunioku et al., 2001; Kushima and Hatanaka, 1992], besides mediating inflammation

and degeneration [Kamimura et al., 2003; Mizuno et al., 1994]. In rat PC12 cells (pheochromocytoma cell line), IL-6 at a concentration of 20 ng/ml inhibited the cell death induced by cisplatin [Kunioku et al., 2001]. However, the protective effect of IL-6 in cochlea was so far not explored. In the study on HEI-OC1 auditory cells mentioned above, neutralization of IL-6 with anti-IL-6 antibody neither aggravated nor alleviated cell death caused by cisplatin, which implied neither protective effect nor cytotoxic effect of IL-6 [So et al., 2007].

In the current study, exposure to cisplatin resulted in evident loss of hair cells in OC fragments, and OHC damage seemed to progress from base to apex and from the third row to the first row, which is consistent with the pattern described in previous reports [Zhang et al., 2003]. Interestingly, adding exogenous IL-6 partially protected the hair cells from cisplatin-induced ototoxicity. Though the loss of OHCs was more severe than that of IHCs, IL-6 appeared to exert a preferential protective effect on IHCs. This differs from previous reports, which implied that otoprotective treatments generally affect OHCs but not IHCs [Yarin et al., 2005; Zhang et al., 2003]. It has been shown that IL-6 at high concentration reduced cell damage in the ischemic-reperfused liver, while at low concentration, IL-6 had only a limited protective capacity [Tiberio et al., 2006]. Nevertheless, the present results suggest a possible protective role of IL-6 in the inner ear, which is a new finding.

Cisplatin has been shown to damage spiral ganglion neurons (SGN) as well as hair cells [Schweitzer, 1993]. In a previous report, cisplatin showed more profound damage to SGN than hair cells [Zheng and Gao, 1996]. The effect of exogenous IL-6 on SGN was not investigated in the current study. Based on the previous evidence demonstrating the neuroprotective effect of IL-6 and the present results demonstrating the otoprotective effect of IL-6 on the hair cells, the next logical step would be to evaluate the influence of IL-6 on the survival of SGN in future studies.

The molecular pathway through which IL-6 exerts its protective activity against various injuries has been analyzed in some other tissues. IL-6 has been shown to activate the transcription factor called signal transducers and activators of transcription 3 (STAT3) in ischemia-reperfusion-injured rat liver [Tiberio et al., 2006]. It has been suggested that the signaling pathway responsible for the anti-apoptotic effect of IL-6 against serum deprivation and chemotherapeutic agents including cisplatin in PC12 cells involves activation of the phosphatidylinositol 3-kinase (PI3K) and STAT3 [Kunioku et al., 2001]. However, the precise mechanism in which IL-6 induces the otoprotective effect in auditory hair cells is still obscure and needs to be clarified in the future.

4.4 Otoprotective role of geldanamycin

4.4.1 Protective effect of HSP70 in inner ear

The inner ear may be subjected to a various types of stress such as aging, acoustic trauma and ototoxic drugs, which result in cell damage and/or death. Sensory hair cells are very susceptible to any injury of the inner ear. Acquired permanent hearing loss is usually associated with a loss of hair cells [Schacht et al., 2008]. In contrast to lower species (fish, bird, etc.), mammals cannot regenerate hair cells [Duan et al., 2002]. Therefore, hair cells are an important target for protective interventions. Consequently, in the present study, investigation of otoprotection is focused on attenuation of direct toxic effects on hair cells.

HSP70, an inducible molecular chaperone, has been found to be up-regulated in the cochlea by potentially damaging types of stress including heat [Dechesne et al., 1992], ischemia [Myers et al., 1992], noise [Lim et al., 1993] and cisplatin [Oh et al., 2000]. Moreover, HSP70 has been indicated to have a protective function in the cochlea [Altschuler et al., 1996]. HSF1, the major transcription factor regulating expression of stress-induced heat shock proteins (e.g. HSP70) is present in rodent cochlea (hair cells, spiral ganglion neurons and stria vascularis) [Fairfield et al., 2002]. The importance of heat shock system was demonstrated using knock-out Hsf1-/- mice, in which HSP induction through Hsf1-dependent stress pathway is eliminated. After exposure to noise, the knock-out mice had greater hearing loss and greater outer hair cell loss than Hsf1+/+ mice, providing evidence for the critical role of HSF1 and HSP in cochlear protection and recovery [Fairfield et al., 2005]. In addition, it has been demonstrated that heat shock protected from aminoglycoside-induced utricular hair cells loss in wild-type mice, but not HSP70 double knock out mice. Moreover, the HSP70-overexpressing utricles of transgenic mice were significantly protected against aminoglycoside-induced hair cell death, compared with utricles from wild-type littermates [Taleb et al., 2008].

4.4.2 Induction of HSP70 as endogenous otoprotective molecules

Heat shock and pharmaceutical induction are two known approaches of inducing HSP70 *in vivo*. It was found that whole-body heat stress produced a substantial increase of HSP70 mRNA and protected mice from acoustic injury [Yoshida et al., 1999]. Sugahara et al. showed that HSP70 was induced by local heat shock (perfusion of hot saline into the middle ear cavity) in cochlear cells of guinea pigs. The induction of HSP70 included cochlear hair cells. Moreover, hair cell loss and loss of the auditory function induced by acoustic overexposure was inhibited by local heat shock [Sugahara et al., 2003].

In clinical settings, induction of HSP70 by pharmaceuticals is more feasible than by heat shock. Recently, geranylgeranylacetone, an anti-ulcer drug, was shown to induce HSP70 in various tissues including gastric mucosa and cochlea [Hirakawa et al., 1996; Sone et al., 2005]. Geranylgeranylacetone had a protective effect rescuing inner ear from endotoxin-induced inflammation [Sone et al., 2005], acoustic injury [Mikuriya et al., 2005], gentamicin ototoxicity [Sano et al., 2007] and age-related hearing loss [Mikuriya et al., 2008] *in vivo* or *in vitro*.

Resveratrol, a natural compound derived from plants, has been implicated as a therapeutic in a number of diseases including cancer, ischemic injury, cardiovascular and inflammatory diseases [Baur and Sinclair, 2006]. The biological activities of resveratrol include inhibition of lipid peroxidation, free-radical scavenging, and inhibition of platelet aggregation, anti-inflammatory activity, vasorelaxation activity and anti-cancer activity. In addition, resveratrol has a direct neuroprotective effect in PC12 cells [Jang et al., 1997]. Though it was found that administration of resveratrol reduced hearing impairment of rats caused by noise [Seidman et al., 2003], till now there are few reports discussing the possible protection of resveratrol in auditory system.

It was indicated that resveratrol activated the heat-shock promoter and induced the expression of HSP70 in cell lines and in human peripheral lymphocytes, and conferred cytoprotection on those cells against severe heat stress [Putics et al., 2008]. However, in the present study, regardless of concentration used, resveratrol has not induced the expression of HSP70 in OC explants. Although the previous study indicated an otoprotective potential of resveratrol, the exact mechanism was not clarified yet. The present result suggests that the otoprotective effect of resveratrol is not mediated by induction of HSP70. In fact, the present study excluded induction of HSP70 by resveratrol in cochlear tissues, implying that the potential of resveratrol as a HSP inducer may not be universal, but cell type-specific.

4.4.3 Otoprotective role of geldanamycin

Geldanamycin, a benzoquinone ansamycin antibiotic and antitumor drug, protects various cells and tissues from necrotic and apoptotic cell death. The mechanisms of action of geldanamycin involves binding HSP90, consequent release of heat shock factor 1 and finally, induction of heat shock proteins, HSP70 being the major one. *In vivo*, HSP70 expression was found to be up-regulated in the kidney, liver, lungs and heart of geldanamycin-treated mice. In addition, geldanamycin reduced ischemic/reperfusion injury [Harrison et al., 2008]. It was reported that geldanamycin increased HSP70 expression in renal adenocarcinoma cells and protected renal adenocarcinoma cells from oxidative stress. In the central nervous system,

geldanamycin increased HSP70 expression and reduced cell injury and death caused by oxygen glucose deprivation, glutamate-induced excitotoxicity or dopaminergic neurotoxicity [Ouyang et al., 2005; Shen et al., 2005; Xu et al., 2003]. Finally, it has been demonstrated that geldanamycin attenuated cytotoxicity induced by okadaic acid (a protein phosphatase inhibitor) in visual sensory cells [Kaarniranta et al., 2005]. To date, there is no report on auditory system, in terms of a protective effect of geldanamycin.

4.4.3.1 Induction of HSP70 by geldanamycin in OC

Because of very high sensitivity of cochlea to ototoxic substances, geldanamycin was first tested for its possible toxic properties. Although geldanamycin was shown to be toxic in some cells even at a relative low concentration (0.1 μg/ml or 0.18 μM) [Supko et al., 1995], in the present study treatment of OC cultures for 24 h with 2 μM geldanamycin did not cause noticeable hair cell damage. In the current study, geldanamycin up-regulated the expression of HSP70 in OC explants at transcriptional and translational levels. The up-regulation of HSP70 mRNA proceeded ahead of that of HSP70 protein, which suggests that the upregulation of HSP70 took place on transcriptional level and the increase of HSP70 protein was a secondary process. HSP70 protein expression in OC started within 4 h of treatment with geldanamycin and persisted at relative high level until 24 h, corroborating the temporal expression pattern seen in previous studies with hippocampal cell line [Xiao et al., 1999] and visual sensory cells [Kaarniranta et al., 2005].

Fluorescence microscopy indicated that HSP70 induced by geldanamycin is mainly located in OHCs, IHCs within the organ of Corti and in interdental cells of spiral limbus, but not in spiral ganglion cell bodies or nerve fibers. This localization pattern of HSP70 protein is similar to that of HSF1 reported by Fairield [Fairfield et al., 2002], except for the negative expression in spiral ganglion. This localization pattern is also consistent with that of HSP70 mRNA induced by heat shock in a previous study [Gower and Thompson, 1997]. This is in contrast to another report showing that HSP70 protein induced by heat shock was present in all cochlear structures except the organ of Corti [Dechesne et al., 1992]. This inconsistency may be due to different stimuli (heat *vs* geldanamycin) or, even more likely, due to different experimental models used (animal *vs* OC explant).

4.4.3.2 Protective effect of geldanamycin against gentamicin-induced ototoxicity

Gentamicin, which is known to be cytotoxic to hair cells, was used to produce hair cell damage, thereby evaluating the possible protective effect of geldanamycin on damaged hair cells.

In the present study, gentamicin caused more severe hair cell damage in basal part than in apical and medial parts, and more severe damage in the first row of OHCs than in second and third rows, which is consistent with what was previously described in *in vivo* studies [Schacht et al., 2008] and a *in vitro* study on mice cochlea [Ding et al., 2002]. However, gentamicin brought a comparable damage to IHCs and OHCs, contrary to previous report [Ding et al., 2002; Schacht et al., 2008]. Thus, to some extent, the vulnerability of IHCs and OHCs to gentamicin appears to depend on experimental systems used.

The mechanism of gentamicin ototoxicity has been extensively studied. ROS overproduction and the resulting redox imbalance have been demonstrated to be the important mechanism of gentamicin ototoxicity. Priuska et al. have found that gentamicin was able to accelerate the formation of free radicals in the presence of iron salts [Priuska and Schacht, 1995]. There is increasing evidence that hair cells damaged by gentamicin die by an active cell death - apoptosis. Gentamicin treatment has been shown to result in chromatin condensation and DNA fragmentation, hallmarks of apoptosis [Cheng et al., 2003; Forge, 1985]. Moreover, cytochrome *c* release from the mitochondria into the cytoplasm and activation of caspase-8, caspase-9 and caspase-3 were observed in gentamicin-damaged hair cells, suggesting that auditory hair cell death is a result of gentamicin exposure in a caspase-dependent pathway [Cheng et al., 2003; Mangiardi et al., 2004]. In addition to caspase-mediated apoptosis, the c-Jun N-terminal kinase (JNK) apoptotic pathway is also implicated in gentamicin ototoxicity. It has been demonstrated that gentamicin-induced ototoxicity leads to JNK activation and apoptosis of inner ear hair cells *in vivo* [Ylikoski et al., 2002].

Based on increasing understanding of the mechanism of ototoxicity and survival, protective strategies and interventions became possible. In past several decades, a lot of effort has been put into the field of protection from ototoxicity and a number of bioactive molecules have been studied to evaluate the possible preventive role on gentamicin-induced ototoxicity. Growth factors (brain-derived neurotrophic factor (BDNF) [Takumida et al., 2003] and erythropoietin [Monge et al., 2006]), radical scavenger (D-methionine) [Takumida et al., 2003], antioxidants (alpha-tocopherol and tiopronin) [Takumida et al., 2003], caspase inhibitor (x-linked inhibitor of apoptosis protein (XIAP) [Tabuchi et al., 2007]), calpain inhibitor (leupeptin) [Ding et al., 2002], cytochrome *c* inhibitor (minocycline) [Corbacella et al., 2004], iron chelators (deferoxamine) [Mostafa et al., 2007], and argon [Yarin et al., 2005] showed protective effects to varying extent against gentamicin-induced ototoxicity.

In the present study, geldanamycin was found to attenuate gentamicin-induced hair cell loss. The most straightforward interpretation on this finding is that the protective effect of

geldanamycin against gentamicin-induced hair cell loss is the consequence of cytoprotective action of HSP70 induced by geldanamycin. Harrison et al. proposed a pathway of geldanamycin-mediated neuroprotection based on a finding that geldanamycin exerts neuroprotection by the destabilization of the Hsp90-HSF1 complex, induces nuclear translocation of HSF1 which in turn binds to the Hsp70 promoter and enhances the synthesis of HSP70 mRNA [Harrison et al., 2008].

Protective effects of HSP70 may be attributed to its critical function as a molecular chaperone. This function involves refolding of misfolded and thus toxic proteins in cytoplasm and reducing protein aggregation. Furthermore, previous studies have demonstrated that HSP70 is able to inhibit apoptosis in a variety of systems by interfering with some apoptotic-signaling cascades, which have been indicated to be involved in the mechanism of ototoxicity. HSP70 has been shown to strongly inhibit JNK activation [Mosser et al., 1997] and to reduce mitochondrial cytochrome *c* release to cytosol [Tsuchiya et al., 2003]. In addition, HSP70 has also been reported to prevent pro-caspase-9 recruitment to apoptosome complex, thus blocking the assembly of a functional apoptosome and activation of caspase-9 [Beere et al., 2000]. Remarkably, HSP70 was reported to inhibit processing of caspase-3 [Mosser et al., 1997] and inhibit cell death even after caspase-3 has been activated [Jaattela et al., 1998]. This previous evidence can be used to explain the protective effect of HSP70.

In this work, protective effect of geldanamycin was observed in gentamicin-exposed OC explants pretreated for 4 h with geldanamycin and in those treated with geldanamycin and gentamicin simultaneously. When OC explants were subjected to gentamicin after geldanamycin pretreatment, HSP70 protein in OC explants induced by geldanamycin had reached a relatively high level, which might provide protection against gentamicin-induced damage. Interestingly, in another case OC explants were subjected to gentamicin and geldanamycin concurrently and HSP70 had not accumulated to a high level yet in the initial stage, geldanamycin produced equivalent protective effects. This may be due to the fact that gentamicin exerts an ototoxic effect relatively late, thus HSP70 induced by geldanamycin in relatively early phase may provide a protective effect to hair cells. The present results imply the possibility for a successful post-injury protection provided by geldanamycin against gentamicin-induced ototoxicity. It has already been demonstrated that geldanamycin provided post-exposure neuroprotection in HT22 hippocampal cell line against glutamate-induced oxidative toxicity [Xiao et al., 1999]. It is necessary to define the therapeutic window for geldanamycin in a future study, which, from a clinical perspective, is more attractive than prevention of ototoxicity.

4.4.3.3 Differential protection of OHCs and IHCs by geldanamycin

As shown in the "Results" section, even though HSP70 expression was induced by geldanamycin in both outer and inner hair cells, and despite the fact that outer and inner hair cells appeared to be equally susceptible to gentamicin, the protective effect of geldanamycin was observed only in outer hair cells but not in inner hair cells. These results suggest that the mechanism of outer and inner hair cell loss may differ between these two cell types, and that in the outer hair cells but not in inner hair cells, the ototoxic effect of gentamicin can be inhibited by geldanamycin and HSP70. It is also possible that other targets of geldanamycin mediate the protection. It has been shown that geldanamycin not only increased the expression of HSP70 but also the expression of HSP90 and HSP27 in COS-1 cells [Sittler et al., 2001] and renal cells [Harrison et al., 2008]. HSP90 and HSP27 have been shown to suppress the aggregation of various proteins and facilitate degrading unwanted and/or harmful proteins, thereby acting as general protective chaperones [Garrido et al., 2006; Neckers and Ivy, 2003]. They can also inhibit apoptosis by preventing the assembly of apoptosome [Garrido et al., 2006; Pandey et al., 2000]. It has been demonstrated that HSP90 and HSP27 were induced in inner ear hair cells by heat shock [Cunningham and Brandon, 2006; Sugahara et al., 2003]. It is unknown whether HSP90 and HSP27 were induced by geldanamycin in the present study, which will be determined in further investigations.

Nevertheless, based on the present results, the protective effect of geldanamycin against ototoxic hair cell death may be attributed to the actions of HSP70 induced by geldanamycin. Further experiments are needed to reveal the molecular mechanisms underlying the otoprotective role of geldanamycin, which may provide insights for therapeutic approaches aimed at preventing ototoxicity induced by various ototoxic agents.

4.4.3.4 Prospect of otoprotection provided by geldanamycin analogues

The administration of geldanamycin in clinical settings is limited due to its high hepatotoxicity [Supko et al., 1995]. In addition, geldanamycin has shown to be incapable of crossing the blood brain barrier [Hay et al., 2004], which is very similar to blood labyrinth barrier [Inamura and Salt, 1992]. These disadvantages led to the development of geldanamycin analogues. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is an analogue chemically derived from geldanamycin. 17-AAG is considered less hepatotoxic and more stable than geldanamycin and has been shown to cross the blood brain barrier [Egorin et al., 2001]. It is currently in an early stage of clinical trials as a chemotherapeutic agent [Goetz et al., 2003]. Another analogue, 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), is

more water-soluble than 17-AAG and has excellent bioavailability, thus is more practicable in preclinical models [Smith et al., 2005]. 17-AAG and 17-DMAG have been shown to induce HSP70 in a variety of cells and provided protective effects [Harrison et al., 2008; Herbst and Wanker, 2007; Smith et al., 2005; Wetzler et al., 2007]. Although there is no evidence so far that indicates *oto*protective effect of these two geldanamycin analogues, 17-AAG and 17-DMAG may be supposed to have protective roles in the inner ear, which should be validated in future studies.

5 Summary

It has been known that inflammation and inflammatory cytokines are involved in pathogenesis of many types of cochlear disorders including ototoxicity. Stress proteins such as HSP70 are induced in response to various stresses in the inner ear and presents otopretective effects. My dissertation was dedicated to study ototoxic and otoprotective properties of selected substances in the inner ear cells and tissues.

In the first part of my work, I studied the gene expression profile of inflammatory cytokines, HSP70 and prestin in the organ of Corti during organotypic culture. I found that exposure to salicylate (2.5 mM) upregulated the number of transcripts encoding IL-6, HSP70 and prestin in the organ of Corti. These studies were complemented by expression study using an auditory epithelium cell line, which yielded similar results. The overall results suggested that salicylate may work as an ototoxin not only by directly influencing motility of outer hair cells (as previously described) but also by affecting gene expression. Interestingly, changes in transcription detected by quantitative real time RT-PCR were not followed by changes in translation, as per Western blot or ELISA of IL-6 and HSP70.

From the first part of my work, I knew that the transcription of IL-6 increases after exposure to salicylate in the organ of Corti during organotypic culture. In addition, the reports of others showed cochlear increase in IL-6 during trauma-, noise- or cisplatin-induced injury. That is why in the second part of my work, I studied the influence of exogenous IL-6 on ototoxicity in the organ of Corti explant cultures. I discovered that the exogenous IL-6 (up to 90 ng/ml) had no ototoxic properties when applied on its own, as determined by cochleogram. Moreover, when IL-6 (30 ng/ml) was added to OC explants together with cisplatin (15μM for 48 h), it had partially protected hair cells from cisplatin-induced ototoxicity. The mechanism of the otoprotective effect induced by IL-6 in auditory hair cells needs to be clarified.

In the last part of my work, I studied otoprotection induced by chemical stimulators of heat shock protein 70. To date, resveratrol and geldanamycin were demonstrated to induce HSP70 production. Based on data showing unequivocally that intracellular endogenous HSP70 is otoprotective, I used the two substances mentioned above to induce HSP70 in the organ of Corti explant cultures. The induction of HSP70 by resveratrol or geldanamycin was analyzed by RT-PCR and ELISA. Resveratrol (up to 200 μM) has not induced HSP70 expression in the organ of Corti explant. In contrast, geldanamycin (2 μM) induced HSP70 expression in OC cultures at both mRNA and protein levels. Fluorescence microscopy revealed that HSP70 induced by

geldanamycin was observed mainly in hair cells and interdental cells of spiral limbus, but not in either spiral ganglion cells or nerve fibers. Next, I studied the otoprotective properties of geldanamycin using a standard ototoxic antibiotic gentamicin (500 μM). In gentamicin-damaged OC explants, geldanamycin significantly reduced the loss of outer but not inner hair cells. The differential protective effect of geldanamycin between outer and inner hair cells indicated that HSP70-independent pathway may contribute to the protective effect of geldanamycin. I observed the otoprotective effect of geldanamycin in OC explants pretreated with geldanamycin and in those treated simultaneously with geldanamycin and gentamicin. Further experiments are needed to reveal the molecular mechanisms underlying the otoprotective role of geldanamycin, which may provide insights for therapeutic approaches aimed at preventing ototoxicity induced by various ototoxic agents.

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7 Abbreviation

β-ME	β-mercaptoethanol
17-AAG	17-Allylamino-17-demethoxygeldanamycin
17-DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
BSG	buffed saline glucose solution
Ct	threshold cycles
DABCO	1,4-diazabicyclo[2.2.2]octane
dB	decibel
DNA	deoxyribonucleic acids
DPOAE	distortion product otoacoustic emission
EDTA	ethylenediamine tetraacetic acid
eIF2α	α-subunit of eukaryotic translation initiation factor 2
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM	Gentamicin
GM-CSF	granulocyte-macrophage colony stimulating factor
HRP	horseradish peroxidase
HSC70	70 kDa heat shock cognate protein
HSF	heat shock factor
HSP	heat shock protein
HSP70	70 kDa heat shock protein
IκB	inhibitor of NF-κB
IFN-γ	interferon-γ
IHC	inner hair cell
iHSP70	inducible HSP70
IL-1	interleukin-1

IL-18	interleukin-18
IL-6	interleukin-6
IQR	interquartile range
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
mRNA	messenger ribonuclear acid
NFκB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
NP-40	nonyl phenoxylpolyethoxylethanol-40
OAE	otoacoustic emission
OC	organ of Corti
OHC	outer hair cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PERK	protein kinse R-like endoplasmic reticulum kinase
PI3	phosphatidylinositol 3-linase
PMSF	phenylmethylsulfonyl fluoride
qPCR	quantitative polymerase chain reaction
rhIGF-I	recombinant human insulin-like growth factor-1
RNA	ribonucleic acid
ROS	reactive oxygen species
rS16	ribosomal protein S16
RSV	resvaretrol
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEM	standard error of mean
SGN	spiral ganglion neuron
STAT3	signal transducers and activators of transcription 3
TLR	toll-like receptor
TMB	tetramethylbenzidine

TNF-α	tumor necrosis factor-alpha
TRITC	tetramethyl rhodamine isothiocyanate
VOT-E36	ventral otocyst-epithelial cell line clone 36
XIAP	X-linked inhibitor of apoptosis protein

Lebenslauf

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.

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Erklärung

„Ich, Yang Yu, erkläre, dass ich die vorgelegte Dissertation mit dem Thema *Gene and protein expression patterns in the rat inner ear during ototoxicity and otoprotection* selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

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