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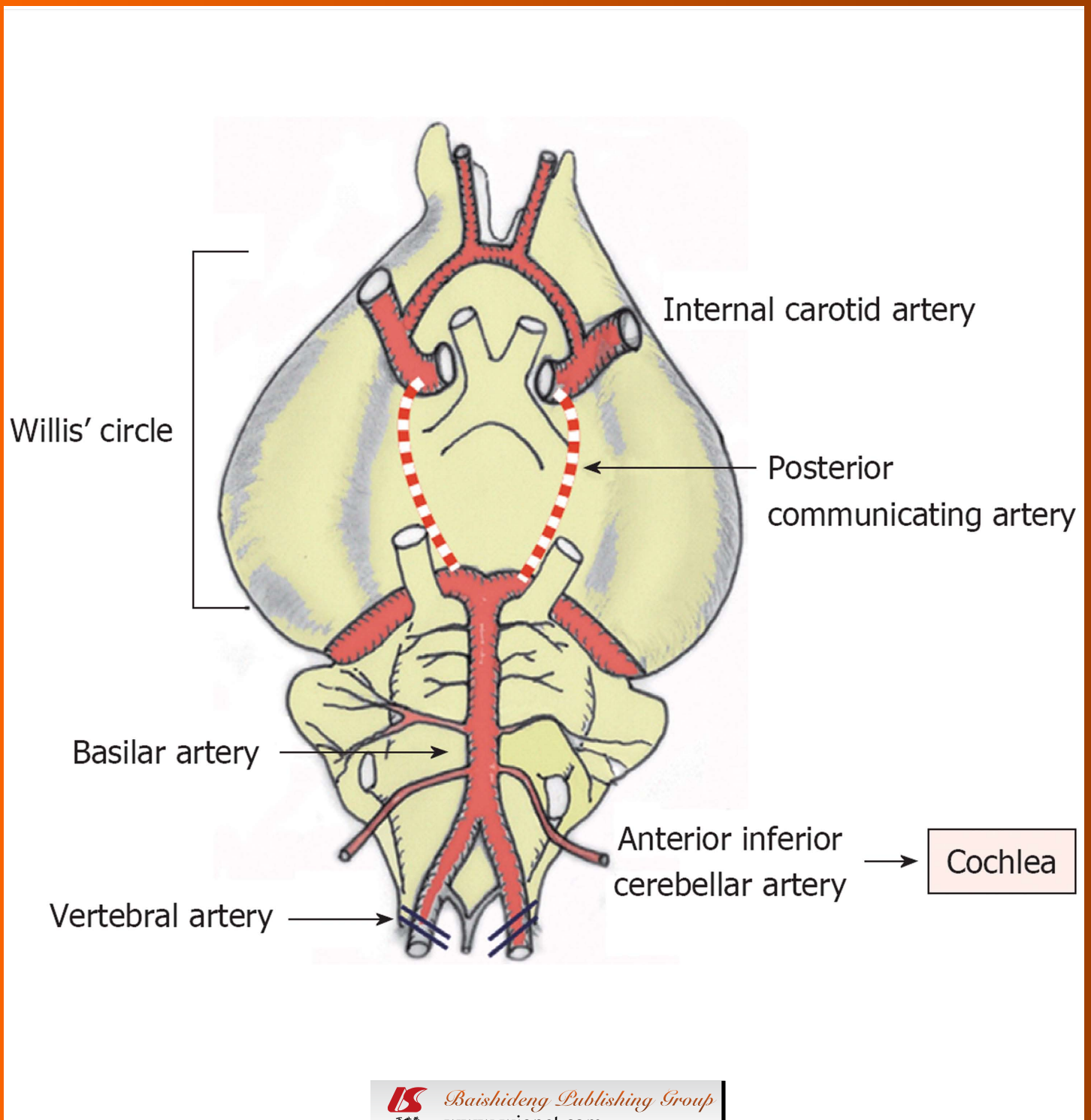

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World Journal of Otorhinolaryngology (*World J Otorhinolaryngol*, *WJO*, online ISSN 2218-6247, DOI: 10.5319) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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EDITORIAL OFFICE
Jin-Lei Wang, Director
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World Journal of Otorhinolaryngology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjotorhinolaryngol@wjgnet.com
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REVIEW

Experimental study of transient cochlear ischemia as a cause of sudden deafness

Kiyofumi Gyo

Kiyofumi Gyo, Department of Otorhinolaryngology, Head and Neck Surgery, Ehime University, Shizukawa, Toon City, Ehime Prefecture 791-0295, Japan
Author contributions: Gyo K solely contributed to this paper.
Correspondence to: Kiyofumi Gyo, MD, Professor, Department of Otorhinolaryngology, Head and Neck Surgery, Ehime University, Shizukawa, Toon City, Ehime Prefecture 791-0295, Japan. kiyofumi@m.ehime-u.ac.jp
Telephone: +81-89-9605365 Fax: +81-89-9605368
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Abstract

The etiology of sudden deafness or idiopathic sudden sensorineural hearing loss (ISSHL) remains unclear. Over the past 15 years, we have investigated the mechanisms of ischemic-induced hearing loss using a gerbil model of transient cochlear ischemia. In the gerbil, cochlear ischemia can be induced by occluding the bilateral vertebral arteries simultaneously at the neck, because the posterior communicating arteries of the Circle of Willis close spontaneously around 1 mo after birth. When 15 min ischemia was loaded on this animal, permanent hearing loss of about 25 dB and the death of hair cells, especially inner hair cells were induced. These pathological changes were mainly due to lack of an energy source, glutamate excitotoxicity, and the production of free radicals, especially superoxide and nitrous oxide species. Ischemic damage could be prevented by various procedures, such as cooling the cochlea, intratympanic administration of insulin-like growth factor 1 or AM-111 (an anti-apoptotic agent), and systemic administration of prednisolone (steroid), edarabone (free radical scavenger), ginsenoside Rb1 (Kanpo), hematopoietic stem cells, glia-cell derived neurotrophic factor, and liposome-encapsulated hemoglobin (artificial red blood cells). We also found that the cochlea was protected by the ischemic tolerance, indicating that minor cochlear ischemia alleviates or prevents inner ear damage in subsequent severe cochlear ischemia. As ISSHL usually occurs suddenly, with no preceding sign or symptom, we suggest that most ISSHL cases are caused by circulatory disturbance, probably at the stria vascularis.

Key words: Sudden deafness; Occlusion of vertebral artery; Mongolian gerbil; Loss of inner hair cell; Ischemic tolerance; Cochlear hypothermia

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INTRODUCTION

Sudden deafness, also called idiopathic sudden sensorineural hearing loss (ISSHL), is a disease of inner ear causing acute hearing loss of unknown etiology. It occurs in approximately 30 cases per 100 000 people a year in Japan, most frequently involving those 50-60 years of age. Presently, ISSHL is considered a symptom of various diseases, including circulatory disturbances, viral infection, endolymphatic hydrops/labyrinthine membrane rupture, and disruption of endolymphatic homeostasis triggered by stress hormones and other hormones as well. As such hearing loss usually occurs suddenly, with no preceding sign or symptom, many investigators have suggested that ISSHL is caused by acute interruption of cochlear blood flow, and steroids and vasodilator agents are often prescribed for the treatment of this disease. Recently, many scientific papers have been published supporting this vascular theory, including circulatory disturbances, as a cause of ISSHL. Suckfüll[1] reported that plasma fibrinogen was raised in patients with ISSHL, suggesting increased blood coagula-

tion. Fortunately, their hearing impairment improved following low-density lipoprotein apheresis. De Felice *et al*[2] reported a strong correlation between a non-functioning posterior communicating artery (PCA) of the Circle of Willis and the incidence of ISSHL. Because the inner ear is nourished solely by the labyrinthine artery, a branch of the basilar artery, a non-functioning PCA may increase the risk of disturbing the continuous blood supply to the cochlea. Large-scale statistical analyses have demonstrated that ISSHL is a strong risk factor for stroke[3] and cardiovascular disease[4]. Due to recent advances in gene analysis technology, various single nucleotide polymorphisms have been found to be closely associated with ISSHL incidence[5-8]. In Japan, Hato[9] showed that the *PRKCH* gene, an expression gene of protein kinase C, was associated with the incidence of ISSHL as well as stroke[10]. The allele ratio of G→A in the *RPKCH* gene is 2.0 in ISSHL and 1.7 in lacunar stroke. Using three-dimensional fluid-attenuated inversion recovery magnetic resonance imaging, Yoshida *et al*[11] reported high signal areas in the cochlea of 31 of 48 patients with ISSHL, suggesting a high concentration of protein or hemorrhage in the cochlea. They noted that hearing prognoses of such patients were poor compared to those without a high signal. These findings are all consistent with the vascular theory of the etiology of ISSHL.

Over the past 15 years, we have investigated the mechanism(s) of ischemia-induced hearing loss using a gerbil model of transient cochlear ischemia. Because this animal can live long after the induction of transient ischemia, experimental studies were undertaken to assess cochlear histopathology, the mechanism(s) of ischemic cochlear damage, and responses to various treatment modalities. We also found that the cochlea was protected by the mechanism known as ischemic tolerance. In this phenomenon, minor cochlear ischemia alleviates or prevents inner ear damage in subsequent severe cochlear ischemia. Here, we present our experimental data concerning transient cochlear ischemia, report the findings of ischemic tolerance in the cochlea, and demonstrate the therapeutic effects of various treatment modalities.

ANIMAL MODEL OF TRANSIENT COCHLEAR ISCHEMIA

Because the nourishing artery of the cochlea comes from the basilar artery, transient cochlea ischemia is difficult to induce without damaging the brain and other neuronal tissues. Indeed, such experiments were previously considered not feasible in small animals because of technical difficulties. Using a technique called experimental hindbrain ischemia[12], we succeeded in making a chronic animal model of transient cochlear ischemia using the Mongolian gerbil. In this animal, the posterior communicating arteries of the Circle of Willis characteristically close spontaneously around 1 mo after birth. As the cochleae receive their blood supply solely from the vertebral arteries in the adult, transient cochlear ischemia is readily induced by obstructing the bilateral vertebral arteries at the neck[13] (Figure 1).

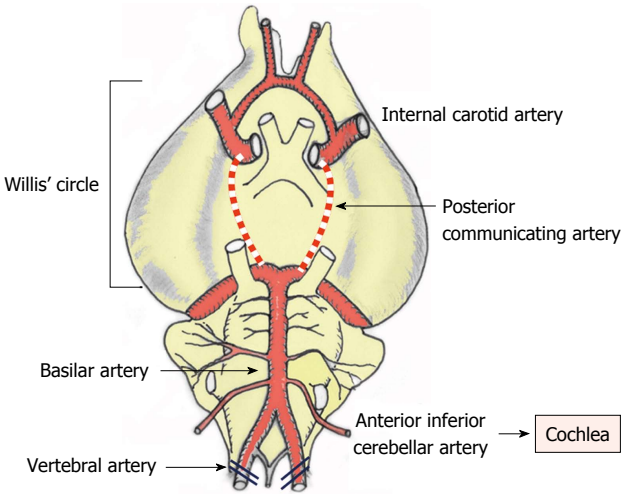

Figure 1 Brain arteries in the gerbil. The posterior communicating arteries of the Circle of Willis close at around 1 mo after birth. Thus, cochlear ischemia can be induced by occluding the bilateral vertebral arteries at the neck in adult animals.

Under general anesthesia, the vertebral arteries were exposed bilaterally and dissected free from the surrounding connective tissue through a ventral transverse incision of the neck. Silk threads (4-0) were loosely looped around each artery, and ischemia was induced in the bilateral cochleae by pulling the ligatures with weights of 5 g for 5 or 15 min. The threads were subsequently removed to allow reperfusion, which was confirmed by observation using an operating microscope (Figure 2). As cochlear damage was minor with 5 min ischemia[14], we used 15 min ischemia in the following studies (Figure 3). The hearing of the animal was assessed by recording electrocochleogram, auditory brainstem responses (ABR), or distortion product otoacoustic emission, depending on the purpose of the study.

ISCHEMIC DAMAGE IN THE COCHLEA

Blood supply to three regions of the cochlea and their ischemic damages

Ischemic damage of the cochlea can be divided into three regions: the lateral, middle, and modiolar regions (Figure 4). The lateral region includes the stria vascularis and spiral ligament, while the middle region is composed of the hair cells and supporting cells in the organ of Corti. The modiolar region is located in the center of the cochlea, and includes the spiral ganglion. The three regions of the inner ear receive arterial blood supply from the labyrinthine artery, *via* the spiral modiolar artery. According to rabbit experiments using microspheres, 82% of cochlear blood flow distributes to the lateral region, 9% to the middle region, and 9% to the modiolar region. In rats, the distribution is 57%, 19%, and 24%, respectively[15]. These findings suggest that blood supply to the lateral region is the largest among the three regions, although the distribution ratio differs by animal species.

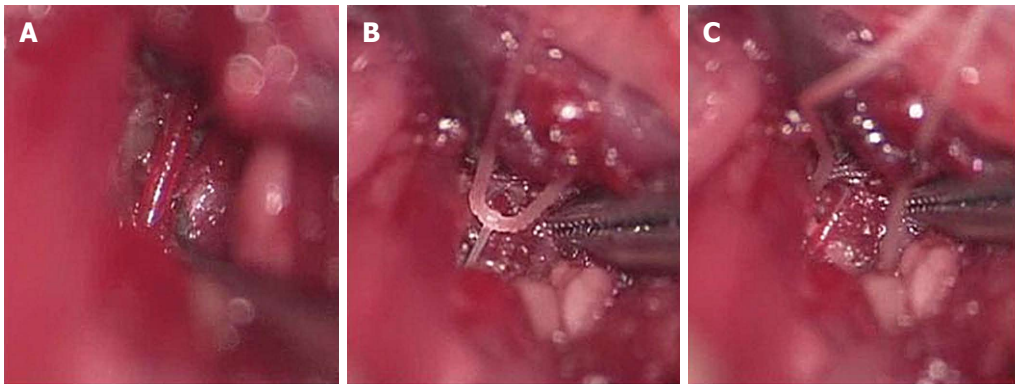

Figure 2 Transient interruption of cochlear blood flow. A: Exposure of the vertebral artery; B: Interruption of cochlear blood flow by pulling the silk thread looped around the artery; C: After inducing transient ischemia, the thread was released and removed to allow recirculation. Originated from[33], with permission.

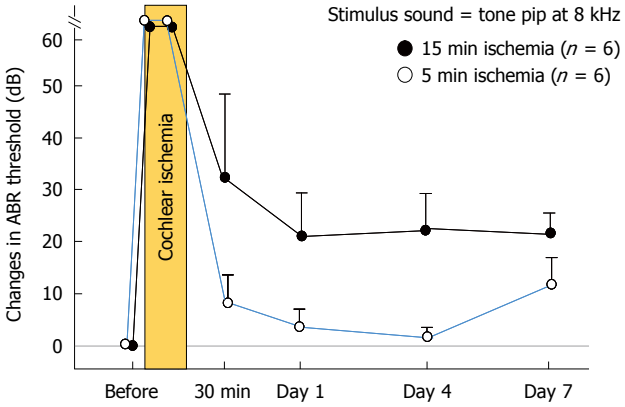

Figure 3 Changes in auditory brainstem response threshold after transient cochlear ischemia. Auditory brainstem response (ABR) threshold before vertebral artery occlusion was defined as 0 dB. With 5 min ischemia, the ABR threshold at 8 kHz recovered almost completely on days 1 and 4, but became slightly worse on day 7, likely due to delayed neural cell death. With 15 min ischemia, the ABR threshold was almost constant after day 1. It remained constant in the range of 20-30 dB. The vertical scale indicates grade of hearing loss, expressed as change in ABR threshold.

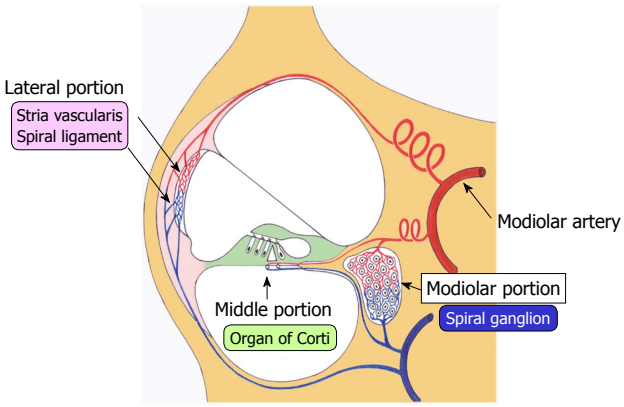

Figure 4 Blood supply to the cochlea. Blood supply to the cochlea *via* modiolar artery can be divided into three regions: the lateral, middle, and modiolar regions. Blood supply to the lateral region, consisting of the stria vascularis and spiral ligament, was the largest among the three regions, supplying more than 80% of total cochlear blood in the rabbit.

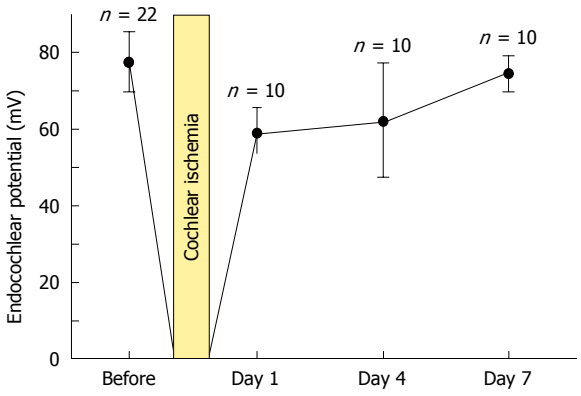

Figure 5 Sequential changes in endocochlear potential after transient cochlear ischemia. Each point indicates the mean voltage of the endocochlear potential. Vertical bars show one standard deviation. Originated from[16], with permission.

Lateral region (stria vascularis and spiral ligament): The main function of the stria vascularis is to constantly supply K^+ into the scala media through an ion channel that consumes ATP as an energy source. According to the K^+ recycling theory, K^+ in the scala media is absorbed by hair cells through mechanical ion channels on the surface of stereocilia called tip links or side links. The channels open and close corresponding to the vibration of the basilar membrane. Once absorbed, K^+ facilitates Ca^{2+} release from stores into the cytoplasm and causes depolarization of the hair cell. Following firing of the hair cells, K^+ released to the outside is then absorbed by the surrounding support cells. It flows laterally, through a gap junction between the supporting cells in the direction of the stria vascularis, where it is again released to the scala media *via* ATP. Because the ion channel at the stria vascularis needs a large amount of ATP, interruption of the blood supply to this area impairs ATP production, resulting in failure of K^+ transport into the scala media. In this way, transient ischemia causes an energy shortage at the stria vascularis and induces decreased endocochlear potential (EP).

As shown in Figure 5, the decrease in EP following 15 min ischemia was reversible. In normal conditions, the EP value was 80.0 mV (n = 22). With ischemia, EP was markedly decreased, to around -20 mV. On the following

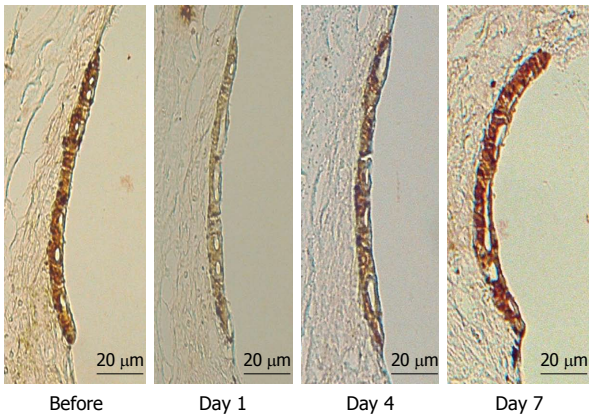

Figure 6 Immunostaining of Na^+-K^+ ATPase in the stria vascularis before and 1, 4, and 7 d after ischemic insult. Immunostaining of Na^+-K^+ ATPase decreased markedly on day 1, and improved slightly on day 4. It recovered to preischemic levels on day 7.

day, it recovered to about 60 mV. It returned to normal by day 7. This indicates that disturbed function of the lateral region returns to normal by day 7[16].

To investigate what happened in and around the stria vascularis, we performed histological staining with hematoxylin and eosin, which revealed no apparent change during the course of recovery. However, immunostaining of Na^+-K^+ ATPase, a marker of the Na/K-pump, and of connexin 26, a marker of gap junctions, showed that the levels of these markers were reduced on days 1 and 4, but recovered to preischemic levels on day 7 (Figure 6). Transmission electron microscope (TEM) studies demonstrated that water retention was prominent in the interstitial layer of the scala tympani on day 1, which became milder on day 4, and almost disappeared on day 7[17] (Figure 7). These histological findings were consistent with the sequential changes in the EP value that recovered on day 7.

Middle region (organ of Corti): In the organ of Corti, ischemic damage was more severe in the inner hair cells (IHCs) than in the outer hair cells (OHCs): the cochlear pathology differed from that of other inner ear diseases, such as acoustic trauma and aminoglycoside ototoxicity, that cause severe damage mainly to the OHCs. Figure 8 shows a typical finding at the basal turn 7 d after ischemia, stained with rhodamine-phalloidin and Hoechst 33342. IHC-predominant damage was also seen by scanning electron microscopy as shown in Figure 9. Percentages of IHC and OHC losses at the three turns of the cochlea are summarized in Figure 10. Loss of IHC was extensive at the basal and second turns, but not at the apical turn. In contrast, no such difference, by turn, was observed in OHCs. This indicates that the underlying mechanisms of ischemic damage apparently differ somewhat between IHCs and OHCs.

TUNEL staining (Figure 11) and TEM studies showed that the loss of IHCs was due to apoptosis, triggered by ischemic insult. Sequential counting of IHCs showed that the rate of IHC loss increased gradually until day 3. Then it remained constant[18] (Figure 12).

Modiolar region (spiral ganglion): HE findings in the spiral ganglion are shown in Figure 13. Loss of the ganglion neurons started on day 4 and progressed until day 7. Figure 14 shows immunofluorescent findings for Bax, an apoptosis-promoting protein, in the spiral ganglion. It was expressed on day 1, but not on day 7. TEM observations indicated that nuclei of the spiral ganglion cells (SGCs) underwent condensation or segmentation, suggesting cell death by apoptosis (Figure 15). The number of SGCs after transient ischemia is shown in Figure 16. A cell decrease was prominent between day 4 and day 7, suggesting delayed cell death in the spiral ganglion[14,19].

Time course of ischemic damage in the three regions of the cochlea: As shown in Figure 3, the ABR threshold at 8 kHz increased 20-30 dB on the next day after 15 min ischemia. The increase remained unchanged or changed a little thereafter. This indicated that hearing loss of an experimental animal became stable after 1 d of ischemia. Meanwhile, time course of ischemic damage was somewhat different among cochlear regions (Figure 17). In the lateral region, severe ischemic damage occurred immediately after the insult, which recovered gradually to a preischemic level within a week. In the middle region, loss of hair cells progressed slowly until day 3, then the decrease stopped. Ischemic damage was more severe in IHCs than in OHCs. Death of IHCs was due to activation of the apoptotic process; it was maximal 12 h after the insult. In the modiolar region, neuronal damage progressed more slowly and steadily. The number of SGCs decreased most prominently between days 4 and 7. Degeneration of the SGCs occurred initially from the ischemic insult, but later by secondary degeneration, corresponding to IHC death.

These findings suggest that when hearing loss is unchanged or slightly recovered after ischemic insult, the degenerating site gradually shifts to other regions.

Mechanisms of ischemic cochlear damage

Although the blood supply to the cochlea was stopped only for 15 min, the effects were much larger than expected. At least three mechanisms were thought to be related to the cochlear damage.

Energy supply deletion: The energy source of the cochlea is ATP, produced from glucose and oxygen *via* the process of glycolysis. Thus, transient cochlear ischemia causes depletion of the energy supply, which induces cochlear damage. According to Thalmann *et al*[20], the ATP concentration decreases rapidly in the stria vascularis and the spiral ganglion, but the decrease is gradual in the organ of Corti. This is probably because glucose and oxygen dissolved in the endolymph are used slowly by hair cells and supporting cells of the organ of Corti. The time needed to decrease the ATP concentration from normal (16 mmol/kg) to a low level (below 2 mmol/kg) after death takes 3 min in the stria vascularis, 15 min in the spiral ganglion, and 60 min in the Corti organ.

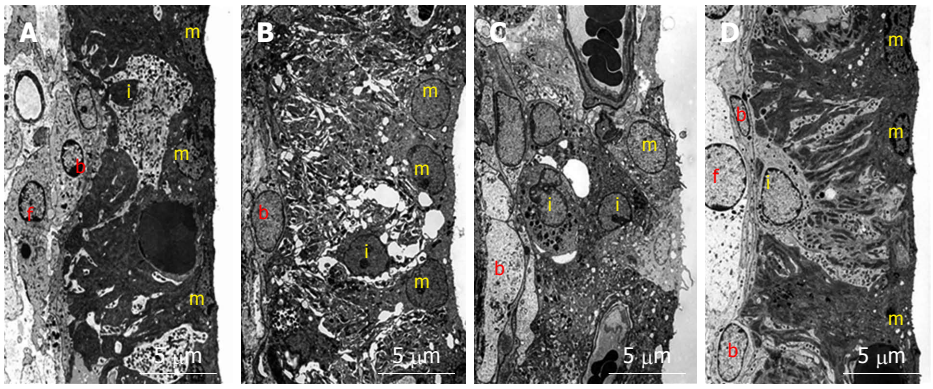

Figure 7 Transmission electron microscopy findings in the stria vascularis before and 1, 4, and 7 d after ischemic insult. A: Marginal cells on the medial aspect of the stria vascularis showed extensive branching processes with intermediate cells. The basal cells, located on the lateral aspect of the stria vascularis, connected to the intermediate cells and type 1 fibrocytes of the spiral ligament by means of gap junctions; B: Vacuoles were seen in marginal cells. The intercellular space increased and intermediate cells seemed to have shrunk; C: Vacuoles persisted in marginal cells. The intermediate cells were still shrunken, although the intercellular space was smaller than that on day 1; D: The intercellular space was no longer enlarged, although a few small vacuoles were found in marginal cells. The extensive branching processes appeared to have a normal shape. m: Marginal cell; i: Intermediate cell; b: Basal cell; f: Type 1 fibrocyte.

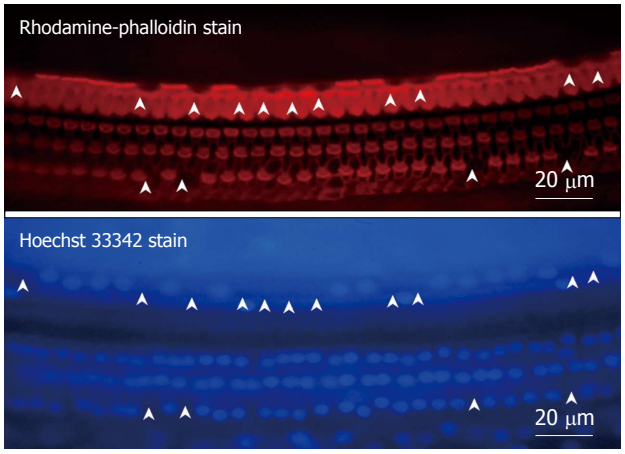

Figure 8 Fluorescence microscopic findings of the organ of Corti 7 d after ischemic insult. The specimen was stained with rhodamine-phalloidin and Hoechst 33342. Three rows of outer hair cells (OHC) and a single row of inner hair cells (IHC) could be observed, and the cell loss was more severe in IHC than OHC. Arrowheads indicate loss of hair cells.

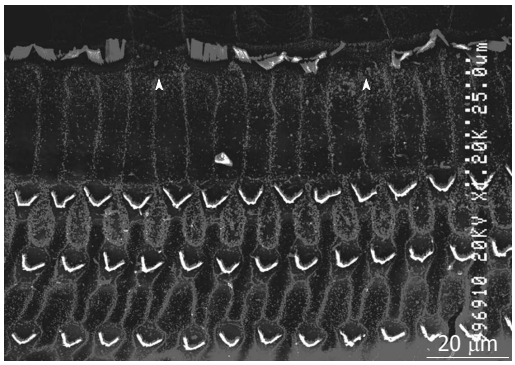

Figure 9 Scanning electron microscopy findings of the organ of Corti 7 d after ischemic insult. Arrowheads indicate damaged inner hair cells.

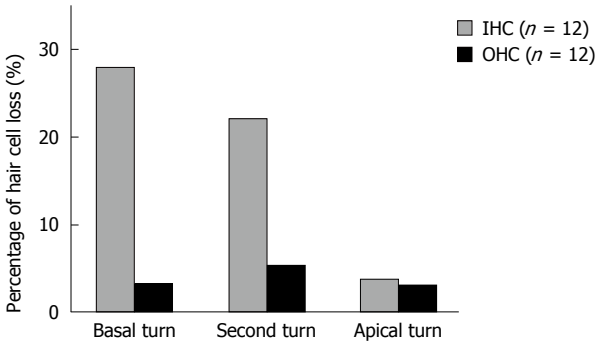

Figure 10 Percentages of hair cell loss at three turns 7 d after ischemic insult. Loss of inner hair cells (IHC) was more severe at the basal and second turn than at the apical turn. No such difference by turn was recognized in the loss of outer hair cell (OHC).

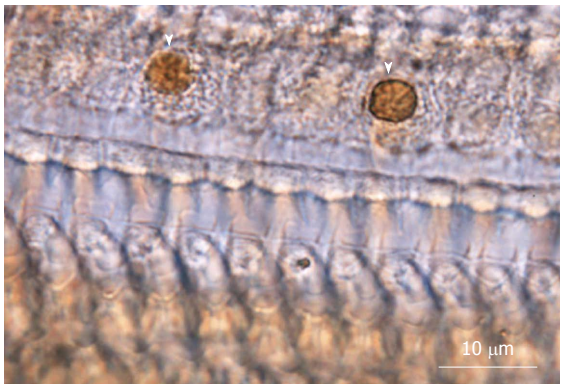

Figure 11 TUNEL staining of inner hair cells. TUNEL staining was positive in inner hair cells (IHC), suggesting cell death due to apoptosis. The arrowhead indicates TUNEL-positive IHC. Originated from[18], with permission.

Free radicals: Free radicals such as superoxide and nitrous oxide (NO) species are produced in the course of ischemia/reperfusion processes. They induce destruction of cell membranes. As superoxide disappears so quickly after production, direct measurement of its concentration in the inner ear is not feasible. We investigated the production of superoxide and oxygen free radicals using

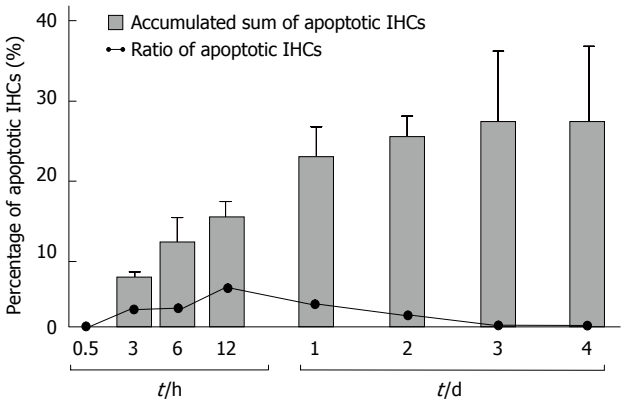

Figure 12 Daily incidence of inner hair cell apoptosis and its cumulative sum after ischemic insult. Incidence of inner hair cells (IHCs) apoptosis was maximal 12 h after ischemic insult. It did not occur after day 3. Vertical bars indicate one standard deviation. Originated from[18], with permission.

edarabone, a free radical scavenger, originally developed as an anti-stroke agent. If free radicals are present, administration of edarabone alleviates their toxic effects dose-dependently. According to our previous study[21], administration of edarabone 1 h after ischemia significantly prevented the increase in the ABR threshold (Figure 18); hair cell loss was also prevented (Figure 19). These findings suggest that free radicals were produced after ischemic insult, and administration of edaravone prevented the toxic effects on the cochlea. Edaravone inhibits activation of the lipoxygenase pathway in the arachidonic acid cascade, which in turn prevents overproduction of superoxide anions. In addition, it scavenges NO directly in a dose-dependent manner.

NO plays an important role regulating vasodilatation and protecting neuronal tissues (Figure 20). When physical stress such as transient ischemia occurs, NO is produced in excess, due to the enzymatic activity of inducible NO synthase (iNOS). On the other hand, large amounts of superoxide are produced in the process of reperfusion after transient arterial occlusion. When NO reacts with superoxide, harmful free radicals such as nitrite (NO_2^-) and peroxynitrite (NO_3^-) are produced. Peroxynitrite causes cell membrane disintegration through lipid peroxidation. Figure 21 shows NO_2^- and NO_3^- concentrations in the perilymph at the scala tympani of the basal turn. Concentrations increased markedly on days 1 and 4, and returned to normal levels on day 7. Immunostaining showed that iNOS expression was prominent at the stria vascularis, spiral ligament, organ of Corti, and the spiral ganglion. The iNOS expression decreased gradually after ischemic insult but was still evident on day 7 (Figures 22 and 23).

Glutamate: Glutamate is a neurotransmitter at the synapse between IHCs and the primary afferent auditory nerve. When sound comes into the inner ear, K^+ enters and accumulates in IHCs, causing depolarization of the IHCs. Glutamate is released into the synaptic cleft in response to the firing of IHCs. After depolarization of IHCs, glutamate is absorbed by the surrounding supporting cells and IHCs, and is transformed into glutamine by enzymatic activity of the glutamate-aspartate transporter. Then glutamine is transferred to IHCs and encapsulated by vesicles, where it is transformed again into glutamate (Figure 24). In this way, glutamate is recycled around the synapses of IHCs[22,23].

When transient cochlear ischemia is induced, the glutamate recycling system is disturbed because ATP is not produced. In that situation, glutamate in the synaptic cleft is not absorbed, but flows out to the extracellular space. Figure 25 shows sequential changes in the glutamate concentration in the scala tympani after ischemic insult. The increase in glutamate concentration was marked but soon subsided after reperfusion. Histological findings revealed vacuolar formation in the synaptic cleft of IHCs as a result of the transient cochlear ischemia[24] (Figure 26). Furthermore, administration of AMPA (glutamate agonist) caused vesicle formation around synapses of IHCs, resembling the histological findings for transient cochlear ischemia[25]. Vesicle formation was more prominent when higher concentrations of AMPA were administered (Figure 27). A glutamate antagonist, DNQX, prevented IHC damage induced by cochlear ischemia. Such ischemic damage was not seen in OHCs.

Interactions of various mechanisms in ischemic cochlear damage: When blood supply to the cochlea is stopped completely, all structures in the inner ear are destined to die as a result of energy depletion. If the ischemia is transient, superoxide is produced in excess following recovery of the blood supply. Furthermore, iNOS is induced at the site of the lesion and facilitates excessive production of NO. By reacting with superoxide, NO is transformed into NO_2^- or NO_3^-. These are strongly toxic and damage cell membranes. Ischemic insult also induces glutamate ototoxicity, which is considered a major cause of IHC death. Figure 28 shows the proposed interacting mechanisms of ischemic cochlear damage.

ISCHEMIC TOLERANCE IN THE COCHLEA

Ischemic tolerance is a preconditioning phenomenon that is activated by mild stressors, and allows survival when exposed to subsequent potentially lethal stressors. After first being reported in the brain[26], this cytoprotection phenomenon has been found in other organs, such as the heart, liver, and spinal cord. It became widely recognized as a pertinent and important process in understanding how the brain protects itself against ischemia. At present, the underlying mechanism(s) of ischemic tolerance remain(s) unclear. According to Kirino[27], there are two main mechanisms of ischemic tolerance. First, there is a cellular defense mechanism that arises by posttranslational modification of proteins or by expression of new proteins *via* a signal transduction system. These cascades of events may strengthen the influence of survival factors or may inhibit apoptosis. Second, there is a cellular stress response and the synthesis of stress proteins, lead-

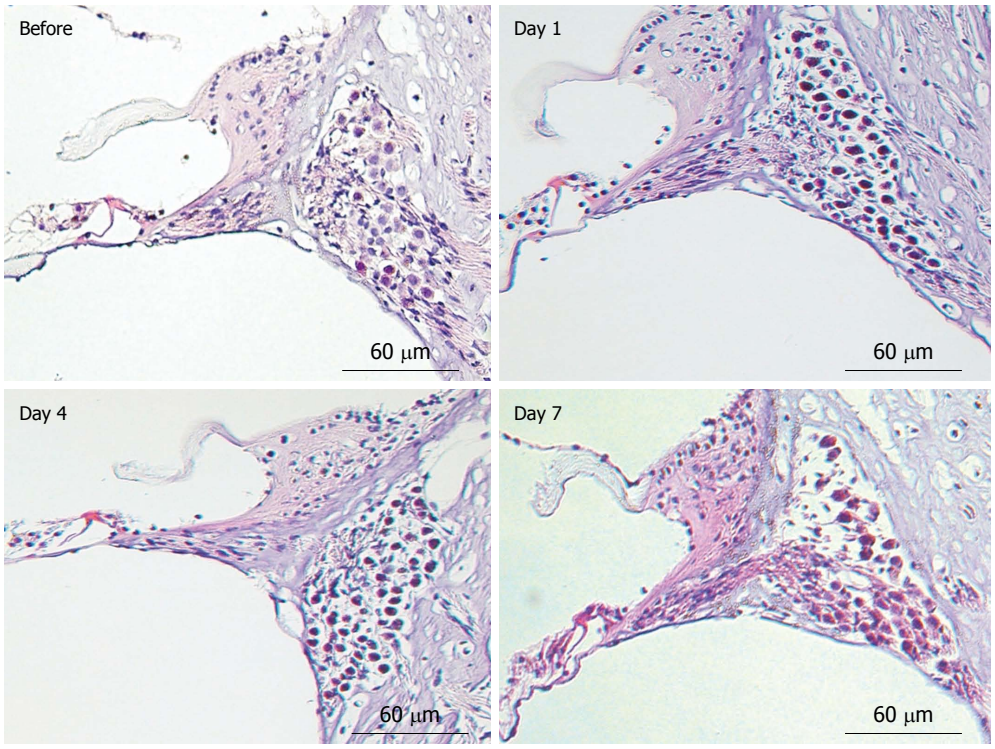

Figure 13 Histological findings of the organ of Corti and the spiral ganglion before and 1, 4, and 7 d after ischemic insult (hematoxylin and eosin staining). Originated from[19], with permission.

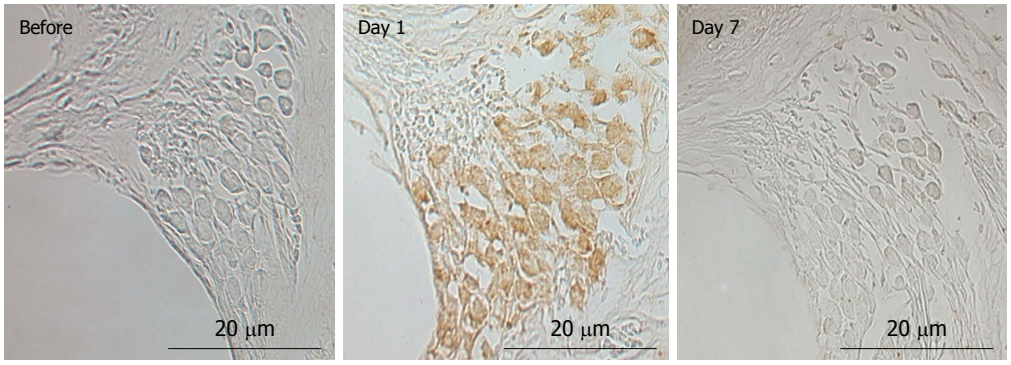

Figure 14 Immunostaining of Bax at the spiral ganglion before and after ischemic insult. Bax was expressed on day 1, but disappeared by day 7. Originated from[19], with permission.

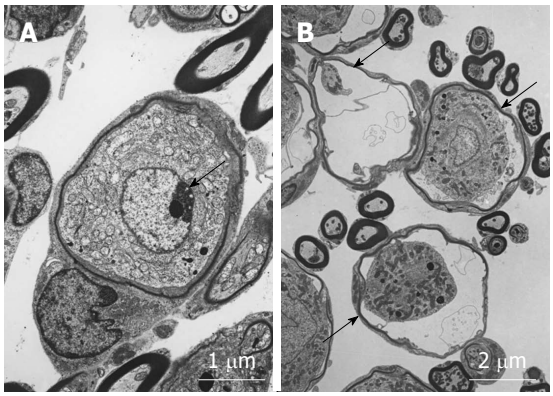

Figure 15 Transmission electron microscopy findings of spiral ganglion cells 1 dafter ischemic insult. A: Nucleus of the spiral ganglion cell underwent chromatin condensation (arrow) and segmentation, suggesting cell death due to an apoptotic process; B: The spiral ganglion cells appeared shrunken (arrows).

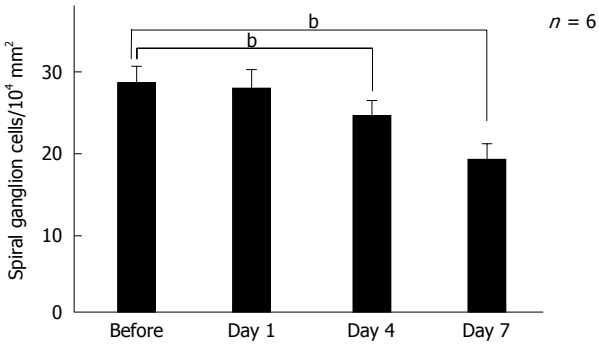

Figure 16 Sequential changes in the number of spiral ganglion neurons after ischemic insult. The number of spiral ganglion cells decreased gradually after ischemic insult. It did not change on day 1, but decreased on days 4 and 7. Each point indicates the mean number of the spiral ganglion neurons per 10^4 mm^2 before and 1, 4, and 7 d after ischemia. Vertical bars indicate one standard deviation. $^bP < 0.01$ *vs* the control group.

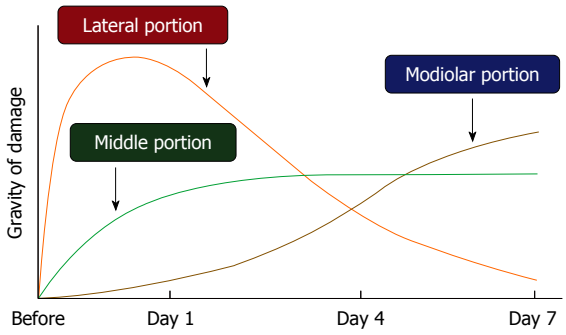

Figure 17 Sequential progression of ischemic damage in three regions of the cochlea. In the lateral region, including the stria vascularis and spiral ligament, ischemic damage occurred immediately after the insult, which recovered gradually to be almost normal on day 7. In the middle region, loss of inner hair cells progressed for the first 2-3 d; thereafter, no further deterioration occurred. In the modiolar region, damage to the spiral ganglion cell was minor for a few days, but became prominent thereafter. The vertical scale indicates grade of damage.

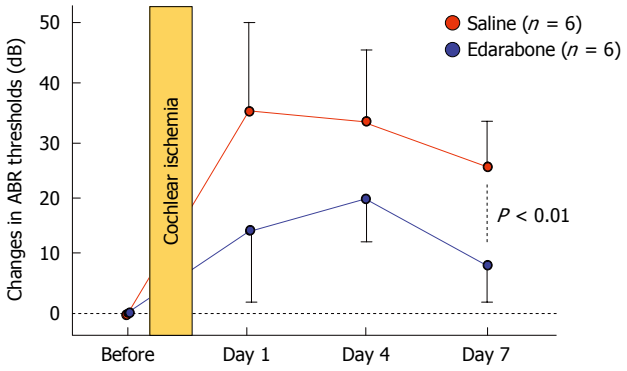

Figure 18 Edaravone ameliorates elevation of auditory brainstem responses threshold after ischemic insult. Originated from[21], with permission.

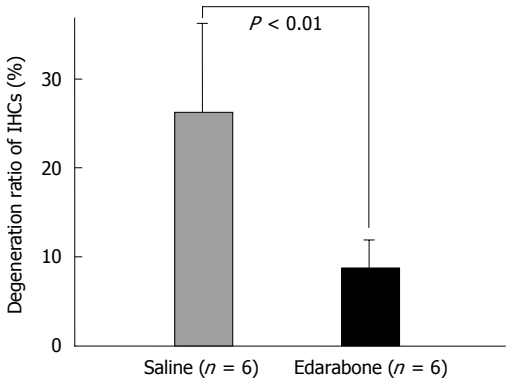

Figure 19 Edaravone prevents inner hair cell loss after ischemic insult. IHCs: Inner hair cells. Originated from[21], with permission.

ing to an increased capacity for health maintenance inside the cell. These proteins work as cellular chaperones by unfolding misfolded cellular proteins.

Presently, the greatest drawback of the vascular theory as an etiology of ISSHL is that it does not explain why recurrence and bilateral incidence of ISSHL are very rare. This issue may be resolved by studying ischemic tolerance in the cochlea. Using an animal model of transient

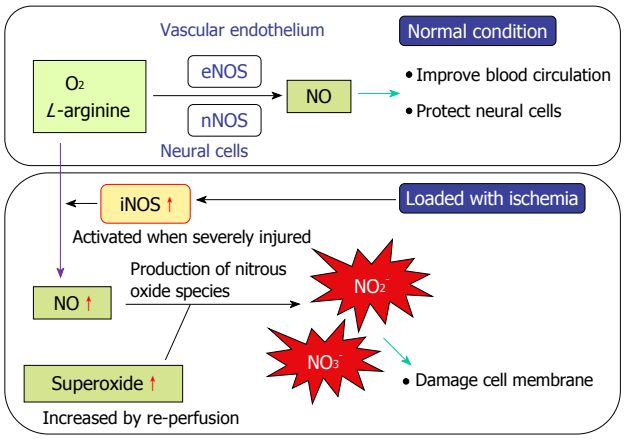

Figure 20 Mechanisms of nitrous oxide and free radical production. In a normal environment, nitrous oxide (NO) not only improves blood circulation by dilating blood vessels, but also protects neural cells against ischemic damage. Production of NO is regulated by enzyme activities of endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS). When transient ischemia occurs, production of NO is facilitated by the expression of inducible NO synthase (iNOS). As superoxide species increase in response to reperfusion, the excessive amounts of NO react with superoxide, resulting in the production of nitrite (NO_2^-) and peroxynitrite (NO_3^-). Free radicals are strong toxins that cause destruction of the cell membrane, resulting in cell death.

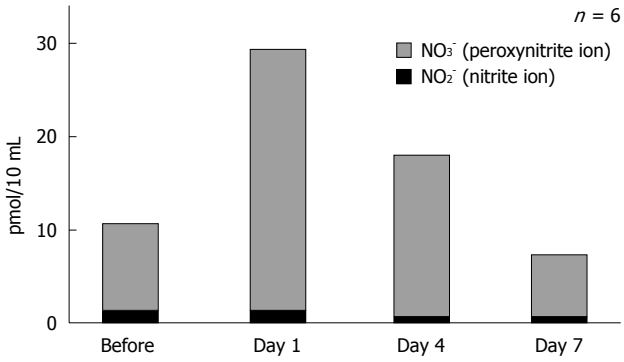

Figure 21 Concentration of nitride and peroxynitride in the scala tympani after ischemic insult. The levels of nitrogen oxides (NO_2^-), especially peroxynitride (NO_3^-), increased significantly on day 1 after ischemia, and then decreased gradually thereafter. Originated from[17], with permission.

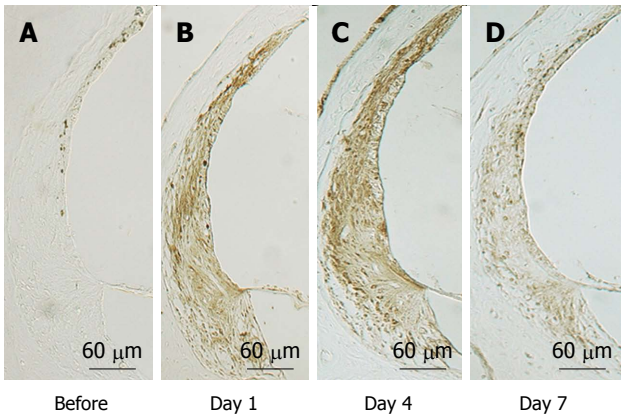

Figure 22 Immunostaining of the lateral wall of the cochlea for inducible nitrous oxide synthase before (A) and 1 (B), 4 (C), and 7 d (D) after ischemia. The stria vascularis and the spiral ligament showed marked immunostaining of inducible nitrous oxide synthase on days 1 and 4, which decreased by day 7.

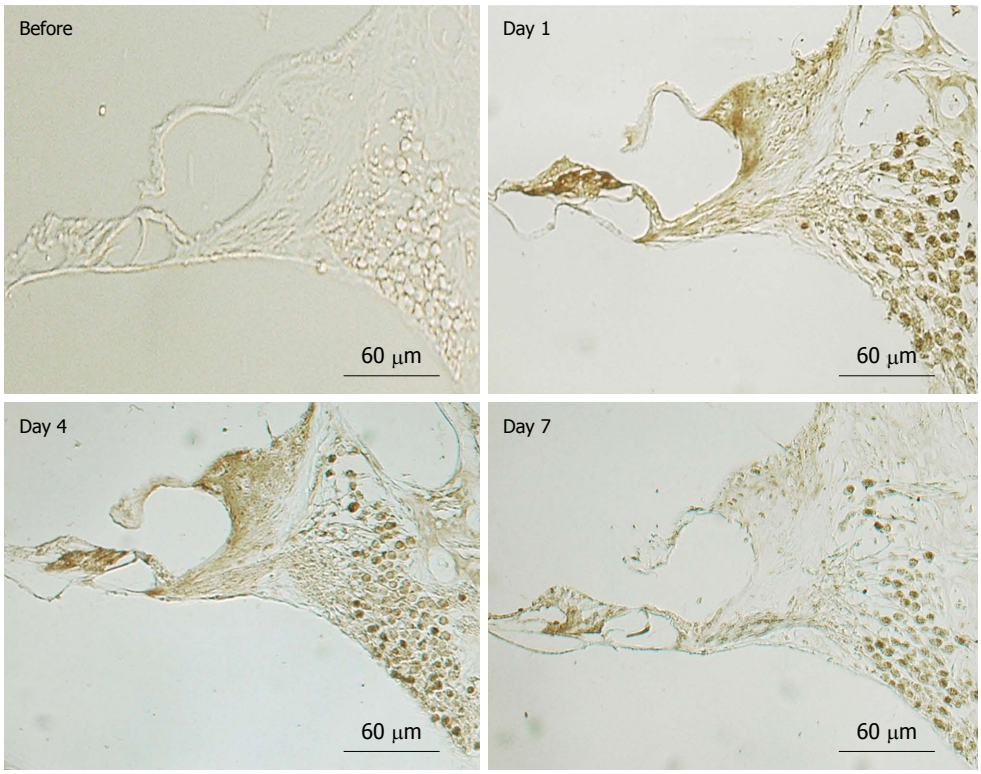

Figure 23 Immunostaining of the organ of Corti and the spiral ganglion for inducible nitrous oxide synthase before and 1, 4, and 7 d after ischemia. Immunostaining for inducible nitrous oxide synthase was observed in the inner hair cells, spiral ganglion cells, and spiral limbus on days 1 and 4. It was obviously decreased on day 7, but the synaptic area underneath the outer hair cells and the spiral ganglion cells was still positively stained. Originated from[17], with permission.

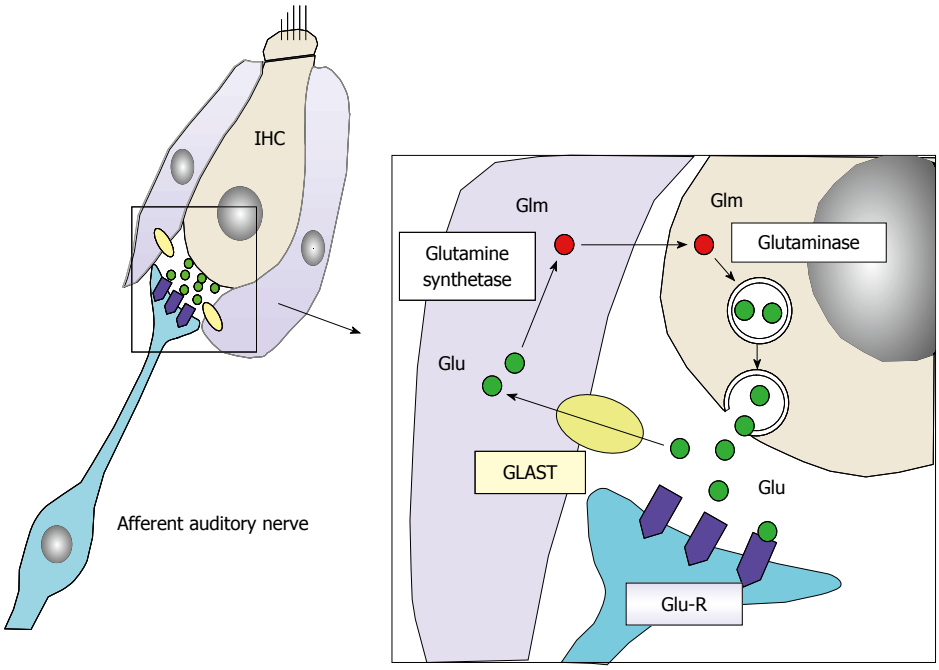

Figure 24 Schematic drawing of glutamate recycle system as a neurotransmitter between inner hair cells and the primary auditory neuron. Glutamate is an excitatory neurotransmitter between inner hair cells (IHCs) and primary auditory neurons. It is released into the synaptic cleft in response to depolarization of IHCs. After stimulating a primary auditory neuron by binding to a glutamate receptor (Glu-R), it is absorbed by the surrounding supporting cells (inner phalangeal cell and inner pillar cells) by means of glutamate-aspartate transporter (GLAST). It is transformed into glutamine, and then transported to IHCs and stored in a vesicle until the next depolarization of the IHC. In this way, the glutamate is recycled.

cochlear ischemia, we investigated whether ischemic tolerance existed in the cochlea[28]. The animals were divided into two groups: the single ischemia group and the double ischemia group. In the single ischemia group, animals

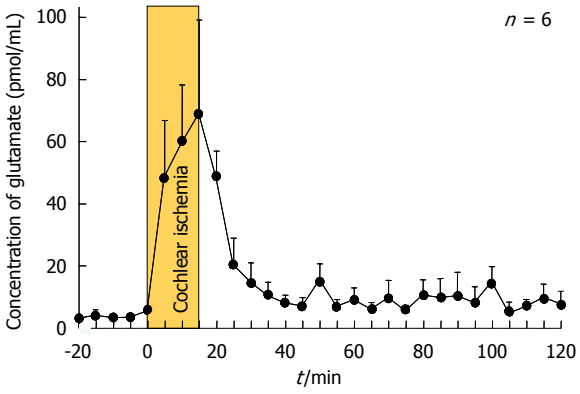

Figure 25 Glutamate concentration in the scala tympani after transient cochlear ischemia. Originated from[30], with permission.

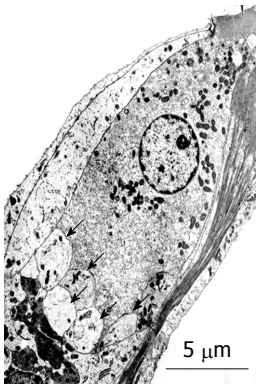

Figure 26 Transmission electron microscopy findings for inner hair cells after exposure to ischemia. Many vesicles formed at the synaptic cleft (arrows). Originated from[30], with permission.

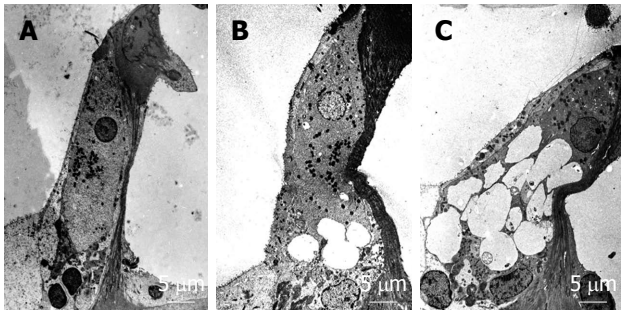

Figure 27 Dendritic terminals in synaptic contact with inner hair cells at the basal turn following administration of (A) artificial perilymph, (B) 50 mmol/L α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, or (C) 200 mmol/L α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate. Swelling of the dendritic terminals was not observed after injection of artificial perilymph. Although swelling of the dendritic terminals was observed at both concentrations of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), the extent of swelling was more pronounced with 200 μmol/L than with 50 μmol/L AMPA. Originated from[25], with permission.

were subjected to 15 min ischemia. In the double ischemia group, animals were subjected to 2 min ischemia 2 d before 15 min ischemia. Figure 29 shows the sequential changes in ABR thresholds in the two groups. There was no change in ABR threshold on day 1 in the single ischemia group. On day 3, 15 min ischemia was induced in both groups. As shown in this figure, hearing loss on days

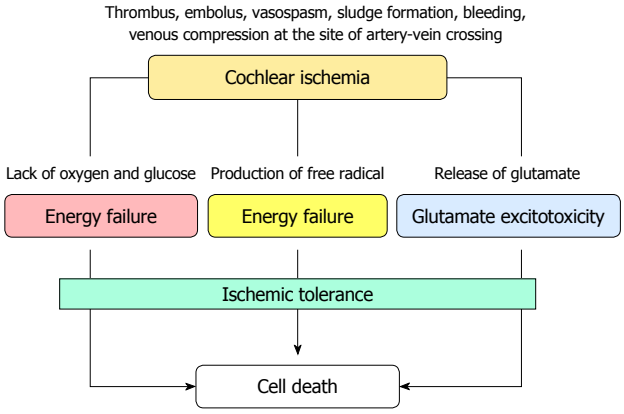

Figure 28 Suggested mechanism of cell death after transient cochlear ischemia. Cell death is considered to result from energy failure, production of free radical species, and glutamate excitotoxicity. Cell death is prevented, to some extent, by the mechanism of ischemic tolerance.

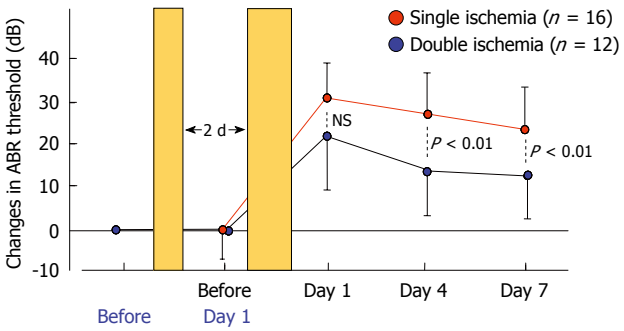

Figure 29 Auditory brainstem responses threshold shifts after single and double cochlear ischemia. Temporal profiles of the shift in the mean auditory brainstem responses (ABR) threshold in the single ischemia group (n = 16) and the double ischemia group (n = 12). All values are presented as mean ± SD. Preconditioning sublethal ischemia for 2 min significantly suppressed the elevation of the ABR threshold with subsequent lethal ischemia for 15 min. Originated from[28], with permission.

4 and 7 was more severe in the single ischemia group than in the double ischemia group ($P < 0.05$). Figure 30 summarizes the ratios of degenerated IHCs in the single and double ischemia groups. IHC loss was more severe in the single ischemia group, whereas fewer cells died in the double ischemia group. These findings suggest that ischemic preconditioning ameliorated ischemia-induced hearing impairment and loss of IHCs.

These results suggest that the rare recurrence of ISSHL might be due to ischemic tolerance in the cochlea. Unlike the immune system generally, this phenomenon does not work for long; the effect persists at most 7 d in brain ischemia. To assess whether this effect could be extended for longer periods, repeated minor ischemia would be necessary. If long-persisting ischemic tolerance can be induced by repeated minor ischemia, this phenomenon might be useful as a method for protecting the cochlea from ischemic damage.

TREATMENT OF ISCHEMIC HEARING LOSS

We have investigated various treatment modalities using

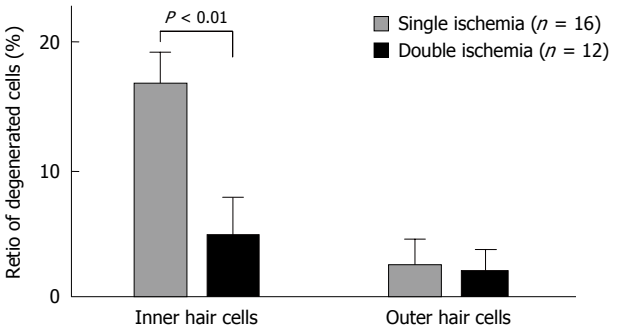

Figure 30 Loss of inner and outer hair cells after single and double cochlear ischemia. Pretreatment with sublethal cochlear ischemia reduced inner hair cell (IHC) damage 7 d after lethal cochlear ischemia. In the double ischemia group (n = 12), the proregion of defects in IHCs was significantly lower than that in the single ischemia group (n = 16). On the other hand, there was no statistically significant difference in the amount of outer hair cell loss between the two groups.

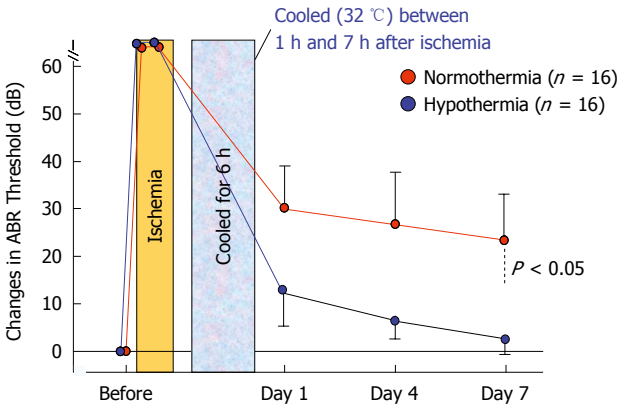

Figure 31 Sequential changes in auditory brainstem responses thresholds after ischemic insult in normothermic and mildly hypothermic animals. Mild hypothermia (32 ℃) from 1 to 7 h after ischemic insult prevented elevations in auditory brainstem responses threshold. The differences between the two groups were statistically significant (P < 0.05).

the animal model, including hypothermia and topical or general administration of test agents. Therapeutic hypothermia is already used as a medical treatment for ischemic brain injuries. Topical administration of test agents by placing them on the round window membrane is an effective way to deliver a medicine into the inner ear. As the amount of agent absorbed from the middle ear is limited, the incidence of possible side effects may also be minimized. We have investigated two agents, insulin-like growth factor 1 (IGF-1) and AM-111, using this method. Systemic administration was performed by administering the various test agents intravenously or intraperitoneally to investigate their protective effects in ischemic damage. Agents tested were prednisolone (steroid hormone), edarabone (antioxidant), prosaposine-derived synthetic peptide (saposine), ginsenoside Rb1 (gRb1) (Kanpo), liposome-encapsulated hemoglobin (LEH), glial cell-derived neurotrophic factor (GDNF), and hematopoietic and neural stem cells.

Hypothermia

The effects of post-ischemic mild hypothermia on ischemic cochlear damage were investigated by changing the timing and duration of cooling[29,30]. Animals were subjected to mild hypothermia (32 ℃) following transient cochlear ischemia. They were divided into six groups, based on the start and end of hypothermia after reperfusion (n = 16 for each group). As shown in Figure 31, post-ischemic mild hypothermia effectively alleviated hearing impairment and hair cell loss when it was applied 1-7 h after reperfusion[31]. The protective effects were more prominent with earlier and longer application of hypothermia. Mild hypothermia 6-9 h after reperfusion did not prevent ischemic damage to the cochlea.

Intratympanic administration

IGF-1: We tested the protective effects of recombinant human IGF-1, applied locally with a gelatin-hydrogel, against ischemic cochlear damage in gerbils. IGF-1 or distilled water (control) immersed in gelatin-hydrogel was applied to the round window membrane through the otic bulla 30 min after ischemic insult (n = 6 for each group). Local administration of IGF-1 significantly reduced the elevation of ABR threshold at 8 kHz on days 1, 4, and 7 after ischemic insult. A histological study also showed that the survival rate of IHCs 7 d after ischemia increased after administration of IGF-1 with the hydrogel. As the gelatin hydrogel dissolved slowly in the body, IGF-1 was released continuously and transported into the inner ear. These findings suggest that local application of IGF-1, in gelatin hydrogel, may prevent ischemic damage to the cochlea[32].

AM-111 (anti-apoptotic agent): AM-111, a cell-permeable peptide inhibitor of c-Jun N-terminal kinase, was investigated for its protective effects against ischemic damage of the cochlea. After induction of transient cochlear ischemia, 10 μL AM-111 at a concentration of 1, 10, or 100 μmol/L in a hyaluronic acid gel formulation was applied to the round window 30 min after the insult (n = 6 for each group). Treatment effects were evaluated by ABR and histology of the inner ear. In controls, transient cochlear ischemia caused a 25.0 ± 5.0 dB increase in the ABR threshold at 8 kHz, and a decrease of 13.3% ± 2.3% in IHCs at the basal turn on day 7. Ischemic damage was mild at 2 and 4 kHz. When the animals were treated with AM-111 at 100 μmol/L, cochlear damage was significantly reduced: the increase in ABR threshold was 3.3 ± 2.4 dB at 8 kHz, and the IHC loss was 3.1% ± 0.6% at the basal turn on day 7. The effects of AM-111 were concentration-dependent: 100 μmol/L was more effective than 1 μmol/L or 10 μmol/L. Direct application of AM-111 in a gel formulation to the round window effectively prevented acute hearing loss due to transient cochlear ischemia[33].

Systemic administration

Prednisolone (steroid): The effects of prednisolone on ischemia-induced cochlear damage were investigated. After

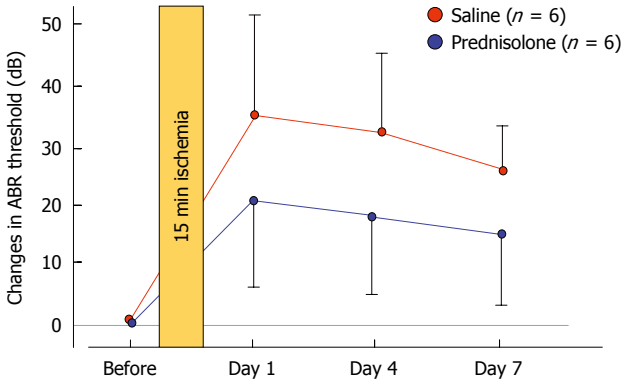

Figure 32 Effects of prednisolone on the shift of auditory brainstem responses threshold after transient cochlear ischemia. Hearing was assessed before and 1, 4, and 7 d after ischemic injury. An increase in auditory brainstem responses threshold that was observed on day 7 was lower in the prednisolone-treated group than in the control group, although the difference was not statistically significant. Originated from[34], with permission.

inducing 15 min ischemia, animals were treated by intraperitoneal injection of prednisolone (1 mg/kg) or physiological saline (control) (n = 6 for each group). Sequential changes in hearing were evaluated by recording ABR before and 1, 4, and 7 d after treatment. The increase in ABR threshold on day 7 was 24.2 ± 8.6 dB in control animals but 14.2 ± 9.2 dB in prednisolone-treated animals (Figure 32). Histological staining for hair cells using rhodamine-phalloidin and Hoechst 33342 showed that the percentage of IHC loss at the basal turn was 26.5% ± 11.4% in the control and 5.3% ± 3.0% in the prednisolone-treated group. These results indicate that prednisolone protects against inner ear damage caused by ischemic insult, even when administered after the insult[34].

Edarabone (antioxidant agent): Edaravone, a free radical scavenger, has potent protective effects on ischemic damage. Edaravone (1 mg/kg, *iv*) or saline was administered 1 h after ischemia (n = 6 for each group). In animals treated with saline, the ABR threshold shift was 24.1 ± 4.2 dB and there was a 26.5% ± 11.4% decrease in the number of IHCs. In contrast, in animals treated with edaravone, the threshold shift was 7.5 ± 4.2 dB and only 8.8% ± 3.5% of IHCs were lost. These results suggest that edaravone protects against ischemic damage of the inner ear following transient ischemia[21].

Prosaposin-derived synthetic peptide: A peptide resembling the neurotrophic region of prosaposin (18-mer peptide, PS-pep) was synthesized artificially and administered subcutaneously four times: immediately and 1, 2 and 3 d after induction of transient cochlear ischemia (n = 6 for each group). On day 7, the ABR threshold shift was 33.3 ± 16.3 dB in animals treated with saline, while it was 12.5 ± 8.2 dB in animals treated with 2.0 mg/kg PS-pep. This alleviation was not seen in animals treated with 0.2 mg/kg PS-pep or saline. Histological examinations conducted on day 7 showed that higher doses of PS-pep significantly alleviated IHC loss, whereas a low dose did not. In addition, an increase in the anti-apoptotic factor bcl-2 was also noted in the IHCs of animals treated with higher doses of PS-pep. These findings suggest that PS-pep prevents hearing loss and cochlear damage due to transient cochlear ischemia by activating an anti-apoptotic pathway[35].

gRb1 (Kanpo): gRb1 is a Kanpo medicine that has protective effects on ischemic brain damage, in addition to other various effects such as regeneration of blood vessels, activation of plasmins, and release of corticosteroids. Using this agent, on day 7 after ischemia, the percentage of SGCs decreased to 67.5% from the preischemic baseline in the basal turn in the control group, whereas it was 90.2% in the gRb1-treated group. Immunohistochemical staining showed TUNEL-positive reactions in the SGCs, with fragmented nuclei. We also investigated the protective effects of gRb1 against ischemic injury in the cochlea. On day 7, the ABR threshold shift in the gRb1-treated group was 14.2 ± 3.8 dB and that in the control group was 22.5 ± 2.9 dB. Furthermore, loss of IHCs in the gRb1-treated group was 8.6% ± 2.6% and that in the control group was 26.5% ± 11.4%. These differences were statistically significant. These findings indicate that gRb1 prevents hearing loss caused by ischemic insult[19].

LEH: LEH was originally developed as an artificial red blood cell (RBC). The experimental animals were randomly assigned to receive 2 mL/kg of low-affinity LEH (l-LEH, P_{50} = 40 mmHg), high-affinity LEH (h-LEH, P_{50} = 10 mmHg), homologous RBCs, or saline 30 min before transient cochlear ischemia (n = 6 for each group). Sequential changes in hearing were assessed by recording ABR at 8, 16, and 32 kHz 1, 4, and 7 d after ischemic insult, and then the animals were sacrificed for histological studies. The ABR study showed that h-LEH was more protective than l-LEH in suppressing hearing loss, in contrast to RBCs or saline treatment. In the morphological study, loss of IHCs was most effectively protected against by h-LEH. These findings suggest that pretreatment with h-LEH is significantly more protective than l-LEH in mitigating hearing loss and underlying pathological damage, in contrast to transfusion or saline infusion 7 d after transient cochlear ischemia[36].

GDNF: GDNF promotes the survival and differentiation of dopaminergic neurons, and is able to prevent apoptosis of motor neurons induced by axotomy. We assessed the utility of an adenoviral vector expressing GDNF (Ad-GDNF) in ischemia-reperfusion injury of the gerbil cochlea. The vector was injected through the round window 4 d before ischemic insult. The distribution of a reporter transgene was confirmed throughout the cochlea, from the basal to the apical turn, and Western blot analysis indicated significant upregulation of GDNF protein 11 d following virus inoculation. Hearing ability was assessed by sequentially recording electrocochleogram, and the degree of hair cell loss was evaluated in specimens stained

with rhodamine-phalloidin and Hoechst 33342. On day 7 after ischemia, the shift in compound action potentials threshold in electrocochleogram and IHC loss were markedly suppressed in the Ad-GDNF group, compared to the control group. These results suggest that adenovirus-mediated overexpression of GDNF may be useful for protection against hair cell damage, which otherwise eventually occurs after transient ischemia in the cochlea[37].

Hematopoietic stem cells: Transplantation of hematopoietic stem cells (HSCs) is considered a potential approach for promoting the repair of damaged organs. We investigated the influence of HSCs on progressive hair cell degeneration after transient cochlear ischemia. After induction of ischemia, animals were treated with an intramuscular injection of HSCs. This procedure prevented degeneration of IHCs and ameliorated hearing impairment. In addition, the protein level of GDNF in the organ of Corti was upregulated after cochlear ischemia and treatment with HSCs augmented this upregulation of GDNF. Furthermore, HSCs injected into the cochlea remained in the perilymphatic space, although they did not transdifferentiate into cochlear cell types or fuse with injured hair cells after ischemia. This suggests that HSCs have therapeutic potential, possibly through paracrine effects. Based on these findings, we concluded that intramuscular injection of HSCs may be a potential new therapeutic strategy for hearing loss[38].

Neural stem cells: Neural stem cells are multipotent progenitor cells that show self-renewal activity. We assessed the use of neural stem cells for ameliorating ischemia-reperfusion injury in the gerbil cochlea. Neural stem cells were injected into the inner ear through the round window 1 d after ischemic insult. Immunostaining for nestin showed that the distribution of neural stem cells was concentrated within the organ of Corti. Seven days after ischemia, the injury-induced shift in ABR and progressive IHC damage were markedly reduced on the neural stem cell-transplanted side. These results suggest that the transplantation of neural stem cells is therapeutically useful for preventing the damage to hair cells that occurs after transient ischemia of the cochlea[39].

CAUSES OF ISSHL

Presently, vascular theory as an etiology of ISSHL remains speculative, because cochlear pathology is difficult to assess in live humans. Figure 33 illustrates the possible causes of ischemic damage in the cochlea. Many risk factors, such as hyperlipoproteinemia, hyperglycemia, obesity, smoking, and stress, have been correlated with the incidence of ISSHL. However, the real cause(s) of this disease remain(s) unknown, primarily because of technical difficulties. As 30%-40% of ISSHL patients heal spontaneously, permanent occlusion of the major nourishing artery would not seem to be a major cause of ISSHL. Instead, we believe that ISSHL is caused by transient or local ischemia, caused by microcirculatory disturbances in the stria vascularis.

Table 1 Clinical features of idiopathic sudden sensorineural hearing loss and those of branch retinal vein occlusion

	ISSHL	Branch retinal vein occlusion
Annual incidence in Japan	35 000	30 000-50 000
Age preponderance (yr)	50-60	60
Sex difference	No	No
Incidence	Sudden	Sudden
Background disease	Unknown	Arteriosclerosis, diabetes mellitus
Involved site	Stria vascularis?	Retinal vein
Bilateral incidence	Rare	2%-4%
Recurrence	Rare	Rare
Spontaneous healing	30%-50%	30%-50%
Effects of steroid	Effective	Effective

ISSHL: Idiopathic sudden sensorineural hearing loss.

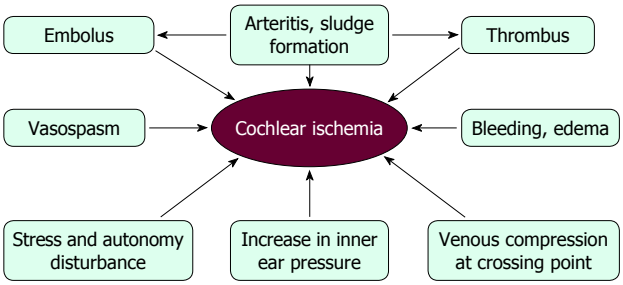

Figure 33 Possible mechanisms of cochlear ischemia that might result in idiopathic sudden sensorineural hearing loss.

Arterial-venous compression is a known cause of branch retinal vein occlusion, which is the second most common cause of blindness in America. ISSHL has similar clinical characteristics to BRVC, as shown in Table 1. If arterial-venous compression is a cause of ISSHL, the capillary network in the stria vascularis would be the most likely site of the lesion. As ATP production occurs mainly in the lateral region, even a minor circulatory disturbance could cause ischemic damage in the cochlea.

CONCLUSION

Using adult gerbils, we induced transient cochlear ischemia without a craniotomy. Ischemic insult caused mild hearing loss and sporadic loss of hair cells, especially IHCs. Mechanisms of induced cochlear damage included depletion of the energy supply, production of free radicals, and glutamate ototoxicity. We found that the cochlea shows ischemic tolerance, which may explain why recurrence is rare in ISSHL. We also reported the results of various treatment modalities, such as hypothermia and the topical and general administration of test agents.

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BRIEF ARTICLE

Elective regional lymphadenectomy for advanced auricular squamous cell carcinoma

William R Ryan, Chase M Heaton, Steven J Wang

William R Ryan, Chase M Heaton, Steven J Wang, Division of Head and Neck Surgery, Department of Otolaryngology, University of California, San Francisco, CA 94115, United States
Author contributions: All authors were involved in data gathering, data synthesis, and manuscript preparation.
Correspondence to: Chase M Heaton, MD, Division of Head and Neck Surgery, Department of Otolaryngology, University of California, San Francisco, CA 94115,
United States. cheaton@ohns.ucsf.edu
Telephone: +1-650-3876807 Fax: +1-415-8857171
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Abstract

AIM: To investigate the rate of occult lymph node disease in elective parotidectomy and neck dissection specimens in patients with advanced auricular cutaneous squamous cell carcinoma (cSCC).

METHODS: At a single institution, from 2000 to 2010, 17 patients with advanced auricular cSCC were considered high risk for occult regional parotid and/or neck nodal metastases and, thus, underwent an auriculectomy and elective regional lymphadenectomy (parotidectomy and/or neck dissection). Indications for elective regional lymphadenectomy were large tumor size, locally invasive tumors, post-surgical and post-radiation recurrence, and being an immunosuppressed patient. We determined the presence of microscopic disease in the regional (parotid and neck dissection) pathology specimens.

RESULTS: There were 17 advanced auricular cSCC patients analyzed for this study. Fifteen (88%) patients were men. The average age was 69 (range: 33 to 86). Ten (59%) patients presented with post-surgical recurrence. Five (29%) patients presented with post-radiation recurrence. Four (24%) patients presented with both post-surgical and post-radiation recurrence. Four (24%) patients were immunosuppressed (2 (12%) were liver transplant patients, 2 (12%) were chronic lymphocytic leukemia patients, and 1 (6%) was both). The subsite distribution of cSCC included helix (3, 18%), antihelix (2, 12%), conchal bowl (7, 41%), tragus (2, 12%), and postauricular sulcus (3, 18%). Four (24%) patients presented with multifocal auricular cSCC. No patients had bilateral disease. All patients were confirmed to have cSCC on final pathology. The tumors were well (5, 29%), moderately (10, 59%), and poorly (2, 12%) differentiated SCC. The average size of the cSCC tumor was 2.9 cm (range: 1.7 to 7 cm). Twelve (70%) tumors were greater than 2 cm. Six (35%) patients underwent partial auriculectomy. Eleven (65%) patients underwent total auriculectomy. Eight (47%) patients underwent elective parotidectomy and elective neck dissections; 3 (18%) underwent only elective parotidectomy; 3 (18%) underwent only an elective neck dissection; 2 (12%) underwent an elective parotidectomy and therapeutic neck dissection; and 1 (6%) underwent a therapeutic parotidectomy and an elective neck dissection. None of the elective parotidectomy or neck dissection specimens were found to contain any malignant disease. All therapeutic parotidectomy and neck dissection specimens contained metastatic SCC. Fourteen (82%) underwent parotidectomy. Of these, 10 (71%) underwent superficial parotidectomy whereas 4 (29%) underwent total parotidectomy. Fourteen (82%) underwent neck dissections [levels Ⅱ/Ⅴa (1, 7%), levels Ⅱ/Ⅲ/Ⅴa (2, 14%), levels Ⅰ/Ⅱ/Ⅲ/Ⅴa (2, 14%), and complete levels Ⅰ-Ⅴ (9, 64%)]. Three (18%) underwent concurrent temporal bone resections for tumor extension from the auricle. The average follow-up for our patients was 44 mo (range: 4 to 123 mo). At the time of the review, 6 (35%) patients were alive and 11 (65%) had passed away.

CONCLUSION: This study suggests that, in patients with advanced auricular cutaneous SCC, elective regional lymphadenectomy is not necessary. However,

furtherprospective studies are necessary to assess the necessity.

Key words: Advanced auricular squamous cell carcinoma; Elective lymphadenectomy; Elective parotidectomy; Elective neck dissection; Occult regional metastases

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INTRODUCTION

Advanced auricular cutaneous squamous cell carcinoma (cSCC) has conventionally been treated with partial or total auriculectomy accompanied by a regional lymphadenectomy, which often includes a parotidectomy and/or a neck lymph node dissection for clinically apparent or occult lymph node disease. However, this treatment paradigm is controversial and without solid data[1]. Auricular cSCC is generally defined as advanced when it has one or more of the following features: large tumor size, increased depth, locally invasive tumors (cartilage and beyond), multifocal disease, clinically apparent lymph node metastases, post-surgical recurrence, post-radiation recurrence, and when the patient is immunosuppressed[2-5]. In patients with auricular cSCC, the practice of performing therapeutic lymphadenectomy follows the logical reasoning of excising known disease whereas the practice of performing elective lymphadenectomy is done out of perceived increased risk of occult nodal disease and a known higher mortality rate associated with advanced auricular cSCC[2,6,7].

The skin of the auricle has lymph drainage that flows to the pre-auricular/parotid, infra-auricular/neck, and post-auricular nodal basins[8,9]. Several studies claim a higher rate of metastasis with auricular cSCC compared to other sites on the head and neck[10-14]. Studies show that, for external auditory canal and auricular cSCC, the range of involvement in the parotid or neck lymph nodes is 7.9% to 17.5%[5,7,14,15]. The rate of regional metastases for other sun exposed skin sites (including the head and neck) ranges from 2% to 5%[6]. The 5 years mortality rate directly attributable to regional metastases from cSCC can be as high as 56.6% to 66.7% even after combined surgical and radiation therapy[6,7].

With advanced auricular cSCC, elective lymphadenectomy is performed in these cases in an effort to maximize therapy, increase the chance of cure, and reduce the chance of locoregional recurrence. However, the incidence of occult parotid and neck lymph node malignant disease is currently unknown. Thus, we developed a study of patients with advanced auricular cSCC and clinically negative parotid and neck lymph nodes to determine the rate of occult microscopic lymph node disease in the elective parotidectomy and neck dissection.

MATERIALS AND METHODS

The Committee on Human Research at University of California, San Francisco approved this study. All patients gave informed consent for the operation they underwent.

We performed a retrospective chart review at our single institution from a database of all non-melanoma skin cancer patients treated surgically from 1997 to 2010. We found 17 patients with auricular cSCC and no suspicious regional (parotid or neck) lymphadenopathy on physical exam or imaging [by computed tomography (CT) or magnetic resonance imaging (MRI)] who underwent auriculectomy (partial or total) and elective regional lymphadenectomy (superficial or total parotidectomy and/or neck dissection to any extent) from 2000 to 2010. We included only patients with auricular cSCC confined to or originating from the helix, antihelix, conchal bowl, tragus, antitragus, postauricular sulcus, or lobule and those extending from these areas to the external auditory canal or temporal bone. We excluded patients who had isolated external auditory canal or temporal bone cSCC without auricular involvement or cSCC from other head and neck subsites.

Indications for elective regional lymphadenectomy retrospectively appeared to be large tumor size, locally invasive tumors (into cartilage, the external auditory canal, parotid, or the temporal bone), multifocal auricular disease (multiple lesions involving nonadjacent auricular subunits), recurrence after previous surgery, recurrence after previous radiation, and being immunosuppressed (including transplant or chronic lymphocytic leukemia patients). Additionally, we found elective neck dissections performed for suspicious parotid lymphadenopathy and elective parotidectomy performed for suspicious neck lymphadenopathy.

For each patient, we determined the following factors: gender, age, prior surgical or radiation treatment, immunosuppression, the preoperative radiology performed (CT or MRI) to determine lymph node disease, the extent of a surgical resection they underwent (partial *vs* total auriculectomy, superficial *vs* total parotidectomy, extent of neck dissections (levels 1, 2, 3, 4, and/or 5), temporal bone resection, and other procedures performed), the final surgical pathology results for the auricle tumor excision, the parotidectomy, and the neck lymphadenectomy, the size of the primary auricular tumor, the grade of the malignancy, if they subsequently underwent post-operative radiation treatment, and the length of follow-up from the surgical treatment, and their mortality/survival.

RESULTS

Table 1 displays the characteristics and treatment experience in the 17 advanced auricular cSCC patients analyzed for this study.

Table 1 Characteristics and treatment experience of the 17 advanced auricular cutaneous squamous cell carcinoma patients

Pt.	Age (yr)	Sex	Tumor size (cm)	Sub-site	Prior Rx	Auriculx type	Parotidx type	Neck diss. extent	Parotid, neck diss. path	Post-op XRT	F/u (mo)
1	71	M	3.0	AH	S, XRT	Total	Elective Superficial	-	Neg, -	-	28
2	86	M	2.5	AH	-	Partial	-	Elective (Ⅱ/Ⅲ/Va)	-, Neg	-	61 (D)
3	69	M	4.1	P	S, XRT	Total	Elective Superficial	Elective (complete)	Neg, Neg	-	80 (D)
4	69	M	1.6	C	S	Total	Elective Superficial	Elective (Ⅰ/Ⅱ/Ⅲ)	Neg, Neg	-	43 (L)
5	56	M	1.3	C	S	Total	Elective Superficial	Elective (complete)	Neg, Neg	Yes	48 (L)
6	68	M	7.0	P	-	Total	Elective Total	Elective (complete)	Neg, Neg	Yes	70 (D)
7	78	M	6.0	T	S, XRT	Total	Elective Total	Elective (complete)	Neg, Neg	-	10 (D)
8	33	F	1.9	C	-	Total	Elective Total	Therapeutic (complete)	Neg, Pos	-	61 (D)
9	70	M	1.7	H	S	Partial	Therapeutic Total	Elective (complete)	Pos, Neg	Yes	96 (L)
10	71	M	1.7	C	-	Partial	Elective Superficial	Therapeutic (complete)	Neg, Pos	Yes	123 (L)
11	86	M	2.0	P	S, XRT	Total	Elective Superficial	-	Neg, -	-	53 (D)
12	69	F	2.5	T	-	Partial	Elective Superficial	Elective (Ⅰ/Ⅱ/Ⅲ)	Neg, Neg	Yes	5 (D)
13	61	M	3.0	H	S	Partial	Elective Superficial	Elective (complete)	Neg, Neg	Yes	LTF
14	65	F	2.4	C	XRT	Total	-	Elective (complete)	-, Neg	-	10 (D)
15	78	M	4.2	C	S	Total	Elective Superficial	Elective (Ⅱ/Ⅲ/Va)	Neg, Neg	Yes	5 (L)
16	84	M	2.0	H	-	Total	-	Elective (Ⅱ/Va)	-, Neg	-	11 (D)
17	65	M	3.0	C	-	Total	Elective Superficial	-	Neg, -	Yes	4 (L)

M: Male; F: Female; AH: Antihelix; P: Posterior sulcus; C: Concha bowl; T: Tragus; H: Helix; Rx: Treatment; S: Surgery; XRT: Radiation treatment; D: Deceased; L: Live; LTF: Lost to follow-up.

Fifteen (88%) patients were men. The average age was 69 years (range: 33 to 86 years). Ten (59%) patients presented with post-surgical recurrence. Five (29%) patients presented with post-radiation recurrence. Four (24%) patients presented with both post-surgical and post-radiation recurrence. Four (24%) patients were immunosuppressed 2 (12%) were liver transplant patients, 2 (12%) were chronic lymphocytic leukemia patients, and 1 (6%) was both). Six (35%) patients had preoperative CT; 6 (35%) had preoperative MRI; and 5 (29%) had no preoperative imaging. The subsite distribution of cSCC included helix (3, 18%), antihelix (2, 12%), conchal bowl (7, 41%), tragus (2, 12%), and postauricular sulcus (3, 18%). Four (24%) patients presented with multifocal auricular cSCC. No patients had bilateral disease.

All patients were confirmed to have cSCC on final pathology. The tumors were well (5, 29%), moderately (10, 59%), and poorly (2, 12%) differentiated SCC. The average size of the cSCC tumor was 2.9 cm (range: 1.7 to 7 cm). Twelve (70%) tumors were greater than 2 cm. Six (35%) patients underwent partial auriculectomy. Eleven (65%) patients underwent total auriculectomy.

Eight (47%) patients underwent elective parotidectomy and elective neck dissections; 3 (18%) underwent only elective parotidectomy; 3 (18%) underwent only an elective neck dissection; 2 (12%) underwent an elective parotidectomy and therapeutic neck dissection; and 1 (6%) underwent a therapeutic parotidectomy and an elective neck dissection. Six surgeons including Ryan WR, Wang SJ performed the operations (along with 3 different temporal bone surgeons).

None of the elective parotidectomy or neck dissection specimens were found to contain any malignant disease. All therapeutic parotidectomy and neck dissection specimens contained metastatic SCC.

Fourteen (82%) underwent parotidectomy. Of these, 10 (71%) underwent superficial parotidectomy whereas 4 (29%) underwent total parotidectomy. Fourteen (82%) underwent neck dissections [levels Ⅱ/Va (1, 7%), levels Ⅱ/Ⅲ/Va (2, 14%), levels Ⅰ/Ⅱ/Ⅲ/Va (2, 14%), and complete levels Ⅰ-Ⅴ (9, 64%)]. Three (18%) underwent concurrent temporal bone resections for tumor extension from the auricle. One (6%) patients underwent a concurrent condyle resection and infratemporal lymph node dis-

section (for therapeutic purposes).

Eight (47%) underwent post-operative radiation.

The average follow-up for our patients was 44 mo (4 to 123 mo). At the time of the review, 6 (35%) patients were alive and 11 (65%) had passed away.

DISCUSSION

This study shows a 10-year experience by 6 different surgeons at one institution of 17 patients with advanced auricular cSCC all of whom had negative elective lymphadenectomy specimens on final surgical pathologic analysis. This absolute result calls into the question the need for elective parotidectomy and neck dissection in the cases of advanced auricular cSCC.

This study was partly inspired by and corroborates Osborne *et al*[16] in their study of advanced auricular cSCC and elective parotidectomy. Osborne *et al*[16] found that, in 19 patients, none of the elective parotidectomy specimens performed for advanced auricular cSCC had any positive final surgical pathology. We found the same results with elective parotidectomy and, uniquely, the same results for the elective neck dissections.

Elective lymphadenectomy is performed for any cancer of the head and neck when the predicted risk of occult nodal disease reaches a certain threshold of 15% to 20%. An elective lymphadenectomy is performed to avoid unsalvageable neck disease and improve survival accepting the fact that some unnecessary surgery will be performed. However, our data showing no nodal metastases in 12 patients who underwent elective neck dissection suggests that elective regional lymphadenectomy for auricular cSCC may not necessarily be beneficial.

Limiting the use of elective parotidectomy and neck dissection could reduce the cost, time, and potential morbidity associated with these operations. The total time associated with the parotidectomy and neck dissection includes operating time, hospital stay, and recovery at home. Both procedures require drain placement and a hospitalization of one to several days. Parotidectomy surgery carries the risks of temporary or permanent facial nerve injury (with possible corneal keratitis, facial droop, asymmetric smile, and oral incompetence), hematoma, seroma, salivoma, cellulitis, abscess, skin flap loss, gustatory sweating (Frey's Syndrome), unwanted indentation in the face, unwanted incision, and perincisional, great auricular, and auriculotemporal nerve injury-associated numbness. Neck dissection, depending on the extent performed, carries the addition risks of unwanted neck sensory dysfunction, neck soft tissue defect if the sternocleidomastoid muscle is removed, chylous fistula/leak, and motor nerve injury to the spinal accessory, marginal mandibular, hypoglossal, vagus, superior laryngeal, phrenic, sympathetic, and brachial plexus nerves. In addition, conceivably, a total auriculectomy could be performed under local anesthesia preventing the need for a general anesthetic and intubation all together in select cases.

The data on cSCC in this study correlates with the body of evidence showing the lack of benefit for elective lymphadenectomy for higher stage malignant melanoma. Several studies show with different thicknesses of malignant melanoma in different parts of the body that elective lymphadenectomy (including parotidectomy and neck dissections in some studies) brought no measurable increase in locoregional control, disease-specific survival, or overall survival[17-19]. Thus, possibly a similar management protocol could be relevant to advanced cases of cSCC with regards to the use of sentinel lymph node biopsy for assessing the need for elective lymphadenectomy[20].

This study is by no means a complete denouncement on the use of elective lymphadenectomy for advanced auricular or head and neck cSCC. There is the risk of leaving occult disease in a patient when a regional lymph node bed is left untreated. Subclinical neck malignancy lymphadenopathy rates in head and neck cSCC are reported as being as high as 35% in 2 studies[21,22]. Two other studies show rates being lower: at 16%[23,24]. Freedlander showed that, in auricular cSCC, 85% of the metastases to the parotid or neck occurred within 1 year of initial auricular excision[14]. Nonetheless, Byers *et al*[3] found no difference in survival between elective and therapeutic neck dissection but did not report the numbers of patients in each category.

A more thorough analysis of the primary tumor final pathology may be the deciding factor for the need for elective lymphadenectomy in auricular cSCC and possibly cSCC for other head and neck sites. In a recent study, Clark *et al*[5] showed that tumors with a depth of invasion > 8 mm had a 56.2% risk of metastatic spread and those with a depth of invasion between 2 and 8 mm and with evidence of cartilage destruction, lymphovascular invasion or a non-cohesive invasive front have a 24.2% risk of metastatic spread. In a meta-analysis, Rowe *et al*[6] showed an increased likelihood of regional metastases with tumor size over 2 cm, depth of invasion over 4 mm, poorly-differentiated grade, perineural invasion, and local recurrence. Given the higher percentages (being over 15%-20%) in auricular cSCC with these features, we agree that elective regional lymphadenectomy is tempting. However, the retrospective nature of these studies calls into question the time relationship between the development of the primary cancer and regional metastases.

The withholding of elective lymphadenectomy for cSCC does not remove the need for close observation. In a patient with poor prognostic risk factors, more frequent follow-up with careful evaluation of the regional lymph node basins is certainly important if an observational strategy is to be implemented. Ultrasound in particular is a promising and accurate method for surveillance of the parotid and neck in these patients which may be used in conjunction or possibly in lieu of the cross-sectional imaging of CT and MRI[25,26].

Our study has limitations. This is a retrospective series at only one institution involving multiple surgeons with different treatment philosophies over a 10-year period with a small sample size. With heterogenous operations being performed and no standard surgical treatment

regimen (superficial or total parotidectomy and different extents of neck dissections being performed), occult lymph nodes could have been missed. The sample size is small reducing its generalizability but does reflect the rare character of this particular clinical scenario. There is no control group to compare regional recurrence or survival rates. However, the use of a comparison untreated group is beyond the scope of the goal of determining the rate of regional nodal basin occult disease.

A prospective randomized controlled trial of advanced cSCC with concern for occult regional metastases would be ideal but difficult to carry out given the multiple subsites of the head and neck and the rare presentation of the disease in this setting. Nevertheless, further studies are necessary to further clarify the extent of occult lymph node involvement in advanced auricular cSCC and other sites in the head and neck and a process by which to appropriately risk stratify patients into undergoing elective lymphadenectomy.

This small sample suggests that, in patients with advanced auricular cSCC, elective regional lymphadenectomy may not be necessary. Larger multi-institutional prospective studies are necessary to assess the necessity of elective regional lymphadenectomy for advanced auricular squamous cell carcinoma.

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We would like to thank the database managers who helped create the head and neck non-melanoma skin cancer database at our institution.

COMMENTS

Background

Advanced auricular cutaneous squamous cell carcinoma (cSCC) has conventionally been treated with partial or total auriculectomy accompanied by a regional lymphadenectomy, which often includes a parotidectomy and/or a neck lymph node dissection for clinically apparent or occult lymph node disease. With advanced auricular cSCC, elective lymphadenectomy is performed in these cases in an effort to maximize therapy, increase the chance of cure, and reduce the chance of locoregional recurrence. However, the incidence of occult parotid and neck lymph node malignant disease is currently unknown. Thus, authors developed a study of patients with advanced auricular cSCC and clinically negative parotid and neck lymph nodes to determine the rate of occult microscopic lymph node disease in the elective parotidectomy and neck dissection.

Research frontiers

Several studies claim a higher rate of metastasis with auricular cSCC compared to other sites on the head and neck. Studies show that, for external auditory canal and auricular cSCC, the range of involvement in the parotid or neck lymph nodes is 7.9% to 17.5%. The 5 years mortality rate directly attributable to regional metastases from cSCC can be as high as 56.6% to 66.7% even after combined surgical and radiation therapy. Current research is attempting to further delineate what surgical management is needed in this disease process.

Innovations and breakthroughs

Very little research has been done in evaluating the incidence of occult nodal disease in advanced auricular cutaneous SCC. Historically, regional lymphadenectomy has been performed electively in these cases. This study concludes that regional lymphadenectomy may not be necessary and hopes to spawn further larger prospective trials.

Applications

This small sample suggests that, in patients with advanced auricular cSCC, elective regional lymphadenectomy may not be necessary. Larger multi-institutional prospective studies are necessary to assess the necessity of elective regional lymphadenectomy for advanced auricular squamous cell carcinoma.

Terminology

Advanced auricular SCC is defined as advanced when it has one or more of the following features: large tumor size, increased depth, locally invasive tumors (cartilage and beyond), multifocal disease, clinically apparent lymph node metastases, post-surgical recurrence, post-radiation recurrence, and when the patient is immunosuppressed.

Peer review

This retrospective study focuses on 17 patients with auricular squamous cell carcinoma. Authors determined the rate of occult lymph node disease in elective parotidectomy and neck dissection specimens. Manuscript is well written.

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CASE REPORT

Endolymphatic hydrops in Meniere's disease secondary to otitis media and visualized by gadolinium-enhanced magnetic resonance imaging

Jing Zou, Ilmari Pyykkö

Jing Zou, Ilmari Pyykkö, Hearing and Balance Research Unit, Field of Oto-laryngology, School of Medicine, University of Tampere, 33520 Tampere, Finland
Author contributions: Zou J reviewed the case history, analyzed the magnetic resonance imaging and wrote the paper; Pyykkö I performed the clinic observation and magnetic resonance imaging.
Supported by The European Community 7th Framework Programme on Research, NanoValid (Contract: 263147)
Correspondence to: Jing Zou, MD, PhD, Associate Professor, Head of Hearing and Balance Research Unit, Field of Otolaryngology, School of Medicine, University of Tampere, Medisiinarinkatu 3, Room C2165a, 33520 Tampere, Finland. jing.zou@uta.fi
Telephone: +358-4-1901307 Fax: +358-3-3641482
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Abstract

Aimed to test the hypothesis that endolymphatic hydrops in Meniere's disease (MD) may be secondary to otitis media, history of a patient who developed MD as a complication of otitis media was reviewed. The inner ear was imaged using a 3.0 Tesla MR system post-intravenous injection of gadolinium-tetraazacyclododecane-tetraacetic acid (Gd-DOTA) in a standard single dosage (0.1 mmol/kg). Both t2-spc-rst-tra-iso (T2-weighted) and heavily T2-weighted 3-dimensional fluid-attenuated inversion recovery magnetic resonance imaging [hT(2)W-3D-FLAIR] sequences were applied. As a result, in the T2-weighted images, the perilymph and endolymph, cerebrospinal fluid surrounding the eighth nerve (N8), and middle ear granulation tissue showed intense signals. In the hT(2)W-3D-FLAIR images, evident enhancement by Gd-DOTA was observed in the middle ear cavity and the perilymphatic compartments of the cochlea. Cochlear endolymphatic hydrops was implicated by the enlarged scala media in the basal turn. In general, the Gd-DOTA uptake in the vestibule was weak, and signs of vestibular endolymphatic hydrops were obvious. The N8 on the diseased side was also significantly enhanced. To conclude, endolymphatic hydrops in MD may be induced by otitis media. Cochlear endolymphatic hydrops in MD secondary to otitis media may not follow the classical pattern.

Key words: Endolymphatic hydrops; Otitis media; Meniere's disease; Magnetic resonance imaging; Sensorineural hearing loss

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INTRODUCTION

Endolymphatic hydrops is the typical pathological finding in Meniere's disease (MD) and has been observed post mortem[1]. Gadolinium-enhanced magnetic resonance imaging (MRI) can definitively diagnose MD and assess endolymphatic hydrops[2]. The etiology of MD is unknown, but immune reactions, viral infections, inflammation, and vascular insufficiency are suspected to contribute to its progression. In some cases, MD may be secondary to chronic otitis media[3]. We speculate that MD cases that are caused by inflammation might have the same mechanisms as immune-mediated inflammation in experimental animals, particularly in the middle ear stimulation that causes endolymphatic hydrops. In guinea pigs, stimulation by keyhole limpet hemocyanin through the middle ear caused endolymphatic hydrops as a result of increased

permeability in the blood-inner ear barrier[4]. However, endolymphatic hydrops in MD that is suspected to be secondary to otitis media has not been observed *in vivo*. The present article describes a patient who developed MD as a complication of otitis media and in whom endolymphatic hydrops was visualized *via* gadolinium-enhanced MRI.

CASE REPORT

In 2008, a 45-year-old man in the outpatient otolaryngology department presented with vertigo, tinnitus, and hearing loss in the right ear. The patient had diabetes, obstructive sleep apnea, unilateral hydronephrosis, and hypertension. In 2000, the patient awoke with vertigo that was provoked by positional changes. In a detailed examination, spontaneous left-beating nystagmus was noted. After performing Epley's maneuver, the vertigo was resolved. The patient did not complain of hearing loss or tinnitus. In 2007, the patient experienced rotary vertigo and vomiting without any noticeable hearing loss after strenuous carpentry work. In a clinical evaluation, the patient showed spontaneous left-beating nystagmus and was diagnosed with right-sided vestibular neuritis. The vertigo faded slowly over the course of several weeks. At the beginning of 2008, the patient experienced tinnitus and hearing loss in the right ear. A closer evaluation detected glue ear on the diseased side. An audiogram showed conductive high frequency hearing loss of 40 dB (Figure 1A). After paracentesis, a tympanostomy tube was inserted and the patient was followed more closely. The hearing loss prevailed (Figure 1B and C), and the spiral CT showed a fluid-filled cellular system in the middle ear. A mastoidectomy was performed, but the middle ear problems did not subside, and the middle ear ventilation did not improve. In an endoscopy of the Eustachian tube, the posterior cushion and the torus tubarius was swollen in a cherry-like manner. The vertigo symptoms worsened, and the patient experienced several weekly vertigo attacks. In February 2011, an inner ear MRI was performed using a 3 Tesla MR System with a 32-channel head coil and an additional ear coil (Siemens Trio-Tim, Erlangen, Germany) with 4 h post-intravenous injections of gadolinium-tetraazacyclododecane-tetraacetic acid (Gd-DOTA). The vertigo attacks continued at the same level of severity. In September 2011, laser Eustachian tuboplasty and exploratory tympanostomy with installment of tympanostomy tube were performed. After the surgery, the patient was nearly asymptomatic except for one mild vertigo attack. Subjectively, the patient's hearing also improved (Figure 1D). At present (June 2012), the patient has no vertigo symptoms.

The MRI measurement was performed on April 5, 2011. Gd-DOTA (500 mmol/L, Guerbet, France) was injected intravenously in a standard single dosage (0.1 mmol/kg). After 4 h, the patient was evaluated with both t2-spc-rst-tra-iso (T2-weighted) and heavily T2-weighted 3-dimensional fluid-attenuated inversion recovery MRI [hT(2)W-3D-FLAIR] sequences. The t2-spc-rst-tra-iso parameters were as follows: SL 0.5, echo time (TE) 132 ms, repetition time (TR) 1610 ms, field of view (FOV) 199 × 199, 380 px 384 s, W 754, C 320, and NEX 2. The hT(2)W-3D-FLAIR images were acquired using the following parameters[5]: SL 0.8, TE 538 ms, TR 10 700 ms, TI 2350, FOV 150 × 180, 270 px 320 s, W 214, C 74, NEX 2. The images were displayed using syngo Fastview software (Siemens Germany) combined with the CS3 program.

In the T2-weighted images, the perilymph and endolymph of the inner ear, cerebrospinal fluid (CSF) surrounding the eighth nerve (N8), and middle ear granulation tissue showed intense signals (Figure 2A-E). There were slightly signal intensity differences between the left [224.5 arbitrary unit (AU)] and right cochlear basal turn (216.5 AU) and the left (239.3 AU) and right vestibulum (236.5 AU). In the hT(2)W-3D-FLAIR images, evident enhancement by Gd-DOTA was observed in the middle ear cavity and the perilymphatic compartments of the cochlea. Cochlear endolymphatic hydrops was implicated by the enlarged scala media in the basal turn (Figure 2B). In general, the Gd-DOTA uptake in the vestibule was weak, and signs of vestibular endolymphatic hydrops were obvious (Figure 2B). The N8 on the diseased side was also significantly enhanced (Figure 2F).

DISCUSSION

This is the first case to show endolymphatic hydrops *in vivo* in a patient with MD secondary to otitis media. In the literature, vestibular pathology secondary to otitis media has been shown by objective measurement of the vestibular function[6,7]. A potential pathway for inflammatory cytokines and even pathogens to enter the vestibular system through the annular ligament across the stapedio-vestibular joint has been hypothesized by Zou *et al*[8] in an *in vivo* MRI study. In the present case, the severity of endolymphatic hydrops was greater in the vestibulum than in the cochlea, which supports the Zou *et al*[8] hypothesis.

One ultrastructural study has shown that the middle ear side of the footplate of the stapes had histopathological changes in patients with otitis media, although the vestibular side remained essentially unchanged[9]. Although involvement of the stapes in otitis media is likely common, the possibility that infection agents or products of inflammation may cross the porous annular ligament into the vestibule must be considered.

Cochlear endolymphatic hydrops located at the basal turn suggested that the round window membrane may also be involved in the passage of pathogens or inflammatory agents into the cochlea. Papp *et al*[10] have reported that chronic suppurative otitis media induced sensorineural hearing loss related to high frequencies. Sensorineural hearing loss at 4 kHz gradually increased according to the duration of the chronic suppurative otitis media and was greater than that of speech frequencies. This result was explained by the closer location of the hair cells that are responsible for high frequency hearing at the base of the cochlea and the round window. According to the present case study and a previous animal study, we further hypothesize that cochlear endolymphatic hydrops in MD

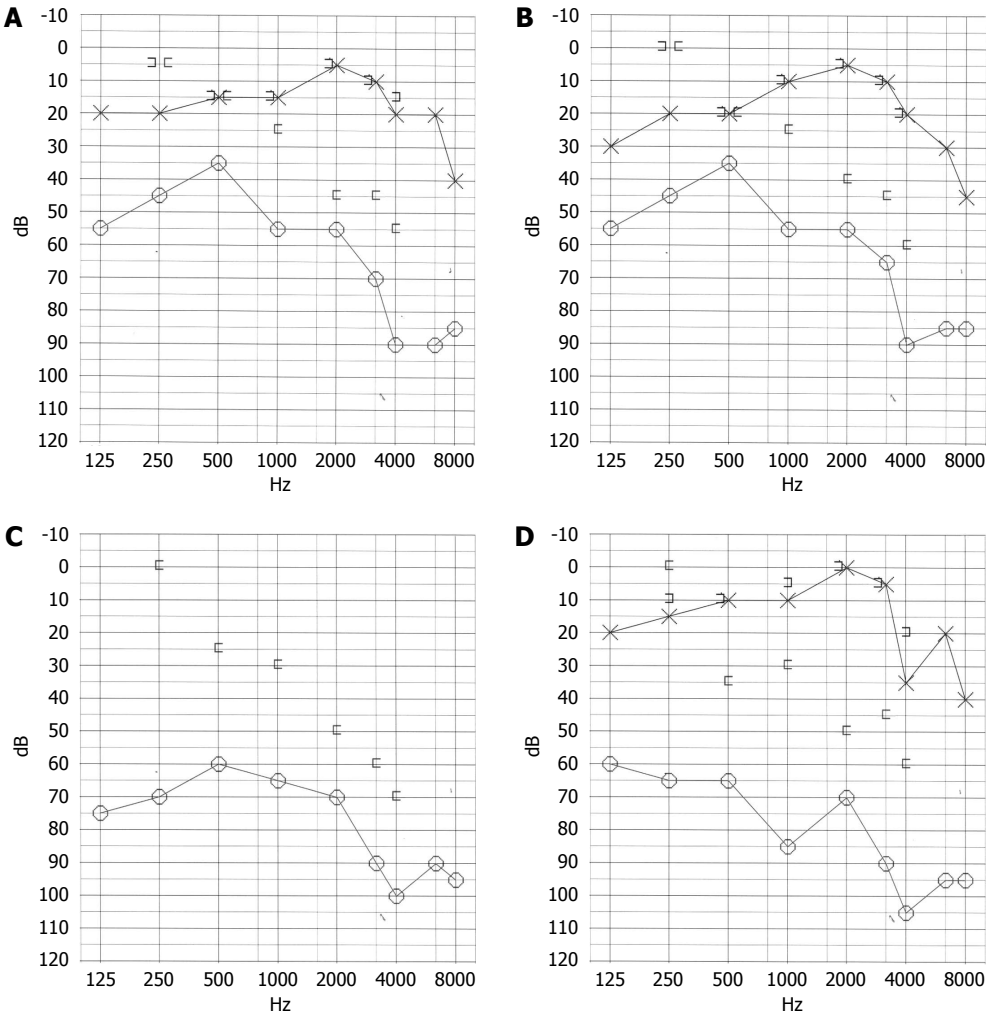

Figure 1 Audiogram showing dynamic change in the hearing thresholds. A: Hearing thresholds at the glue ear diagnosis (8 years post-Benign paroxysmal positional vertigo); B: Hearing thresholds one year after the tympanostomy tube insertion; C: Hearing recovery 2 years after the tympanostomy tube insertion; D: Hearing thresholds at the magnetic resonance imaging study.

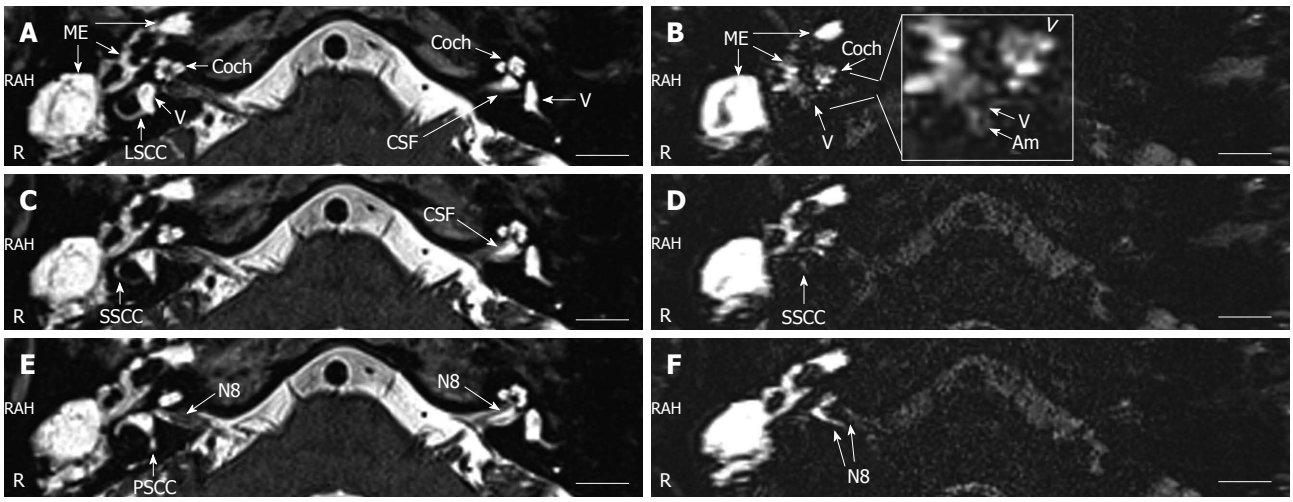

Figure 2 Magnetic resonance imaging of the inner ear acquired with a 3 Tesla machine. In the T2-weighted images (A, C, E), intense signals in the perilymph and endolymph of the inner ear and cerebrospinal fluid (CSF) surrounding the eighth nerve (N8) and the middle ear cavity (ME) were demonstrated. In the heavily T2-weighted 3-dimensional fluid-attenuated inversion recovery magnetic resonance images, evident enhancement by gadolinium-tetraazacyclododecane-tetraacetic acid (Gd-DOTA) was detected in the middle ear cavity and the perilymphatic compartments of the cochlea. Suspected endolymphatic hydrops was indicated by the enlarged scala media at the basal turn [arrowhead in the enlarged window of (B)]. In general, the Gd-DOTA uptake in the vestibule was weak, and endolymphatic hydrops became obvious in the vestibulum (V) and ampulla of the semicircular canal (Am) [enlarged window of (B)]. Gd-DOTA uptake in the perilymph of superficial semicircular canal was detected in the diseased ear (D). No uptake of Gd-DOTA was demonstrated neither in the lateral semicircular canal nor in the posterior semicircular canal. The N8 (in F) on the diseased side showed significant enhancement. LSCC: Lateral semicircular canal; PSCC: Posterior semicircular canal; SSCC: Superior semicircular canal.

secondary to otitis media may not follow the classical pattern and spread from the apex to the basal turn and vestibulum[4].

The observed enhancement of the N8 indicates a local injury of the blood-brain barrier. It has been reported that activated neuritogenic T-cells alter the blood-nerve barrier when entering into the peripheral nerves, which provides circulating demyelinating antibodies access to the endoneurium[11]. Similarly, this process may occur in the N8 if immune reactions overreact.

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15 Morse SS. Factors in the emergence of infectious diseases.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/2218-6247/g_info_20100724224620.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing

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