Changes in guinea pig cochlea after transient cochlear ischemia

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Perturbation of cochlear microcirculation, that is, ischemia is a major cause of hearing impairment. Earlier studies examined the short-term (≤ 7 days) effect of cochlear ischemia. This study characterized the long-term (4 weeks) functional and morphological changes in adult guinea pig cochleae subject to transient ischemia by clamping the labyrinthine artery for 0.25–3 h. Notably, cochlear ischemia for over 1 h caused an increase of auditory brainstem response thresholds and loss of high-frequency hearing, basal-turn hair cells, and spiral ganglions. Auditory recovery may be possible after 30-min ischemia. The extent of the functional and morphological changes depended on the ischemia period, and the changes progressed in extent from the apical to the basal turn in an orderly fashion.
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Introduction

The cochlea is a metabolically active organ that requires a substantial amount of energy to maintain its normal physiological function. As it is an end-artery organ mainly supplied by the labyrinthine artery (a branch of the anterior inferior cerebellar artery) [1], it is sensitive to blood flow disturbances that causes local hypoxia and may result in common otologic disorders, such as noise-induced [2] or sudden sensorineural hearing loss [3].

Although there have been many attempts to characterize how ischemia causes cochlear damage, it has proved difficult to induce ischemia in animals that would permit long-term studies. If any, cochlear ischemia can be induced by occluding the bilateral vertebral arteries through hindbrain approach [4] and compressing the neurovascular bundles in the internal auditory canals through either a skull base approach [5] or occipital approach [6]. Postoperation observation periods in these studies were relatively short (1 week at most [6]) and limited, a full evaluation of the effects of the ischemia experiments was not possible. Indeed, some ischemia-related hearing disorder, for example, sudden sensorineural hearing loss may evolve over a relatively long period of time after the initial acute insult. Early intervention may aid the recovery process in patients of sudden sensorineural hearing loss [7] whose hearing may continue to improve for 1 month after the treatment cessation [8]. Therefore, a study incorporating a long observation period is needed to better understand the functional and morphological cochlear changes caused by ischemia, which is the purpose of this study.

Materials and methods

Adult albino guinea pigs aged between 2 and 4 months (body weight 350–550 g) were used. The use and care of animals in this study were approved by the Institutional Animal Care and Use Committee of the China Medical University (permission number: 97-60-N). Animals were anesthetized by intramuscular injection of a mixture of Zoletil (30 mg/kg, Virbac, Carros, France) and xylazine (10 mg/kg, Bayer, Leverkusen, Germany). A maintenance dose, 50% of the initial dose, was injected intramuscularly every 60 min thereafter. For surgical accessibility and convenience, only the left ear was used for this experiment. The right cochlea was surgically destroyed to avoid acoustical crossover during auditory measurements.

Experimental groups

The animals were divided into the following groups. (i) Control ($n = 6$): no surgery. (ii) Sham operation ($n = 6$): the animals received surgery as described below until the step at which the labyrinthine artery was only momentarily exposed after the overlying dura was excised, and then the wound was closed. (iii) Ischemia groups: the labyrinthine artery was then temporarily occluded with microclamps for 15 or 30 min or for 1, 2, or 3 h (six animals per subgroup). Then the microclamps were released and wounds were closed.

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In each experimental group, four cochleae were prepared for cochlear surface preparation and hair cell counting. Other cochleae were sectioned along paramodiolar axis and stained with hematoxylin/eosin.

Surgical procedures

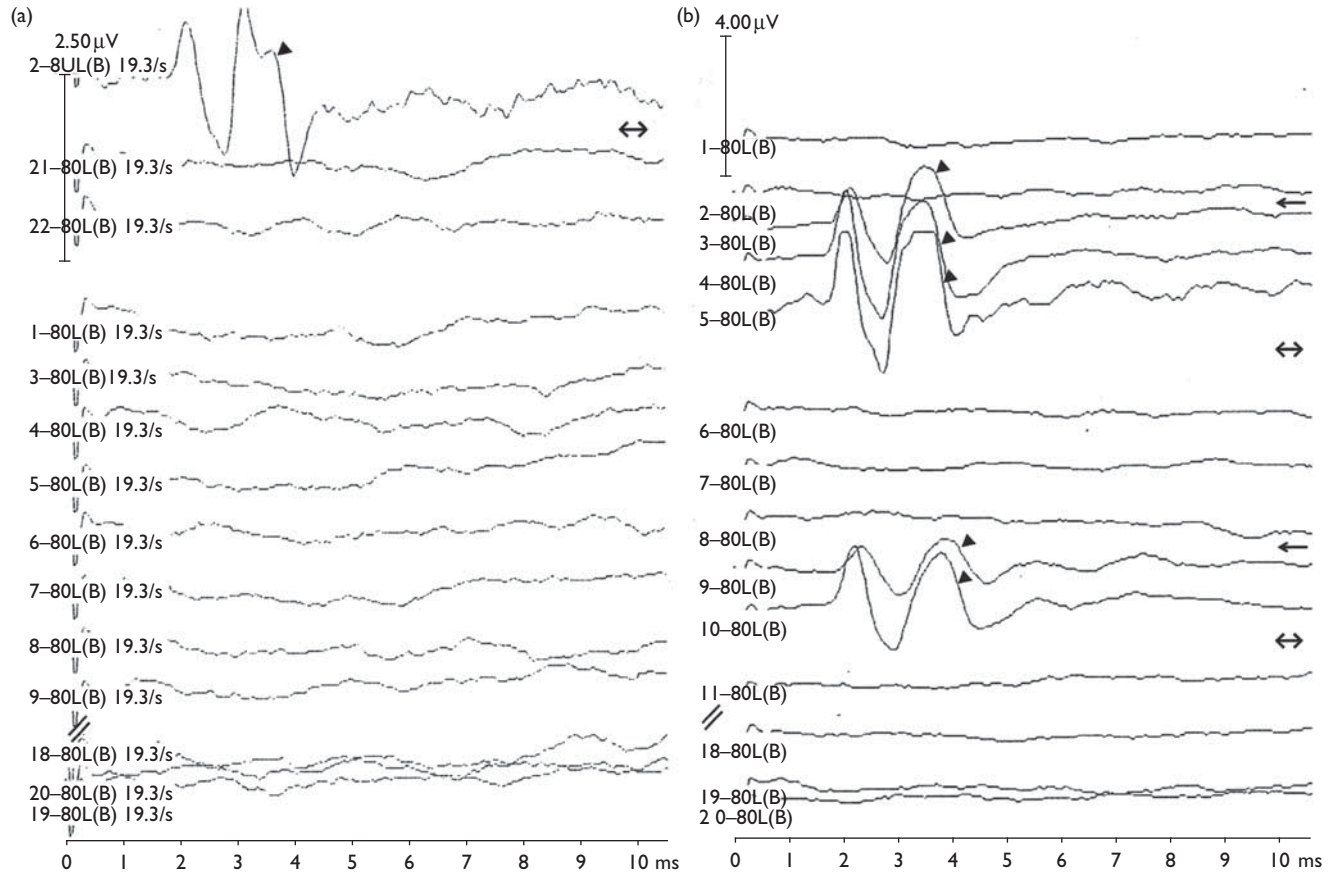
After an animal had been anesthetized, it was placed in the prone position. The skin and subcutaneous myofascial plane of the ear were dissected to expose the mastoid bulla. The cochlea could be directly destroyed by needle penetration and disruption from the opened bulla.

The ventral-approach procedure to the labyrinthine artery has been described [9]. Electrodes were inserted subcutaneously into the left mastoid (anode), right mastoid (cathode), and the back (ground). An earphone was then inserted into the left ear canal to monitor the auditory brainstem responses perioperatively. The skin over the ventral neck was disinfected with 75% alcohol and draped with aseptic dressings. A submental incision of 2 to 3 cm in length was made medially to the mandibular edge. The submandibular gland was separated to expose the digastric muscle and the paracondylar process. Separation of the digastric muscle from the fractured paracondylar process exposed the tympanic bulla. The anterior wall of the tympanic bulla was opened using a rongeur to visualize the basal cochlear turns. Drilling started at the petrous bone, continued medially to the basal turn and anteriorly to the inferior petrosal sinus. A fenestration $\sim 1.5 \times 3.0$ mm was made at the base of the skull, thus the labyrinthine artery was visible under the dura. After the dura was excised, the labyrinthine artery was fully exposed and then closed with V1 microclamps (#00396-01, S&T Microsurgical Instruments, Foster City, California, USA), and cochlear function was thereafter monitored by click auditory brainstem responses at a 120-dB sound pressure level at least every 3 min. Persistent absence of the auditory brainstem response waveform indicated a successful occlusion of the labyrinthine artery (Fig. 1).

Hearing test

Tone burst auditory brainstem responses preoperative and serial postoperative hearing tests were performed in

Fig. 1



Changes in the auditory brainstem response waveforms during surgery. (a) Before the surgery, an apparent auditory brainstem response (ABR) waveform was recorded (top ◀). After the microclamps were applied (↔), the waveform disappeared. (b) The ABR waveform reappeared (◀) when the microclamps were released (←). The waveform did, however, disappear again when the microclamps were reapplied (↔). 'L' indicates the stimulation sound to the 'left' ear, which was the operated ear and 'B' indicates the recording of ABR from bi-aural mode.

a sound-attenuated room. The pure tone bursts were generated with the amplitude specified by a real-time programmable attenuator using Intelligent Hearing System (IHS, Miami, Florida, USA) with ER2 insert earphone, with stimulus frequency at 1, 2, 4, 8, 16, and 24 kHz (0.2 ms rise/fall time and 1 ms flat segment) with maximal output level of 125, 123, 111, 117, 98, and 96 dB sound pressure level. The click/tone bursts were produced by high-frequency transducer in a closed acoustic system through the sound delivery system. Responses for 1024 sweeps were averaged at each intensity level in 10 dB steps and around the threshold in 5 dB steps. Threshold was defined as the lowest intensity at which a clear waveform was visible upon inspection of an evoked trace. Each auditory threshold was compared with the preoperative threshold, which served as the baseline measurement.

For the control, the sham operation and the ischemia groups, serial hearing tests were performed preoperatively, immediately after the operation, on days 1 and 3, and weeks 1, 2, 3, and 4.

Surface preparation of the cochlea and the hair cell counting procedure

At the end of the study, the animals were deeply anesthetized and then killed by decapitation. The left cochleae were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline by perilymphatic perfusion and then immersed in the same fixative for 1 day. The organ of Corti was carefully dissected. The tissues were permeabilized with 0.3% Triton X-100 for 10 min and then were incubated at room temperature with rhodamine-coupled phalloidin (Molecular Probes, Eugene, Oregon, USA) diluted 1 : 200 for 30 min. Strips of the organ of Corti were divided into the four turns, which were mounted on glass slides and examined with a fluorescence microscope (Model Leitz DM RBE; Leica, Wetzlar, Germany) to count the number of hair cells present at each cochlear turn, thereby determining the extent of hair cell loss.

Histopathological examination

Cochlear sectioning along the paramodiolar axis was done followed by hematoxylin/eosin staining. After fixation as described above, the cochleae were decalcified by immersion in 10% ethylenediamine tetra acetic acid for 4 weeks, with gentle stirring at 4°C. The cochleae were then dehydrated, embedded in paraffin, and serially sectioned (4 μm thick) parallel to the modiolar axis. The sections were plated for hematoxylin/eosin staining and examined under a high-power light microscope.

Statistical analysis

The auditory threshold shifts and the percentages of hair cell loss between the control, sham-operated, and ischemia groups were analyzed using the nonparametrical Mann–Whitney *U* test contained in the SPSS program (version 13.0 for Windows, SPSS Inc., Chicago, Illinois, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

Time and dose-dependent changes in auditory threshold shifts caused by cochlear ischemia

The serial auditory threshold shifts found after transient cochlear ischemia of different durations are depicted in Fig. 2. No significant auditory threshold shifts were noted for the control or sham-operated groups. The noise made by the Rongeur or the drilling process seemed to have no obvious affect on the hearing. In the group of the labyrinthine artery clamped off for 15 or 30 min, marked threshold shifts occurred, but returned to preoperative levels within 3 days. Persistent threshold shifts were noted in the groups with artery occlusion for $\geq$ 1 h. In this group, threshold shifts associated with higher frequencies (8–24 kHz) were larger than those associated with lower frequencies (1–4 kHz). It is also true for the 2-h ischemia group showing large-scale threshold shifts that nearly approached the maximum stimulation level in the 3-h ischemia group.

When the threshold shifts were recorded 4 weeks after ischemia (Fig. 3), we found that there are no significant differences between the control, sham-operated, and experimental groups with 15-min or 30-min ischemia. In 1-h ischemia group, marked threshold shifts that decreased as the frequencies increased were found. The degree of discernible threshold shifts was dependent on the duration of ischemia (Fig. 3).

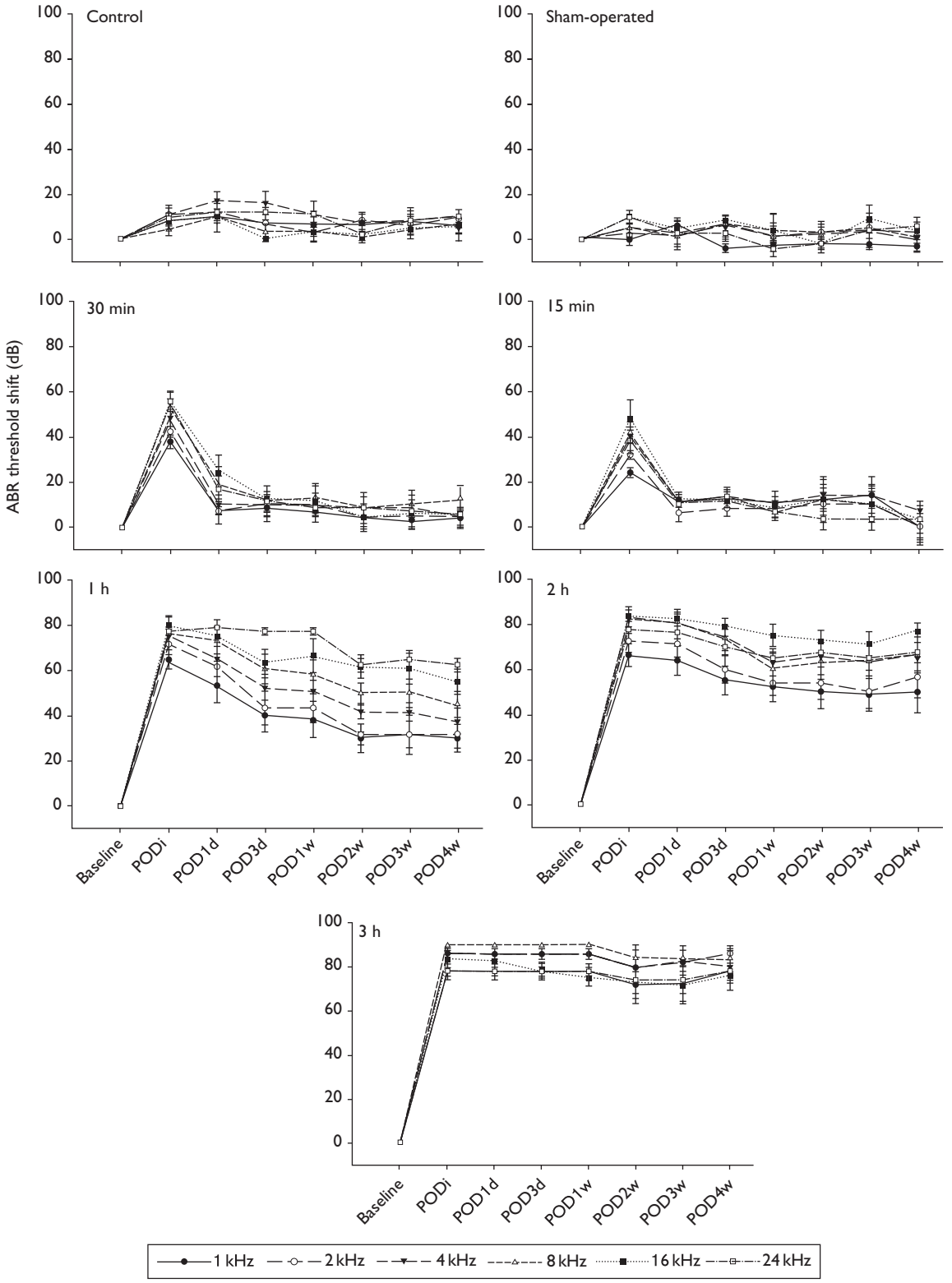
Hair cell loss

The amount of outer hair cell loss increased as the ischemia duration increased, but the cell loss did not increase significantly until the ischemia period was $\geq$ 1 h (Fig. 4a–d). Outer hair cell loss was more severe in the basal turn than that in the apical turn (Fig. 4a–c). A similar result was observed in inner hair cells suffering from 1-h ischemia except the loss of inner hair cells in the upper turns where cell loss was not significant until ischemia duration was $\geq$ 2 h (Fig. 4d). At 4 weeks, after the 2-h ischemia, there was a loss of hair cells, especially the outer hair cells that were nearly absent in the basal turn, although residual hair cells were still present in the upper turns (Fig. 4e).

Histopathological analysis

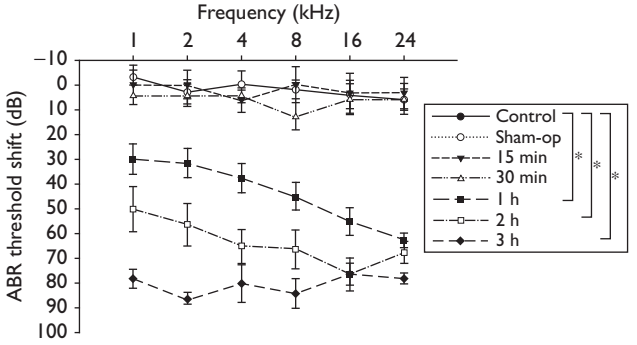
The morphology of spiral ganglia and the organ of Corti was grossly normal at 4 weeks after a 30-min ischemia insult (Fig. 5a and b). Spiral ganglion loss was, however, apparent after 1-h ischemia (Fig. 5c) and became more severe as ischemia period was increased (Fig. 5d). At the same time, the organ of Corti appeared flattened (Fig. 5c and d).

Fig. 2



Auditory threshold shifts after transient cochlear ischemia of different periods. Transient threshold shifts were found for the 15-min and 30-min ischemia groups. Impaired recovery of the auditory threshold shifts was found if ischemia duration $\geq$ 1 h. *y*-axis indicates the auditory threshold shifts in decibels. *x*-axis indicates the timing for serial auditory brainstem response measurement, from baseline, immediately, 1 and 3 days, and 1, 2, 3, 4 weeks after the surgery (PODi, POD1d, 3d, 1w, 2w, 3w, and 4w). All values are presented as mean ± SEM. POD, postoperation day.

Fig. 3



Auditory threshold shifts 4 weeks after cochlear ischemia of different durations. Compared with the control group, significant threshold shifts were found when ischemia lasted $\geq$ 1 h (*$P<0.001$). All values are presented as mean ± SEM.

Discussion

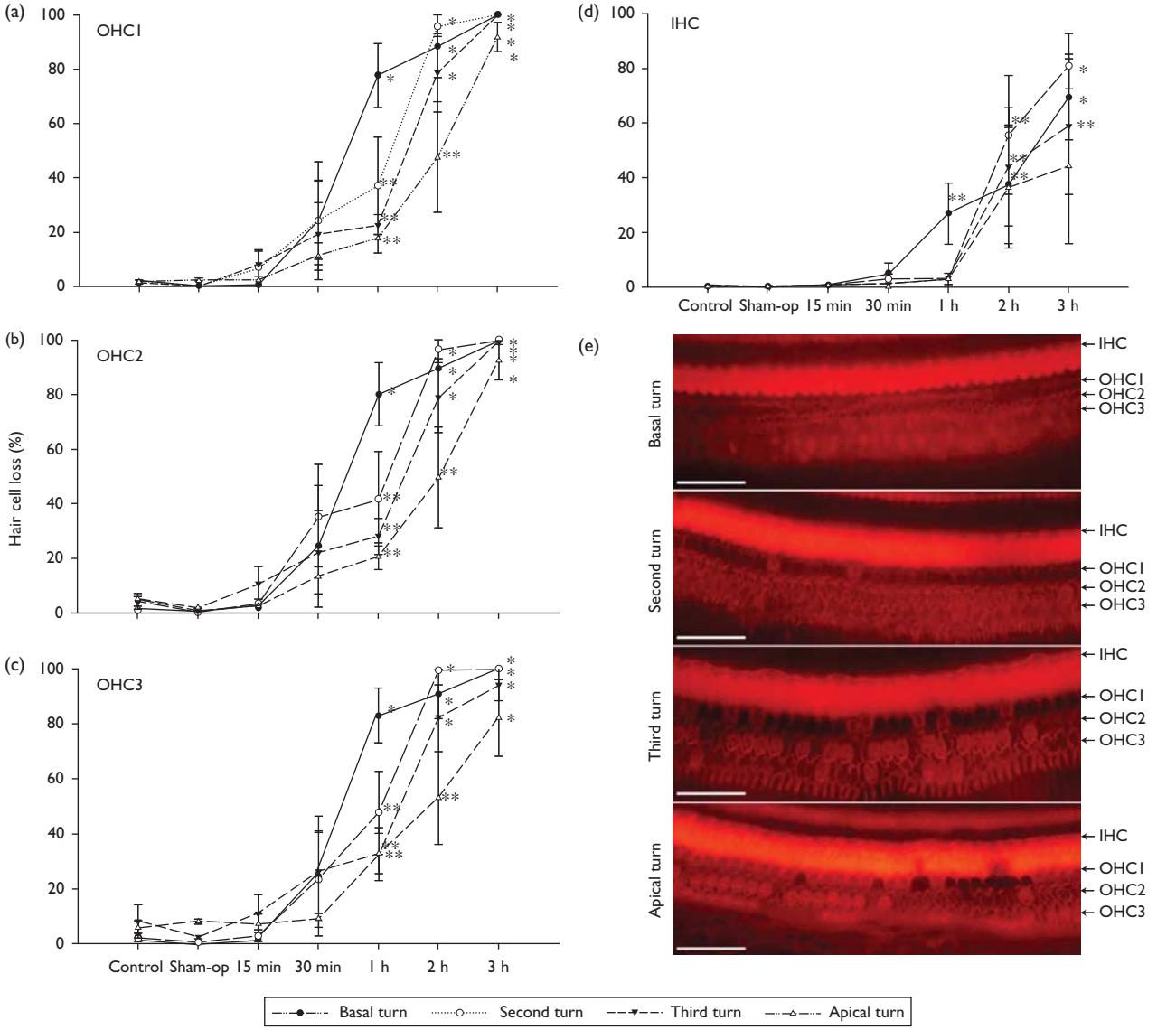
Several different approaches have been developed to study the effects of transient cochlear ischemia in different animals. Each surgical approach has different limitations and applications when used with different species. For example, the hindbrain approach [4] to occlude the bilateral vertebral artery produces reversible and consistent cochlear ischemia in gerbils, but can be used only with gerbils because of their peculiar posterior brain circulation. When bilateral vertebral artery occlusion was used in guinea pigs, the results were inconsistent [10]. Bilateral occlusion of the vertebral artery for $\geq$ 1 h may also damage the brainstem vital nuclei, thereby limiting the ischemia period. The esophagus and pharynx must be removed when the skull base approach [5] is used. Therefore, longer observation periods of more than 1 day may not be feasible. A relatively long observation period (1 week) after transient cochlear ischemia was possible when the occipital approach was used [6]. In the study reported herein, we used the ventral approach to expose the labyrinthine artery. The surgical field was accessed through the auditory bulla, which limited brain damage and preserved the pharynx and trachea. The guinea pigs survived for a relatively long period (4 weeks) after their operations and consequently the long-term observation was possible.

A possible drawback of the ventral approach is that the operative field is very narrow. We could not continuously and directly monitor the ischemia induced by the microclamps using an instrument such as a laser dopplerometry. Moreover, cerebrospinal fluid that leaked from the skull base fenestrum interfered with the continuous measurement of cochlear blood flow and made the measurements variable and inconsistent. Therefore, we monitored the occlusions by serial auditory brainstem responses. The cochlea has a very low oxidative reserve and is very sensitive to interruption of the blood supply; the electrical activity of the cochlea disappears within seconds when the blood supply is interrupted [9]. Serial auditory brainstem response monitoring has been widely used during skull base surgery, for example, acoustic neuroma surgery [11]. Once the cochlear blood flow is interrupted, the entire auditory brain stem waveform disappears [11]. In contrast, when the damage is localized to the auditory nerve or the central auditory pathways in the brainstem or mesencephalon, the classic auditory brainstem response waveforms may instead be altered: late (III–V) waveforms may disappear, whereas early (I and/or II) waveforms remain visible [11]. In agreement with the earlier study [15], when the microclamps used in this study were successfully applied to the labyrinthine artery, the waveforms completely disappeared, but rapidly reappeared after the removal of the microclamps (Fig. 1). Our preliminary studies further verified the occlusion effect by the microclamps using intracardiac perfusion with 3% trypan blue that did not perfuse into the microclamped cochlea, but did into the control cochlea. Therefore, we believe that the microclamps successfully and reversibly occluded the arterial blood supply to the guinea pig cochlea.

The auditory threshold shifts may return to preoperative levels, partially recover, or poorly recuperate when cochlear ischemia was induced for $\leq$ 30 min, $\geq$ 1 h, or $\geq$ 3 h, respectively (Figs 2 and 3). The revival time defined by Perlman and colleagues [9] is the maximum duration of ischemia after which auditory responses can fully return. In this study, it may be up to 30 min for the ischemic cochlea of guinea pig. The recovery of auditory threshold shifts after shorter periods of cochlear ischemia ($\leq$ 30 min) occurred within 3 days. Auditory recovery from the 1-h ischemia did, however, seem to progress up to 2 weeks, which is a time period similar to the clinical course of sudden sensorineural hearing loss that was possibly attributed to ischemic damage [3]. Most patients with sudden sensorineural hearing loss spontaneously recover; although some continue to suffer from variable degrees of hearing loss [7]. The time between the initial insult and the beginning of treatment is a major prognostic indicator of hearing recovery from sudden sensorineural hearing loss. The longer the patients waiting before the medical treatment after the onset of this disease, the poorer the recovery [7]. The hearing status in a substantial number of the abovementioned patients does, however, improve long after the cessation of treatment [8], possibly because of the long recuperation potential, as shown in this study.

Our results also showed that auditory changes had a closer association with the high-frequency range than the low-frequency one. These facts were reflected by morphological evidences in the cochlear surface preparation and the number of phalloidin-stained hair cells. The hair cells in the basal turn seemed to be more susceptible to ischemic damage than those in the upper turn. The present results were comparable with those of the earlier

Fig. 4

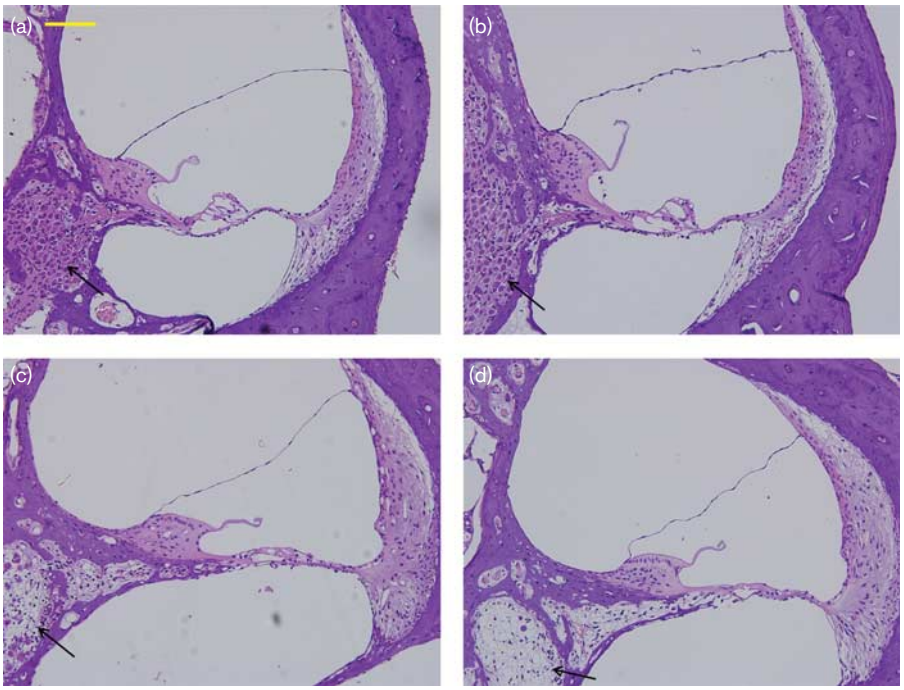


Loss of outer (a–c) and inner hair cells (IHCs) (d) induced by ischemia. Compared with the control group, hair cell loss at the basal turn was significant until ischemia period was $\geq$ 1 h. Inner hair cell loss in the upper turns was not significant unless ischemia was $\geq$ 2 h. All values are presented as mean ± SEM (*P<0.001; **P<0.05). (e) Representative hair cell morphology stained with rhodamine-phalloidin in animals 4 weeks after 2-h ischemia. Complete loss of IHCs and the three rows of outer hair cells (OHC1, OHC2, OHC3) were depicted in the basal turn. Significant loss of hair cells was present in the second turn. Some hair cells were identifiable in the third and apical turns. Bar, 20 μm.

studies using guinea pigs [9] and gerbils [4]. The basal portion of the cochlea has a greater rate of oxygen consumption than the upper apical portion does [12]. Conversely, the energy reserve (especially glycogen) of the organ of Corti follows an inverse base-to-apex distribution: more glycogen in the apical turns than that in the basal turns [13]. Hair cells, particularly the outer hair cells, in the cochlear base used more energy than those in the apex because the former needs to operate at higher frequencies [14]. A base-to-apex gradient of differential intrinsic susceptibility to free radicals has also been reported and conceivably the hair cells in the basal turns are more vulnerable to ischemia-induced damage than those in the apical turns [15]. These data including ours could explain why the apical turn tolerates ischemic damage better than the basal turn does.

In addition to the intrinsic base-to-apical differential susceptibility to ischemia, this study also showed that the outer hair cells of guinea pig were more vulnerable to ischemia than the inner hair cells. At 4 weeks after 15-min ischemic insult, minimal outer hair cell loss occurred, but loss of outer hair cells was apparent after 1-h insult. At the same time, mild loss of inner hair cells was found

Fig. 5



Hematoxylin/eosin-stained cochlear sections. Normal architecture of the organ of Corti, contained abundant spiral ganglia (arrow) in the cochlear section from control (a) and 30-min ischemia group (b). Apparent loss of spiral ganglia (arrow) and distorted organ of Corti were observed in the section from 1-h ischemia group (c). More severe loss of spiral ganglia (arrow) was observed in 2-h ischemia group (d). Bar, 50 μm.

after 30-min ischemic injury, and inner hair cells in the basal turn showed significant loss after 1-h insult (Fig. 4). In guinea pigs, outer hair cells may be more vulnerable to ischemia reperfusion injury and aminoglycoside ototoxicity than inner hair cells [9,16]. With the longer periods of ischemia, both inner and outer hair cells may be affected. However, early loss of inner hair cells rather than outer hair cells after cochlear ischemia may occur in gerbils subjected to ischemia reperfusion by occlusion of the bilateral vertebral artery [4]. A comparable evidence was found in guinea pigs put through a similar surgical approach [17]. Glucocorticoids possess protective effects against cochlear ischemic damage to guinea pig outer hair cells [18] and gerbil inner hair cells [19]. Therefore, the susceptibility differences between inner and outer hair cells to ischemia may reflect species specificity.

In this study, spiral ganglia have also been damaged at 4 weeks after cochlear ischemia (Fig. 5). In addition to direct injury induced by ischemia, secondary spiral ganglion loss occurs after the loss of hair cells especially inner hair cells [20]. The latter, once challenged with external insult, for example, cochlear ischemia, could release excessive glutamate that caused spiral ganglion cell death [21]. In this study, inner hair cell loss was not apparent unless the ischemia was enduring for 1 h or longer. Inner hair cells in 30-min ischemia group remained relatively unaffected. Similar trend was observed in spiral ganglion that showed apparent cell loss in 1-h ischemia group and remained morphologically intact in 30-min group, indicating that the loss of the spiral ganglia was paralleled to inner hair cell loss after ischemia.

Conclusion

Orderly functional and cellular changes in the ischemic cochlea were found and depended on the duration of ischemia. The revival time for guinea pig cochlea after transient ischemia may be up to 30 min. A base-to-apex gradient of ischemia susceptibility existed in the hair cells. The latter and spiral ganglia were the most vulnerable to ischemia after which the loss of spiral ganglia was equivalent to that of inner hair cells.

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The authors report no conflicts of interest relevant to this article.

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