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Original Article

Relationship between changes in the cochlear blood flow and disorder of hearing function induced by blast injury in guinea pigs

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Abstract: The auditory system is the most susceptible to damages from blast waves. Blast injuries always lead to varying degrees of hearing impairment. Although a disorder of the cochlear blood flow (CoBF) has been considered to be related to many pathological processes of the auditory system and to contribute to various types of hearing loss, changes in the CoBF induced by blast waves and the relationship between such changes and hearing impairment are undefined. To observe the changes in the cochlear microcirculation after exposure to an explosion blast, investigate the relationship between changes in the CoBF and hearing impairment and subsequently explore the mechanism responsible for the changes in the CoBF, we detected the perfusion of the cochlear microcirculation and hearing threshold shift after exposure to an explosion blast. Then, an N-nitro-L-arginine-methyl ester (L-NAME, NO synthase inhibitor) solution and artificial perilymph were applied to the round window (RW) of the cochlea before the blast exposure, followed by an evaluation of the CoBF and hearing function. The results indicated that the changes in the CoBF were correlated to the strength of the blast wave. The cochlear blood flow significantly increased when the peak value of the blast overpressure was greater than approximately 45 kPa, and there was no significant change in the cochlear blood flow when the peak value of the blast overpressure was less than approximately 35 kPa. Following local administration of the NO synthase inhibitor L-NAME, the increase in the CoBF induced by the blast was inhibited, and this reduction was significantly associated with the hearing threshold.

Keywords: Cochlea, blood flow, hearing function, blast injury, guinea pigs

Introduction

Blast injuries can occur in normal life, during military acts and in the workplace. They can cause injuries to various organ systems [1]. These injuries differ, ranging from relatively minor to lethal, in accordance with the magnitude of the blast wave [2, 3]. As an air-containing organ, the auditory system is considered to be most susceptible to blast wave-associated damage. Such damages can be full range, affecting the region from the tympanic membrane to the inner ear, and lead to temporary and permanent losses of hearing sensitivity [4]. These structural damages cause conductive hearing loss, sensorineural hearing loss or both [1-3].

In addition to mechanical damage, vascular, metabolic, and chemical changes will produce temporary or permanent hearing impairment. Although CoBF is considered to provide important support for auditory function and is suspected to play an important role in many pathological processes [5-7], the difficulty of measuring CoBF directly in vivo has limited the investigation of the correlation between the change in the blood flow and hearing dysfunction in auditory blast injuries. The effect of treatments that aim to promote blood flow in the cochlea is also uncertain [8, 9]. Therefore, the role of disordered CoBF in hearing impairment induced by a blast still remains ambiguous. Many previous studies have focused on a high level of noise and impulse noise. However, due to differences in the

experimental conditions, criteria and research methods, the results have been varied [10-14]. A better understanding of the CoBF changes will be helpful for the prevention and management of hearing disorders resulting from blast damage.

Materials and methods

Subjects

A total of 45 guinea pigs of both sexes were used, weighing 250-350 g and with normal Preyer's reflexes. The animals were housed under standard laboratory conditions with free access to food and water. Examinations of the tympanic membrane revealed no evidence of pathology. All operative procedures were performed in a state of deep surgical anaesthesia with pentobarbital sodium (30 mg/kg) administered intraperitoneally.

Experimental protocols were approved by the Management Committee of Experimental Animals of Third Military Medical University, China.

Surgery and preparation

The animals were initially anaesthetised with pentobarbital sodium (3 mg/kg intraperitoneal injection). This injection was repeated every 60 min using half of the initial dose. The left bulla was exposed and opened via a retroauricular approach. A round hole (2 mm diameter) was drilled in the upper portion of the bulla to observe the position of the laser Doppler probe, and a small hole (1 mm diameter) was drilled under the observation hole for the insertion of a stainless steel tube (1 mm outer diameter and 0.5 mm inner diameter) that was used to bolster the probe. The tip of the tube was placed on the bony surface of the basal turn of the cochlea. The probe was fixed, and the holes were sealed with dental self-curing resin. The incision was sutured, and the outer tip of the tube protruded beyond the skin. The systemic blood pressure (BP) of all animals was recorded by a pressure transducer (MLT0380/D Reusable BP transducer, AD Instruments Pty Ltd, Australia) through a catheter that was inserted into the left femoral artery before surgery, and the data were collected using a quad bridge (ML118, AD Instruments Pty Ltd). The BP data were recorded and analysed using Chart Software (v5.5.6, Copyright: 1994-2008, AD Instruments Pty Ltd, Austrilia), and after being divided into 5 s segments, each individual segment was averaged and converted to the percentage change.

Histopathology of cochlea

At the end of each experiment, the animals were killed under pentobarbital anaesthesia (30 mg/kg, intraperitoneal) followed by cervical dislocation. The temporal bone was removed, and the bulla was opened immediately. A small hole was created in the apex of the bone shell by a needle, and the round window was opened by the same method. The fixative solution (4% paraformaldehyde, buffered at pH 7.3) was perfused three times into the cochlea through the apical hole using a pipette. Then, the cochleae were immersed in the same fixative solution at 4°C overnight. After fixation, the excess bone around the cochlea was removed, and the cochlea was decalcified in 10% EDTA solution (buffered at pH 7.3) for 5 days. Next, the specimens were embedded in paraffin and sectioned in the horizontal plane at a thickness of 5 μm. The sections were stained with haematoxylin-eosin and studied by light microscopy.

Detonation and measurement of blast wave

To generate the blast wave, a detonator (paper shell, 600 mg cyclotrimethylenetrinitramine, RDX inside) was fixed in an open space at a height of 0.4 meter and detonated by electrodes. The electric pressure transducers were fixed at the same level and distance to record the pressure of the blast waves. A dynamic data acquisition meter (TESTELECTRONIC Inc, Chengdu, China) was used to record the signal from the transducers. The signal was recorded and analysed using DAP Software (v3.01, copyright: 2005.6.6).

Measurement of CoBF by laser doppler

The blood flow of the basal turn of the cochlea was registered with a laser-Doppler blood flow meter (ML191, AD Instruments Pty Ltd, Australia). The needle-shaped probe (0.4 mm diameter) was inserted in the bolster tube until its tip reached the surface of the basal turn of the cochlea. The visible red laser light, with a wavelength of 830 nm, was supplied through

Table 1. Peak value and duration of overpressurea

Distance (m)[b]	n[c]	Peak value (kP[a])	Duration (s)
0.5	3	64.52±4.60[d]	0.348±0.022[e]
0.6	3	46.06± 4.32[d]	0.381±0.018[e]
0.7	3	34.62± 4.75[d]	0.411±0.019[e]
0.8	3	27.07± 6.78[d]	0.440±0.011[e]
0.9	3	23.30± 2.90[d]	0.467±0.008[e]

[a]Results are expressed as mean ± S.E.M. [b]The heads of the transducers were placed at the same distances as the external auditory canal. [c]The tests of the blast wave were repeated three times for each distance. [d]Significantly different between the peak values of the blast waves at each distance ($P<0.05$). [e]Significantly different between the durations of the positive phase at each distance ($P<0.05$).

an optic fibre. The blood flow meter was connected to a Power Lab System (AD Instruments), and the percentage of back scatter (BSC) and the laser Doppler flowmetry output signal were recorded using Chart Software (v5.5.6, Copyright: 1994-2008, AD Instruments). Both the LDF and BSC outputs were factory calibrated to produce a certain voltage output per calibrated unit. The BSC output represents the relative strength of the returned signal.

Evaluation of hearing function

To assess the hearing function, the auditory brain stem response (ABR) was measured. An evoked potential instrument (MK-NMD, MINGKAN Technologies, Chongqing, China) was used to generate acoustic stimuli and subsequently record the evoked potentials. An alternating broadband clicking sound (repetition rate of 11/s) was given to test the ear and used as the stimulus sound, and white noise sound was used as a contralateral masking noise. To record the bioelectrical potentials, subdermal stainless-steel needle electrodes were inserted at the vertex (active), dorsolateral to the measured ear (reference) and the nasal root (ground). The thresholds were determined from a set of responses at varying intensities with 5 dB SPL intervals, and the electrical signals were averaged from 512 to 1024 repetitions. The thresholds at each frequency were verified twice.

Blast exposure and evaluation of CoBF and hearing function

To ensure that the animals were exposed to a suitable intensity of blast overpressure, the animals were placed at distances from 0.5 meter to 0.9 meter at 0.1 meter intervals (3 animals for each distance). The blast overpressures were measured at the same distances and times. The normal LDF was recorded for 5 minutes a half hour before the exposure to the explosion blast, and the same recording process was performed immediately and 1 hour, 3 hours, 1 day and 2 days after the exposure. The ABR was recorded immediately and 1 hours, 3 hours, 1 day and 1 week after the exposure to the explosion blast. The CoBF and ABR of an additional 3 animals were measured at same time points, serving as the blank group.

Measurement of CoBF and evaluation of hearing function after L-NAME infusion and blast exposure

For this experiment, 27 guinea pigs were divided into 3 groups. L-NAME (Beyotime Institute of Biotechnology, China) was dissolved in a 1% artificial perilymph solution (137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 12 mM $NaHCO_3$, and 10 mM glucose; pH adjusted to 7.4 at 37°C) [15]. Group A consisted of 9 animals that received a 2 μl infusion of a 1% L-NAME solution into the RW by a micropipette (75N 5 μl SYR, HAMILTON CO). Group B consisted of the same number of animals that received the same infusion. Group B consisted of 9 animals that received the same dose of artificial perilymph infused into the RW. Both groups A and C were exposed to a blast generated by the same detonator at the distances of 0.5 meter, 0.6 meter and 0.7 meter (3 animals for each distance) 15 minutes after the RW infusion. The CoBF and ABR were measured in all animals of the 3 groups 10 minutes before the blast exposure; the CoBF was recorded immediately, 1 hour, 2 hours, and 3 hours after the blast exposure, and the ABR was measured 2.5 hours after the blast exposure in groups A, B and C.

Statistical analysis

Peak values and durations of the overpressure, BP, data of CoBF, and TS of all groups in each distance were averaged and analyzed using a 1-way analysis of variance (ANOVA). Data of CoBF and TS between group A and group C were averaged and analyzed using independent samples T-test. Correlation between the

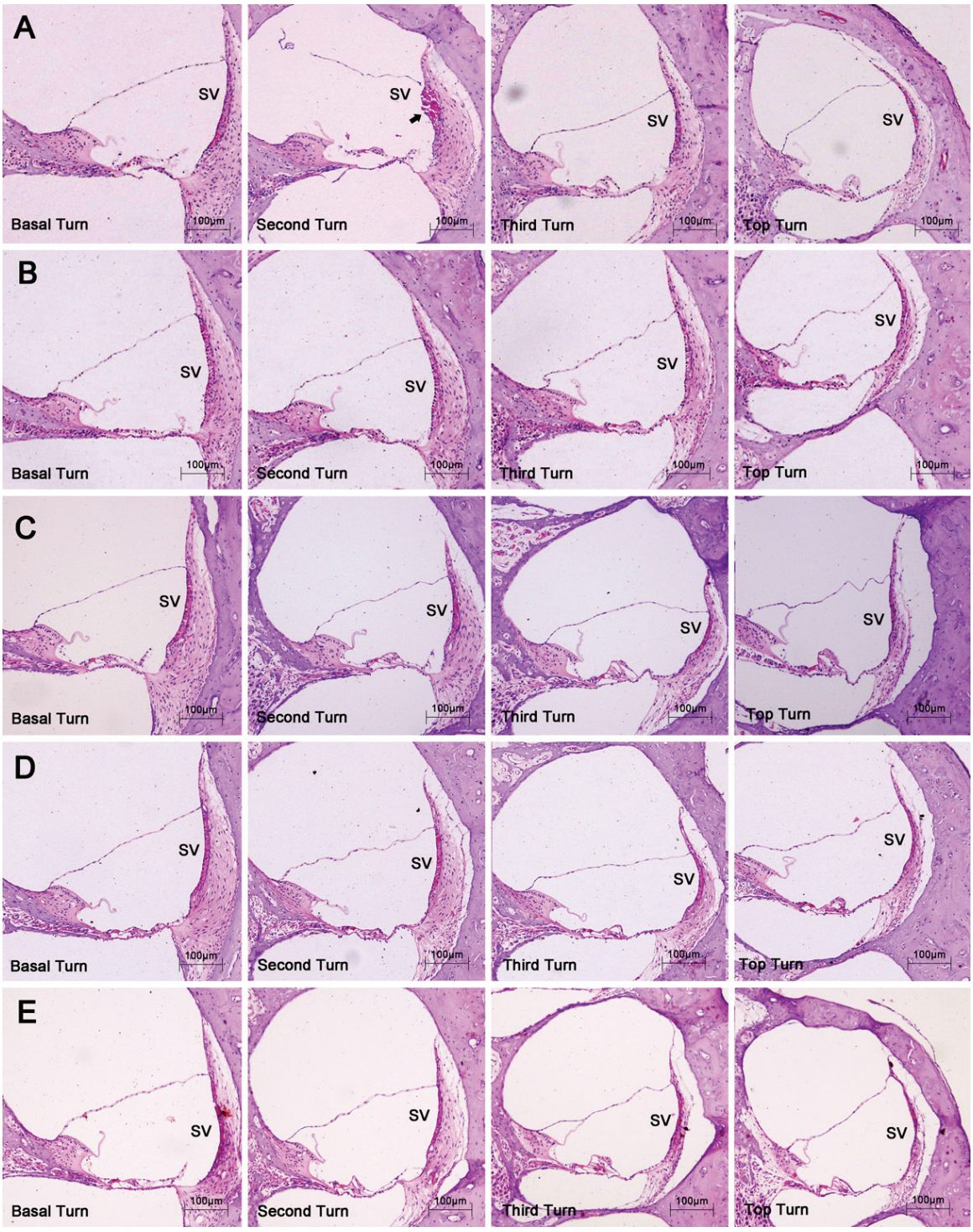

Figure 1. The pathological changes of the inner ear. Haematoxylin-eosin, original magnification ×100. SV, stria vascularis. A, B, C, D and E: show the pathological changes of the four turns of the cochlea at each distance, respectively. The arrow indicates the rupture of the stria vascularis in the second turn.

maximum values of the CoBF baseline and peak values of the blast overpressure was analyzed using bivariate correlation analysis. All analysis processes were conducted using SPSS statistics 17.0. The distribution are reported as mean±S.E.M.

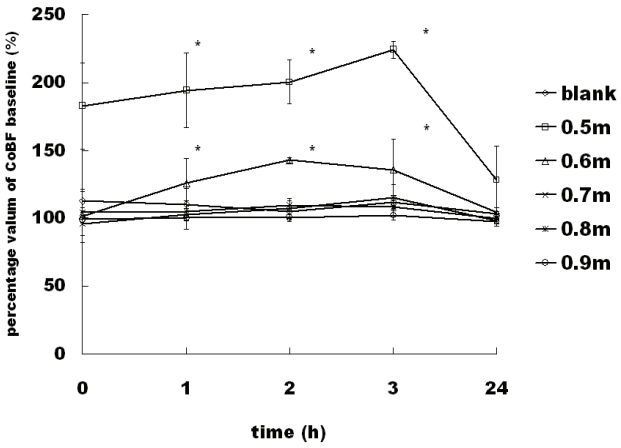

Figure 2. Time course of the cochlear blood flow (CoBF) measured by LDF at different distances. The y axis depicts variations in the percentage of CoBF compared with the initial baseline levels, and the x axis depicts the time. The values are means ± S.E.M.

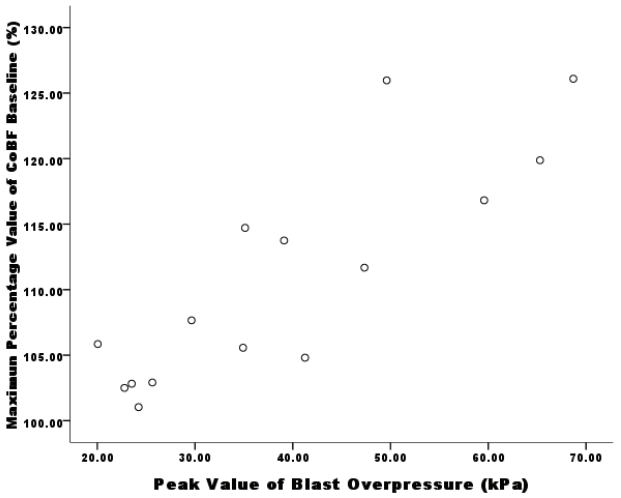

Figure 3. Correlation between the maximum values of the CoBF baseline and peak values of the blast overpressure. The correlation is significant at the 0.01 level (2-tailed) (analysed by SPSS 17.0.0).

Results

The systemic blood pressure, which was recorded until the end of each experiment, showed values within the normal range, and the percentage changes were minimal in all animals.

Pressure changes of the blast waves at different distance

As an open field explosion, free-field blast waves were produced with a characteristic pressure-time history. The intensity of the blast wave decreased with the increase in the distance from the epicentre, and the positive phase duration had the opposite relationship.

Table 1 shows the peak value and duration of the overpressure at each distance. The nearest point had the highest peak overpressure value of 63.64±5.78 kPa and the shortest duration time of 0.348±0.022 s. The farthest distance had the weakest overpressure intensity of 23.34± 1.52 kPa and the longest duration of 0.467±0.008 s.

Histopathological changes of the cochlea

The pathological changes of the cochlea were not significant under the light microscope. Disorder of the organ of Corti was common for all distances. Although the irregular arrangement of sensory cells could be observed for the close distances (0.5 meter) and was relatively distinct in the basal and second turns, the complete detachment of the sensory epithelium was not observed. Structural damage to the later wall of the cochlea was rare, with only one case of rupture of the stria vascularis found in the second turn at the distance of 0.5 meter. **Figure 1** shows the pathological changes of the inner ear for each distance.

Blood flow measured by LDF

After exposure to a blast wave, the cochlear blood flow (CoBF) showed appropriate distance and time-dependent patterns. At close distances (0.5 meter and 0.6 meter), the baseline CoBF increased immediately after the blast exposure, reached a peak value (0.5 meter, 227.76% of the initial level; 0.6 m, 142.79% of the initial level) at 2 to 3 hour (0.5 meter, 3 hours; 0.6 meter, 2 hours) and dropped gradually after reaching the maximum, recovering within 24 hours. As the distance increased, the variations in the CoBF were gentler, and there was no significant difference ($p>0.05$) when the distance was greater than 0.7 meter (**Figure 2**). Bivariate correlation analysis between the maximum value of the CoBF baseline and peak value of the blast overpressure indicated that the correlation was significant at the 0.01 level (2-tailed) (**Figure 3**).

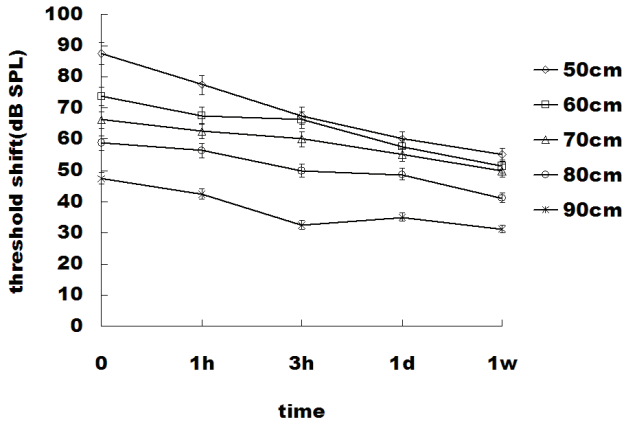

Figure 4. Time course of the hearing threshold shift. The y axis depicts the hearing threshold shift in dB SPL, and the x axis depicts the time. The values are means ± S.E.M. The difference between each distance is significant (P<0.05).

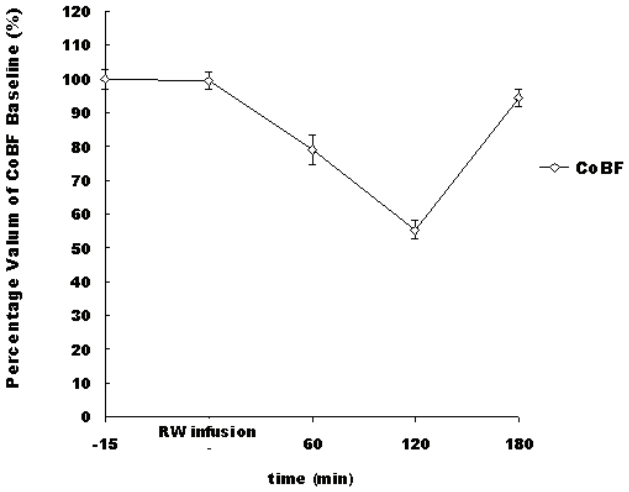

Figure 5. Percentage changes in the CoBF following the administration of 2 μl of 1% L-NAME in the RW. The CoBF decreased after the RW infusion, reached its lowest point at 2 hour, and then recovered after 3 hour. The data points are means±S.E.

Hearing threshold shift after blast

Hearing threshold shifts (TS) were significant with 87.5 dB (0.5 meter) to 47.5 dB (0.9 meter) (P<0.01), immediately after the blast wave exposure for all distances, followed by a gradual recovery course. Although the hearing threshold shift also showed a time-dependent pattern, the recovery courses were not so quick compared with the CoBF changes. The TS remained at 55 dB (0.5 meter) to 31.25 dB (0.9 meter) even 1 week after the blast exposure. At every time point of the test, the TS values were significantly different between each distance (**Figure 4**).

Observation of CoBF and hearing threshold shift after L-NAME infusions and blast exposure

The BP was simultaneously recorded using the same Chart Software after anaesthetisation until the end of the experiment. The results showed values within the normal range, and the percentage changes were minimal in all animals from the 3 groups.

The changes in the CoBF presented different patterns in the 3 groups during the experiment. Following the L-NAME RW administration, the CoBF decreased and reached its lowest baseline value (percentage value of change: 55.3±2.8%) (**Figure 5**) at 2 hours in the animals that were administered L-NAME (group B). This decrease lasted for 3 hours, followed by recovery to the normal level. Varying degrees of the decrease in the CoBF were also detected in the animals that were exposed to the blast at 3 distances after the L-NAME RW administration (group A). Contrasting changes were observed in the animals that were administered artificial perilymph (group C), with the CoBF increasing after the blast exposure (P<0.05) at all 3 distances; these changes lasted for 3 hours. At most of the time points, the differences between groups A and C were significant (**Figure 6**).

The hearing threshold shifts (TS) were significant for all distances between group A (75.0±7.1 dB at 0.5 meter, 70.0±9.1 dB at 0.6 meter, 56.3±4.8 dB at 0.7 meter) and group C (57.5±6.5 dB at 0.5 meter, 58.8±4.8 dB at 0.6 meter, 42.5±8.7 dB at 0.7 meter). Meanwhile, the TS values were minimal (1.3±2.3 dB) between before and 3 hours after the L-NAME solution RW infusion in group B (P>0.05).

Discussion

Blast pressure can produce a wide range of traumas, especially in air-containing structures. The auditory system has been considered the most susceptible organ and can endure serious injuries after a blast explosion [16]. The severity of the resulting hearing dysfunction is

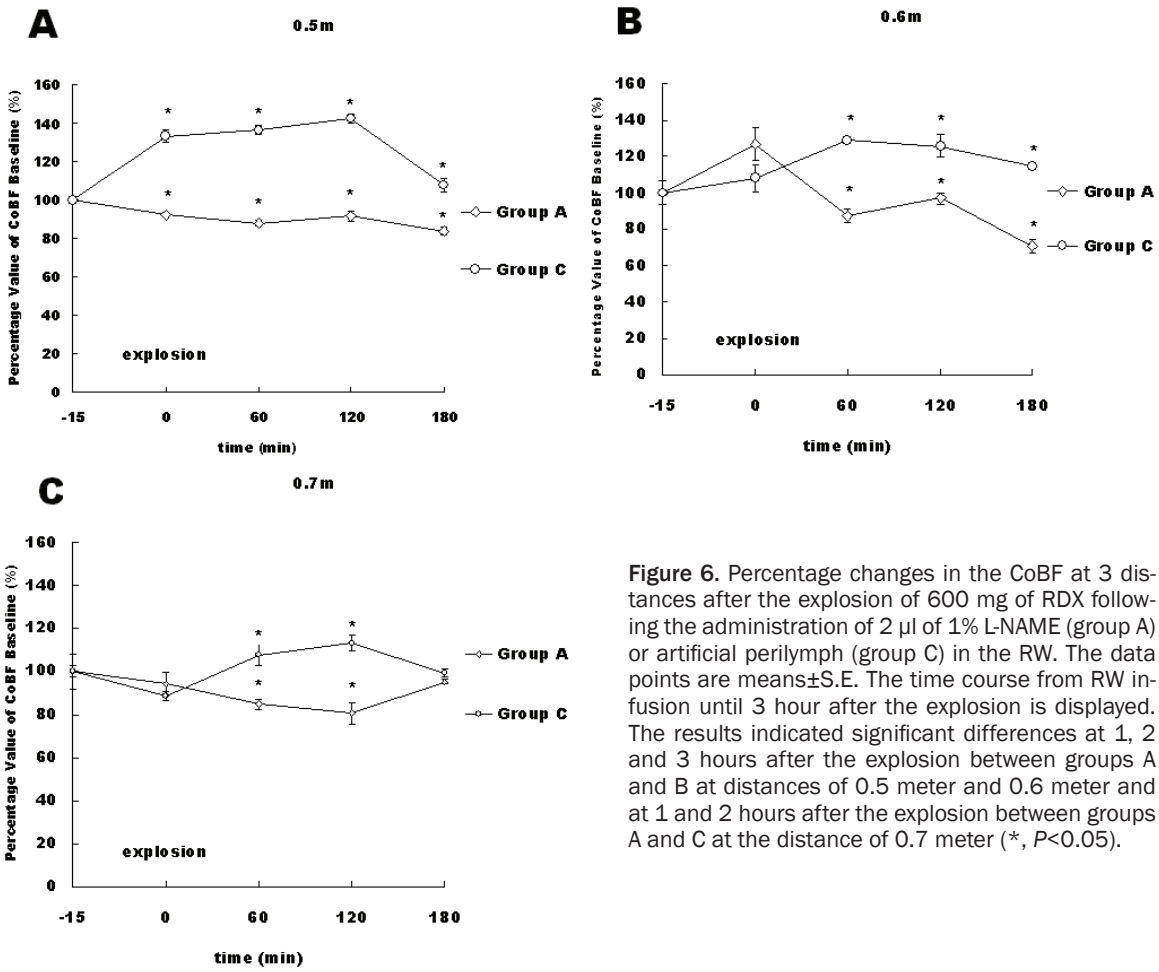

Figure 6. Percentage changes in the CoBF at 3 distances after the explosion of 600 mg of RDX following the administration of 2 μl of 1% L-NAME (group A) or artificial perilymph (group C) in the RW. The data points are means±S.E. The time course from RW infusion until 3 hour after the explosion is displayed. The results indicated significant differences at 1, 2 and 3 hours after the explosion between groups A and B at distances of 0.5 meter and 0.6 meter and at 1 and 2 hours after the explosion between groups A and C at the distance of 0.7 meter (*, $P<0.05$).

related to the distance from the epicentre in a simple open-space explosion. However, the trauma can be fatal if the injuries occur near a violent explosion, and the management of such trauma would focus on life support measures and non-auditory organ systems. Therefore, a moderate intensity of blast is necessary to evaluate injuries of the auditory system individually. In this study, we generated a relatively mild blast with a small power explosive (600 mg RDX) and placed the animals at moderate distances (0.5 meter to 0.9 m) corresponding with the medium intensity peak value of the blast overpressure (63.64±5.78 kPa to 23.34±1.52 kPa). There were no obvious signs of injury to the life support systems in all animals when exposed to this blast range, but tympanic membrane ruptures and damage to the middle ear were observed, as was distortion to the structure of the organ of Corti. However, as the main theme of this study, no distinct damage of the microvasculature structure was detected. The absence of that type of damage suggested that changes in the COBF were more likely to be functional.

Similar to its role in other tissues and organs, the microvasculature is a key component of the cochlea. Although the CoBF is estimated to require only on the order of 1/10000 of the total cardiac output in guinea pigs and 1/1000000 in humans [17], a normal blood supply to the cochlea is critically important for maintaining the inner ear potential and sustaining the production of endolymph. Moreover, sensory hair cells are vulnerable to ischaemia [18, 19]. Although disorders of the CoBF have been considered to be involved in many pathophysiological processes of the inner ear [20-22], the difficulty of direct detection has limited the investigation of CoBF pathological changes, especially after blast injury. Early studies suggested that noise can reduce the cochlear blood flow, decrease the red blood cell density

and increase the aggregation of red blood cells [10, 11, 23]. However, contrasting results have indicated an elevation in the CoBF after exposure to high-intensity noise [24] and blast waves [14]. Other researchers have suggested that changes in the cochlear microcirculation are intensity-related [25]. Such inconsistent results are likely due to the diversity in the experiment conditions and test methods. Although the definitions of impulsive noise and blasts are arbitrary, the distinction in the intensity, transmission pattern and mechanical features between them is apparent. Blast waves always have more intensive variation in the resulting pressure and involve the high speed movement of air over a very short time. Consequently, the pathological changes in the cochlear microcirculation induced by a blast may differ from the changes induced by high-intensity noise or impulse noise. In this study, our findings revealed an obvious relationship between the variations in the CoBF and the strength of the blast. Although increases in the CoBF were observed when the peak value of the blast overpressure was greater than 45.68±6.21 kPa, the CoBF was stabilised when the peak value of the blast overpressure was less than 35.05±4.11 kPa. This observation suggested the existence of a threshold for the blast overpressure peak value to cause CoBF changes. Unlike the hearing threshold shift, the time course of the CoBF changes indicated a rapidly recovering process. The baselines of the CoBF recovered to the normal level within 3 to 24 hours. Taking into consideration the histopathology changes in the cochlea, such results indicated that the structure of the cochlear microvasculature was not as vulnerable to the blast overpressure compared with other structures of the middle and inner ear, indicating strong autoregulatory ability. This autoregulation more likely occurs locally, in agreement with previous studies [17, 26-28].

Although disorders of the CoBF have been considered to be associated with hearing loss in many diseases, the impairment of hearing function induced by a blast involves a few pathologic factors. Most previous studies of blast injuries have focused on the mechanisms involved in the damage to structures of the auditory system and the subsequent hearing loss induced by these damages [29-33]. The role of the CoBF disorder induced by a blast is ambiguous. In this study, we used a NO synthase inhibitor, L-NAME, to inhibit the changes in the CoBF induced by the blast and evaluated the effect of this inhibition on hearing function. As an important regulator of CoBF, nitric oxide (NO) is a potent vasodilator [34-36]. It can cause smooth muscle and pericyte relaxation by activating cGMP and affecting its downstream targets [37] and can also directly inhibit voltage-gated calcium channels, causing smooth muscle cells to relax [38]. This dilation effect can be inhibited by N-nitro-L-arginine-methyl ester (L-NAME), a NO synthase inhibitor [39]. Such an inhibitory effect is dose-dependent, and this effect varies with the regional blood flow in the cochlea via different pathways and administration strategies [40]. In this study, we investigated the changes in the CoBF and evaluated the hearing function after the administration of an L-NAME solution in the RW. A decrease in the CoBF was observed immediately after the administration and lasted for 3 hour, with the lowest baseline value reaching 55.3±2.8% of the initial level at 2 hour after the administration. In the meantime, this local application did not impact the hearing function or systemic blood pressure, suggesting that there is a compensatory range of CoBF with respect to hearing impairment under normal situations. This L-NAME application also inhibited the increase in the CoBF after the blast exposure. In contrast to a normal situation, such inhibition corresponded with a more obvious TS compared with the application of artificial perilymph. These findings suggested that the increase in the blood flow has a positive role in the protection of hearing function, indicating that NO plays an important role in the regulation of the CoBF. Although the CoBF has a relatively wide compensatory range for hearing impairment under normal conditions, the hearing function appears to be more vulnerable to decreases in the CoBF. This observation also implies the importance of blood flow-supporting measures in auditory blast trauma. These findings will be helpful to better understand the CoBF changes and hearing dysfunction caused by auditory blast injuries and provide potential new methods of the prevention and management of these injuries in the clinic.

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