

Research Paper: Bilateral Carotid Artery Occlusion and Cochlear Oxidative Stress and Hearing Loss in Rats



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ABSTRACT

Introduction: This study aimed to evaluate the effects of bilateral carotid artery occlusion on cochlear oxidative stress and hearing status in rats.

Methods: The rats were divided into two sets. The first set was used for electrophysiological recording (click and 4 kHz tone burst auditory brainstem responses and electrocochleography) on the day before surgery and then on the first, fourth, and seventh days after surgery. Animals of the second set were used for biochemical analysis. The cochlea of animals in the second set was collected on the first, fourth, and seventh days after carotids occlusion for biochemical analysis. For the control groups, no carotids occlusion was done. For ischemia induction, both common carotid arteries were occluded for 20 minutes.

Results: Electrophysiological analysis showed that burst auditory brainstem thresholds significantly elevated after common carotid arteries occlusion on the first, fourth, and seventh days after surgery with abnormal electrocochleography results at 75%, 70%, and 85% on the first, fourth, and seventh days after surgery, respectively. The electrophysiological finding confirmed by biochemical results that showed malondialdehyde and nitric oxide levels increased and superoxide dismutase and catalase activities decreased after occlusion in cochlea tissue.

Conclusion: This study showed that bilateral common carotid artery occlusion increases cochlear oxidative stress and induces hearing loss in rats.

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Highlights

- ABR thresholds rose after common carotid arteries occlusion.
- Abnormal electrocochleography results were observed in the first few days.
- Biochemical results confirmed the electrophysiological finding.

Plain Language Summary

This study showed that the auditory threshold (auditory brainstem thresholds) significantly increased after common carotid arteries occlusion on the first, fourth, and seventh days after surgery. The electrocochleography test showed the hydrops in cochlea were abnormal at 75%, 70%, and 85% on the first, fourth, and seventh days after surgery, respectively. Biochemical results also confirmed these results. This study showed that bilateral common carotid artery occlusion increases cochlear oxidative stress and induces a hearing loss in rats.

1. Introduction

Ischemic injury is one of the major causes of hearing loss (Gyo, 2013). Vascular occlusion is thought to be especially important in sudden-onset hearing loss (Gyo, 2013; Koga et al., 2003). Understanding the mechanism of ischemia-reperfusion injury to the cochlea is essential for the development of therapeutic interventions for this type of injury. When the organs are exposed to ischemia, the metabolism changes from aerobic to anaerobic, which results in decreased cellular ATP production and oxidative stress (Bielefeld, Hu, Harris, & Henderson, 2005).

The cochlea is one of the most important parts of the human hearing system because it converts sounds into an electrical message and sends it to the central nervous system (Groves, 2010). Damage to the cochlea results in permanent hearing loss because its cells cannot be replaced (Groves, 2010). To date, a lot of studies have been done to understand the mechanism of damage to the cochlea (such as noise and ototoxic drug-induced cochlear damage, and age-related cochlear degeneration) and many have shown that the common point of injury can be oxidative stress (Sha, Taylor, Forge, & Schacht, 2001). Some studies on the damaged cochlea by the cisplatin and aminoglycoside showed that the use of antioxidants has protective effects (Schacht, Talaska, & Rybak, 2012).

The function of the cochlea is strongly dependent on the supply of food and oxygen by blood flow (Mom, Avan, Romand, & Gilain, 1997). It has been suggested that one of the reasons for reduced hearing loss with the unknown cause can be the loss or reduction of blood flow to the cochlea.

Animal models also show that even one minute stop in the bloodstream of the cochlea can cause damage and hearing impairment (Tabuchi et al., 2010).

In physiological conditions, the cell produces small amounts of reactive oxygen species (ROS) that act as second messengers and have many roles during various cell functions (Fanaei, et al., 2014; Keshtgar et al., 2012). But in the case of ischemic conditions, the production of ROS and other oxidants rises sharply and leads to increased damage from ischemia (Fanaei, et al., 2014; Sun et al., 2018).

Even post-ischemic reperfusion enhances oxidative stress (Sun et al., 2018). Considering that the nerve cells and the hair cells of the cochlea cannot be replaced after injury, it is essential to know the effect of ischemia through oxidative stress on the cochlea for effective treatment.

Previous studies were developed an animal model of transient cochlear ischemia by occluding both vertebral arteries in the gerbil and through this method induced hearing loss (Gyo, 2013). In the present study, we examined the effect of bilateral carotid artery occlusion on cochlear oxidative stress and hearing status in rats. We used auditory brainstem response for hearing assessment and electrocochleography for measuring endolymphatic hydrops in the cochlea. Also, we measured malondialdehyde (MDA) and Nitric Oxide (NO) levels, Superoxide Dismutase (SOD), and Catalase (CAT) activities in rats cochlea.

2. Methods

2.1. Animals

Experiments were carried out on male Wistar rats weighing 250–300 g. The rats were maintained under controlled conditions with the temperature at 22°C–24°C, the relative humidity of 40%–45%, and a 12-hour lighting cycle and permitted ad libitum access to water and standard lab chow.

This study was approved by the Institutional Animal Research Ethics Committee at Tehran University of Medical Sciences for Biochemical Results (grant number 5734) and Iran University of Medical Sciences for Electrophysiological Results (Grant No. 930212524757).

2.2. Experimental design

Animals were divided into two sets. The first set was used for electrophysiology recording and included the control and ischemic groups. The second set was used for biochemical analysis. Animals of the second set were randomly assigned to one of the following groups (10 in each group): 1-day control group, 1-day ischemic group, 4-day control group, 4-day ischemic group, 7-day control group, and 7-day ischemic group. The cochlea of animals in the second set was collected on the first, fourth, and seventh days after bilateral carotid artery occlusion for biochemical analysis. For the control groups, no carotids occlusions were done.

2.3. Surgical procedure

For carotid artery occlusion in ischemic groups, the rats were anesthetized with ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP). Both common carotid arteries were exposed and carefully separated from the vagus nerve and associated connective tissues. Then, atraumatic arterial clamps were applied to each of the arteries to occlude blood flow. Twenty minutes later, the clamps were removed and reperfusion was visually confirmed. Then the rats were allowed to recover and were observed until they resumed movement, drinking, and grooming behavior.

2.4. Threshold estimation with auditory brainstem response

The Auditory Brainstem Response (ABR) was used as a common electrophysiological test for assessing hearing thresholds in rats. The rats were tested one day before and on the first, fourth, and seventh days after surgeries. During the tests, the rats were under anesthesia. Each ear was tested separately and then both ears were

tested. The baseline ABR was used for detecting hearing loss. The surgery was performed in rats with normal hearing with click and 4 kHz tone burst stimuli. The rats with hearing loss or a threshold greater than 20 dBnHL were excluded from the study.

The auditory brainstem response was recorded by Eclipse (EP25 software, intra acoustic). The needle electrodes were placed on the forehead (non-inverting), mastoids (inverting), and tail (ground). Then click and 4 kHz tone burst stimuli were presented at 37.7 Hz with insert phone at maximum intensity (100 dBnHL) and after obtaining the waves and checking the waveform, the intensity decreased in 5 to 10 dB steps. The lowest intensity that replicated wave II was detectably considered as the threshold. About 1500–2000 stimuli are used for detecting the waves near the threshold. The polarity was alternate and the filter setting was 100–3000 Hz. The impedance was under 5 k Ω and the difference between the needles impedance was under 2 k Ω . The amplification was 100000 x.

2.5. Electrocochleography recording

The electrocochleography (EcochG) was recorded with the same instrument and electrode placement. The click stimuli were presented via insert phone to each ear separately at a rate of 11.3 per second and maximum intensity (95–100 dBnHL). The polarity was alternate and 1500 stimuli were used for detecting the replicated waves. The responses were filtered from none to 3000 Hz and the ratio of Summation Potential (SP) to Action Potential (AP) were computed.

Measurement of Malondialdehyde (MDA) and Nitric Oxide (NO) levels, Superoxide Dismutase (SOD), and Catalase (CAT) activities in cochlea tissue

Cochlea samples were obtained one day before and then 1, 4, and 7 days after the beginning of the experiment. They were homogenized in 1 mL of 50 mM phosphate buffer (pH 7.4) with a homogenizer. After that, the homogenized cochlea samples were centrifuged (4° C, 1000 rpm for 10 min) and the supernatant was collected.

2.6. Malondialdehyde (MDA) assay

Measurement of MDA level was done by a commercial chemical colorimetric assay kit according to the manufacturer's protocol (MDA, A003; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). By this kit, MDA-TBA adduct is formed by the reaction of MDA and Thiobarbituric Acid (TBA) under high temperature.

Malondialdehyde (MDA) was measured in acidic media and heat (100°C) colorimetrically at 532 nm. After reagents preparations, the following steps were taken to measure MDA. First, 100 µL of the samples and standards were transferred to the related name test tubes. Then, 100 µL of R4 reagent was added to all tubes. Next, 200 µL of chromogen solution was added. Afterward, the tubes were heated above the mixture for 60 minutes at a boiling water bath to the pink color formation. Then, the tubes were cooled in an ice bath and centrifuged those 10 minutes (5000 rpm). Next, 200 µL of pink color supernatant was pipetted to the microplate. Finally, the absorbance rate was read at 532 nm.

2.7. Nitric Oxide (NO) assay

Measurement of NO level was done by a commercial chemical colorimetric assay kit according to the manufacturer's protocol (NO, A012; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The kit measures total nitrate/nitrite in a simple two-step process. First, it converts nitrate to nitrite utilizing nitrate reductase. Then, Griess reagent was used to convert nitrite to a deep purple azo compound. The amount of the azo chromophore accurately reflects the nitric oxide amount in the samples. The assay procedure was done as below.

Nitrate reductase and enzyme cofactor were added to related wells. The wells were incubated at room temperature for 1 h to convert nitrate to nitrite. Then, 5 µL enhancer was added to standard and sample wells. The wells were incubated at room temperature for 10 minutes. Then, 50 µL Griess reagents of R1 and R2 were added to standard and sample wells, respectively. Finally, the output was measured on a microplate reader at 540 nm.

2.8. Catalase Activity (CAT) assay

Measurement of catalase activity was done by a commercial chemical colorimetric assay kit according to the manufacturer's protocol (CAT, A007-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). After kit reagents preparations, the following steps were taken to measure CAT activity.

First, reagents and samples were equilibrated to room temperature. Then, 10 µL of samples and standards were added to related test tubes. R1 and R2 reagents were warmed at 37°C and then 100 µL of each was added to tubes. Tubes were incubated for 60 s at 37°C. Next, 100 µL of R3 and 10 µL of R4 reagents were added, respectively. Finally, the absorbance rate was read at 405 nm.

2.9. Superoxide Dismutase (SOD) activity assay

Measurement of SOD activity was done by a commercial chemical colorimetric assay kit according to the manufacturer's protocol (SOD, A001; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). This kit uses the superoxide anion for conversion to hydrogen peroxide and oxygen under enzymatic reaction conditions. After kit reagents preparations, the following steps were taken to measure SOD activity. First, 200 µL of the diluted radical detector, 10 µL of standard, and 10 µL of the sample were added per well in related wells on the plate. Then, 20 µL of diluted xanthine oxidase was added to all wells to initiate the reactions. The plate was incubated on a shaker for 30 minutes at room temperature. Finally, the absorbance was read at 450 nm using a plate reader.

2.10. Data analysis

The analyses were done in SPSS V. 17. The t test was performed for each day separately to show any difference between left and right ear results. Then the changes in the hearing were compared on different days for click and 4 kHz stimuli with the paired t test. In EcochG, the SP and AP were obtained, and the SP/AP amplitude ratio was calculated on different days using descriptive analysis such as mean and standard deviation. Biochemical data were analyzed by 2-way ANOVA with repeated measures and further evaluated by Bonferroni post hoc analyses. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Electrophysiological results

The ABR thresholds were obtained from two groups (Figure 1). The ABR mean thresholds were 16.67±3.83 dBnHL for click and 19.38±2.50 dBnHL for 4 kHz tone burst stimuli on the day before surgery (Table 1). The data had a normal distribution and there were no significant differences between left and right ear results in this day or the next days (P>0.05), so in the further analysis, we used both ear results together. Table 2 showed the thresholds minus thresholds of the day before surgery. For click stimuli, the threshold shift increased on the first and fourth days after surgery and then slightly decreased on the seventh day. The differences between ABR thresholds on the day before surgery and the following days after surgery were significant (P<0.05). A similar trend was observed for 4 kHz but the threshold shifts were higher for 4 kHz, especially on the first day, and had less decrease for the seventh day after surgery (Table 2).

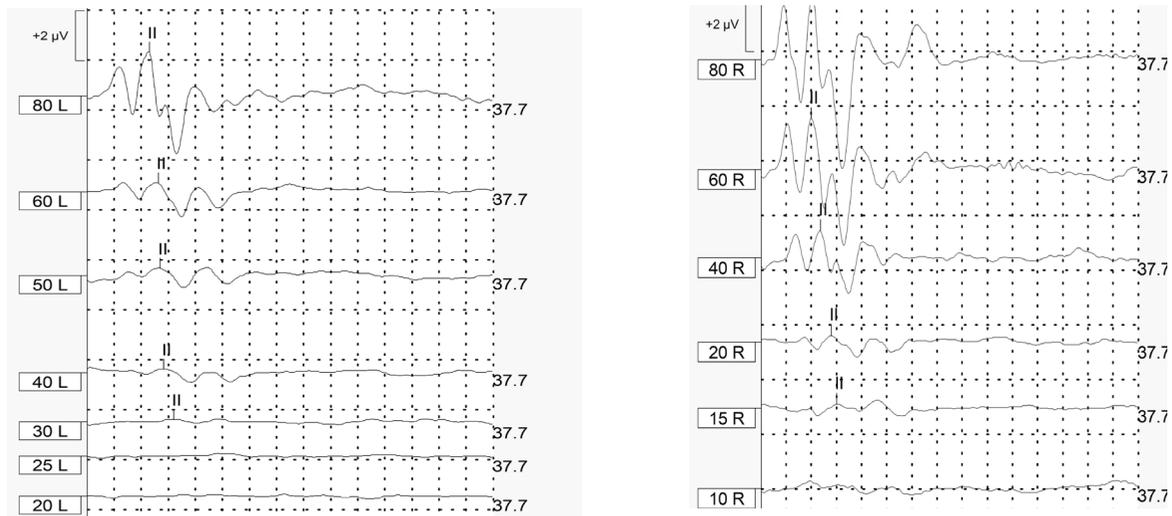


Figure 1. The auditory brainstem response thresholds for two groups: Ischemia (left) and control (right) **NEURSCIENCE**

The SP and AP also were obtained for the two groups (Figure 2). The mean SP/AP ratio on the day before surgery was 0.24 ± 0.11 , but in the analysis of the following days, the ratio beyond 0.44 (two standard deviations beyond the mean ratio) was considered as abnormal. About 75%, 70%, and 85% of cases had an abnormal ratio on the first, fourth, and seventh days after surgery, respectively. The changes between each day after ischemia and the day before surgery were significant ($P=0.00$) but the difference between day 1 and 4, day 4 and 7, and day 1 and 7 were not significant ($P>0.05$). Table 3 presents the SP/AP amplitude ratio on different days.

3.2. Biochemical results

As shown in Figure 3, bilateral carotid artery occlusion significantly increased cochlear NO levels in the ischemic animals on the first and fourth days after ischemia when compared to the sham group ($P<0.05$ and $P<0.001$, respectively). But it markedly decreased on the seventh day after surgery in the ischemia group and had no significant difference with the control group. Ischemia significantly ($P<0.001$) increased cochlear MDA contents on all days after ischemia (Figure 4). Cochlear MDA concentration increased from the first day to the fourth day then decreased on the seventh day. On the other

hand, ischemia significantly decreased cochlear contents of SOD and CAT on all days after ischemia (Figures 5 & 6). Though CAT and SOD concentrations in the ischemic group were significantly lower than the sham group, they increased as the experiment continued till the end process, but their levels were significantly lower ($P<0.001$) than the sham group on all days after ischemia.

4. Discussion

In this study, we examined the cochlear oxidative stress and hearing status after bilateral common carotid artery occlusion in the rats. We evaluated hearing and endolymphatic hydrops by using ABR and electrocochleography, respectively. To evaluate the role of oxidative stress in hearing loss caused by ischemia, we measured NO, MDA, SOD, and CAT levels in the cochlea.

The ABR in rats consisted of five waves and wave II has the lowest thresholds (Alvarado, Fuentes-Santamaria, Jareno-Flores, Blanco, & Juiz, 2012; Church et al., 2012), so this wave usually is used for threshold estimation in rats and their threshold could be as low as 10-20 dBnHL in normal-hearing population. Wave II is used for determining the hearing sensitivity in rats (Alvarado et al., 2012; Church et al., 2012). It mainly originates from the cochlear

Table 1. ABR thresholds for click and 4 kHz tone burst stimuli at day before surgery

Stimuli	Min.	Max.	Mean±SD
Click	10	20	16.67±3.83
4 KHz	10	20	19.38±2.50

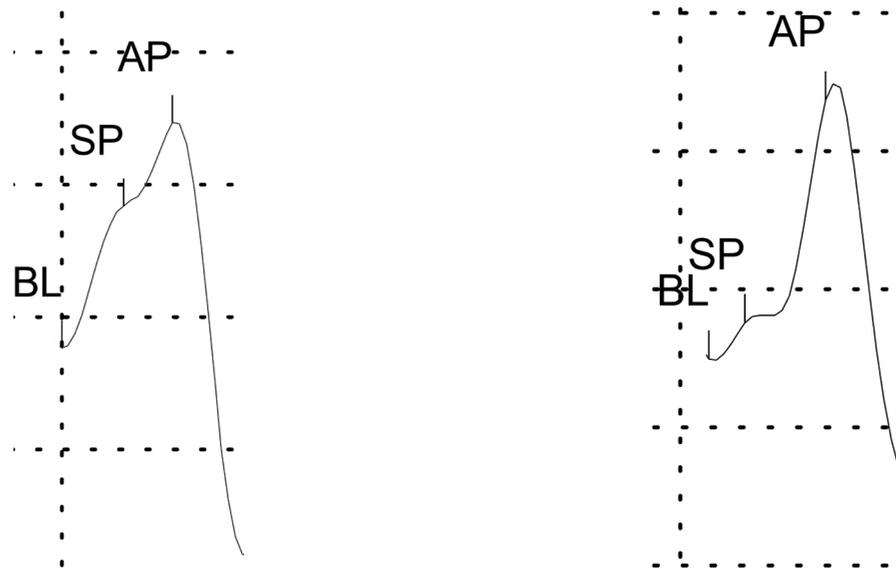


Figure 2. The summation potential and action potential for two groups: Ischemia (left) and control (right) **NEURSCIENCE**

nucleolus and auditory nerve so decreasing of its thresholds may mainly because of the damage to cochlear hair cells (Alvarado et al., 2012; Church et al., 2012). Our results showed that means of ABR threshold in ischemic rats significantly increased after ischemia. In general, the click stimuli represent the threshold of 1 to 4 kHz and by comparing its results with 4 kHz, it is found that the ischemia group had a greater and earlier effect on a higher frequency than lower frequency, so the basal portion of the cochlea is more susceptible to damage induced by ischemia.

Some studies that have induced cochlea damage by paraquat, cisplatin, and aminoglycoside also showed that the basal part of the cochlea is more vulnerable than other parts (Bielefeld et al., 2005; Wong & Ryan, 2015). The greater vulnerability of the basal portion has been related to a lower level of antioxidant defenses (especially glutathione) in the basal hair cells, relative to the apical part (Bielefeld et al., 2005; Sha et al., 2001). There-

fore, during various kinds of injuries to the cochlea, this weaker antioxidant defense ability of the basal part makes it more vulnerable to oxidative stress (Sha et al., 2001; Wong & Ryan, 2015).

The ABR thresholds increased on the first and fourth days and then slightly decreased on the seventh day after the surgery. The results show that ischemia causes hearing loss and small changes from the fourth day to the seventh day may show some recovery or simply represent fluctuation of hearing sensitivity due to endolymphatic hydrops.

The endolymphatic hydrops is a consequence of abnormal production or absorption of endolymph in the cochlea. In electrocochleography, an elevated SP/AP amplitude ratio shows endolymphatic hydrops, and different studies have used the recording of SP and AP and calculating the SP/AP ratio in the animals (Franz & Anderson, 2008; van Deelen, Ruding, Veldman, Huizing, &

Table 2. Threshold shift for different days and stimuli

Day	Stimuli	Min.	Max.	Mean±SD *
1	Click	0	25	6.39±8.87
4	Click	0	50	23.33±14.03
7	Click	0	30	15.00±10.16
1	4 kHz	0	35	17.65±11.19
4	4 kHz	0	80	30.88±19.54
7	4 kHz	0	70	27.33±20.07

* P<0.05.

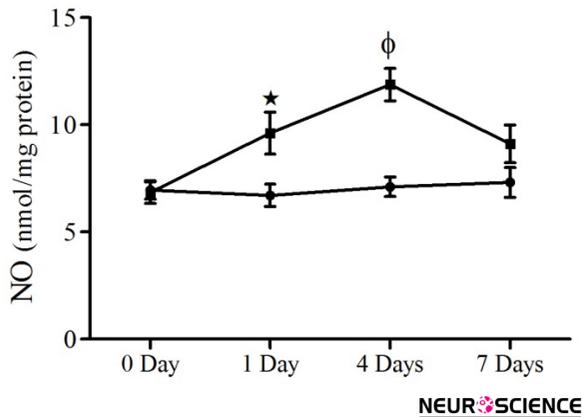


Figure 3. Cochlea nitric oxide levels in the sham and ischemic groups on 1 day before ischemia (0 Day) and on the 1, 4, and 7 days After Ischemia (AI) (Mean±SEM). P<0.05, sham versus ischemia on the first day AI.

† P<0.001, sham versus ischemic group on the fourth day AI.

Smooenburg, 1987). Results showed that SP/AP ratios were abnormal and increased after ischemia that indicates endolymphatic hydrops has been induced by bilateral carotid artery occlusion. A high ratio (up to 85%) of cases had an abnormal SP/AP ratio in different days after surgery and a comparison of ABR and EcochG results showed cochlear damage. So, our results showed that ischemia as a blood supply abnormality can generate endolymphatic hydrops in the cochlea and endolymphatic hydrops could be responsible for hearing loss.

Previous studies revealed that oxidative stress has a key role in cochlear pathogenesis and hearing loss (Du et al., 2015; Fetoni et al., 2013; Poirrier, Pincemail, Van Den Ackerveken, Lefebvre, & Malgrange, 2010). Therefore, oxidative stress status of rat cochlea was assessed in the present study by measuring MDA (as a lipid peroxidation marker (Fanaei et al., 2014; Fanaei et al., 2014) and NO levels that play important roles in the pathophysiology of hearing loss (Poirrier et al., 2010) in cochlear tissue.

Table 3. SP/AP amplitude ratio in different days

Day	Mini.	Max.	Mean±SD
Before surgery	0.7	0.42	0.24±0.11
1	0.7	1.0	0.68±0.28*
4	0.12	1.0	0.65±0.32 *
7	0.10	1.0	0.70±0.28 *

* P<0.05 when compared with day before surgery

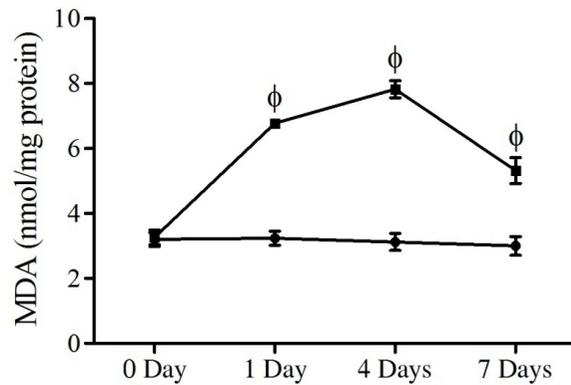
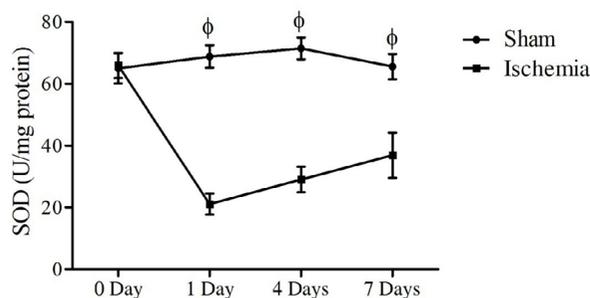


Figure 4. Cochlea Malondialdehyde (MDA) levels in the sham and ischemia groups one day before ischemia (0 Day) and on the first, fourth, and seventh days After Ischemia (AI) (Mean±SEM).

† P<0.001, sham versus ischemic group on the first, fourth, and seventh days AI.

Our results showed that MDA and NO levels significantly increased after ischemia. Besides, after bilateral carotid artery occlusion, SOD, and CAT activities in cochlea tissues considerably reduced.

So, bilateral carotid artery occlusion creates an imbalance between the oxidant and antioxidant process in the cochlea that was caused by the overproduction of ROS and reduction in cochlea antioxidant capacity. Therefore, biochemical data confirmed electrophysiological results that showed hearing loss. Animal models of hearing loss and endolymphatic hydrops have provided a basic scientific understanding of the mechanism of hearing loss for its treatment. These models have shown that hearing loss is accompanied by a cascade of electrophysiological and biochemical changes that contribute to the auditory dysfunction (Gyo, 2013). The most common model to induce hearing loss is transient cochlear ischemia in the Mongolian gerbil (Gyo, 2013; Takeda et al., 2009).



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Figure 5. Cochlea Superoxide Dismutase (SOD) activity in the sham and ischemia groups one day before ischemia (0 Day) and then on the first, fourth, and seventh days After Ischemia (AI) (Mean±SEM).

ϕ P<0.001, sham versus ischemic group on the first, fourth, and seventh days AI.

In adult Mongolian gerbil, the circle of Willis is not connected to the vertebral arteries and cochlear ischemia is induced by transient occluding bilateral vertebral arteries (Gyo, 2013; Yoshida et al., 2007). In our study, we used rats for induction hearing loss through bilateral carotid artery occlusion. The circle of Willis of a rat is connected to vertebral arteries (Speetzen, Endres, & Kunz, 2013). So, in this study for brain hypoperfusion induction, we occluded common carotid arteries that are the main source of blood flow to the brain (Speetzen et al., 2013). This occlusion reduces blood flow to the cochlea and induces ischemia. This model has some advantages such as rat is more available than gerbil and access to carotid arteries is easier than vertebral arteries. Also, bilateral carotid artery occlusion is a model as global cerebral ischemia; in real situations global cerebral ischemia occurs commonly in patients who have a variety of clinical conditions, including cardiac arrest, shock, and asphyxia and in patients undergoing complex cardiac surgery. So, hearing loss is one of the neurologic sequels of global cerebral ischemia. Results of the present study showed that bilateral common carotid artery occlusion increases cochlear oxidative stress and induces hearing loss in rats.

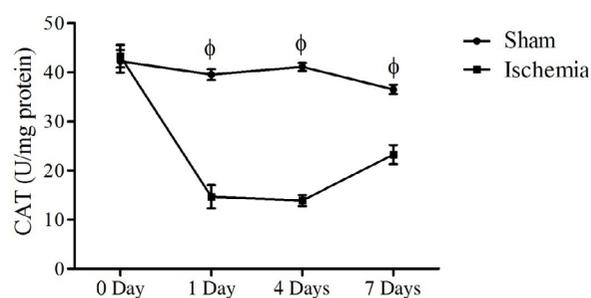
Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Institutional Animal Research Ethics Committee at Tehran University of Medical Sciences.

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Figure 6. Cochlea Catalase (CAT) activity in the sham and ischemia groups one day before ischemia (0 Day) and then on the first, fourth, and seventh days After Ischemia (AI) (Mean±SEM).

ϕ P<0.001, sham versus ischemic group on the first, fourth, and seventh days AI.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

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