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Title: Bilateral Carotid Artery Occlusion Induces Cochlear Oxidative Stress and Hearing Loss in Rat

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Abstract

Introduction: The aim of this study was evaluation the effects of bilateral carotid artery occlusion on cochlear oxidative stress and hearing status in rat.

Methods: The rats were divided into two sets. First set used for electrophysiological recording (click and 4 kHz tone burst ABR and EcochG) at day before surgery and 1st, 4th and 7th days after surgery. Animals of second set that used for biochemical analysis. Cochleae of animals in second set were collected at 1st, 4th and 7th days after carotids occlusions for biochemical analysis. For control groups no carotids occlusion took place. To ischemia induction both common carotid arteries were occluded for 20 minutes.

Results: Electrophysiological analysis showed ABR thresholds significantly elevated after common carotid arteries occlusion at 1st, 4th and 7th days after surgery and abnormal EcochG results at 75%, 70% and 85% in 1st, 4th and 7th days after surgery respectively. Electrophysiological finding confirmed by biochemical results that showed Malondialdehyde and Nitric oxide levels were increased and Superoxide dismutase and Catalase activities decreased after occlusion in cochleae tissue.

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

Key words: Hearing loss, rat, carotid artery, cochlea, oxidative stress, Auditory Brainstem Response.
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 ischemic-reperfusion injury to 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 metabolism, 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). After damage to the cochlea, its cells cannot be replaced, resulting in permanent hearing loss (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 that have damaged cochlea by the cisplatin and aminoglycoside showed that the use of antioxidants have 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 unknown cause can be the loss or reduction of blood flow to the cochlea.

Animal models also show that even one minute loss in the bloodstream of the cochlea can cause damage and hearing impairment (Tabuehi 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, Khayat, 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, Karimian, et al., 2014; Sun et al., 2018).
Even post-ischemic reperfusion also enhances oxidative stress (Sun et al., 2018). Considering that the nerve cells and the hair cells of the cochlea do not have the ability to replace after injury, knowing the effect of ischemia through oxidative stress on the cochlea is essential for effective treatment.

Previous studies 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 effect of bilateral carotid artery occlusion on cochlear oxidative stress and hearing status in rat. We used auditory brainstem response for hearing assessment and electrocochleography for measuring endolymphatic hydrops in cochlea. Also, We Measured Malondialdehyde (MDA) and Nitric oxide (NO) levels, Superoxide dismutase (SOD) and Catalase (CAT) activities in rat cochlea.

**Material and Methods**

**Animals**

Experiments were carried out on male Wistar rats weighing 250–300 g. The rats were maintained under controlled conditions with temperature at 22-24 °C, 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 number 930212524757).

**Experimental design**

Animals were divided into two sets. First set used for electrophysiology recording and include control and ischemic groups. Second set was used for biochemical analysis. Animals of second set were randomly assigned to one of the following groups (10 in each group): 1 day control group, 1 day ischemic group, 4 days control group, 4 days ischemic group, 7 days control group and 7 days ischemic group. Cochleae of animals in second set were collected at 1st, 4th and 7th days after bilateral carotid artery occlusion for biochemical analysis. For control groups no carotids occlusions took place.
Surgical Procedure

For carotid arteries occlusion in ischemic groups, rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) 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 rats were allowed to recover and were observed until they resumed movement, drinking, and grooming behavior.

Threshold estimation with Auditory Brainstem Response

The Auditory Brainstem Response used as a common electrophysiologic test for assessing hearing thresholds in rats. The rat tested at one day before and at 1st, 4th and 7th after surgeries. During the tests the rats were under the anesthesia. Each ear tested separately and both ears were tested. The baseline ABR used for detecting the hearing loss. The surgery performed in rats with normal hearing with click and 4 kHz tone burst stimuli. The rats with hearing loss or a threshold greater than 20dB nHL excluded from the study.

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

EcochG recording

The EcochG recorded with the same instrument and electrode placement. The click stimuli presented via insert phone to each ear separately at a rate of 11.3 per second and maximum intensity (95-100dB nHL). The polarity was
alternate and to 1500 stimuli used for detecting the replicated waves. The responses were filtered at none to 3000Hz 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 cochleae tissue**

Cochleae samples were obtained at one day before and 1st, 4th and 7th days after begin of experiment and then they were homogenized in 1 ml of 50 mM phosphate buffer (pH 7.4) with a homogenizer. After that, homogenized cochleae samples were centrifuged (4°C, 1000 rpm for 10 min) and supernatant was collected.

**Malondialdehyde (MDA) Assay:**

Measurement of MDA level was done by a commercial chemical colorimetrical assay kit according to manufacturer's protocol (MDA, A003; Nanjing Jiancheng Bioengineering Institute, Nanjing, China)). By this kit MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature. 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. 200 μl of Chromogen solution was added. Then, tubes were heated above mixture for 60 minutes at boiling water bath to pink color formation. After that, tubes were cool in ice bath and centrifuged those 10 minutes (5000 rpm). 200 μl of pink color supernatant pipetted to microplate. Finally, absorbance was read at 532 nm.

**Nitric oxide (NO) assay:**

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

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

**Catalase activity (CAT) Assay:**

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

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

**Superoxide dismutase (SOD) activity Assay:**

Measurement of SOD activity was done by a commercial chemical colorimetrical assay kit according to 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, briefly the following steps were taken to measure SOD activity: 200 µl of the diluted Radical Detector, 10 µl of Standard and 10 µl of sample were added per well in related wells on the plate. Then, 20 µl of diluted Xanthine Oxidase was added to all the wells to initiate the reactions. Plate was incubated on a shaker for 30 minutes at room temperature. Finally, the absorbance was read at 450 nm using a plate reader.

**Data analysis**

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

Result

Electrophysiological Results

The ABR thresholds were obtained for two groups (figure 1). The ABR thresholds were 16.67 (±3.83) dBn HL for click and 19.38 (±2.50) dB n HL for 4 kHz tone burst stimuli in day before surgery (Table 1). The data had normal distribution and there were no significant differences between left and right ear result in this day or further days (p>0.05), so in further analysis we used both ear results altogether. Table 2 showed the thresholds minus thresholds of day before surgery. For click stimuli, the threshold shift were increased at 1st and 4th days after surgery and then slightly decreased at 7th. The differences between ABR thresholds in day before surgery with following days were significant (P<0.05). The similar trend observed for 4 kHz but the Threshold shifts were higher for 4 kHz especially at 1st day and had less decrease for 7th day after surgery (Table 2).

The SP and AP also were obtained for two groups (figure 2). The mean SP/AP ratio in day before surgery was 0.24 ±0.11, in analysis of following days, the ratio beyond 0.44 (two standard deviation beyond mean ratio) considered as abnormal. The 75%, 70% and 85% of cases had an abnormal ratio at 1st, 4th and 7th 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, 4 and 7, 1 and 7 were not significant (P value > 0.05). Table 3 showed the SP/AP amplitude ratio in different days.

Biochemical Results

As shown in figure 3, bilateral carotid artery occlusion significantly increased cochlear NO levels in the ischemic animals at 1st and 4th days after ischemia when compared to sham group (p< 0.05 and p< 0.001 respectively). But it markedly decreased at 7th day after ischemia in ischemia group and had not significant difference with control group. Ischemia significantly (p< 0.001) increased cochlear MDA contents at all days after
ischemia (figure 4). Cochlear MDA concentration increased from 1st day to 4th day then decreased to 7th day. On the other hand, ischemia significantly decreased cochlear contents of SOD and CAT at all days after ischemia (figure 5 and 6). Though CAT and SOD concentrations in ischemia group were significantly lower than Sham group, they increased as the experiment continued till the end process, but their levels were significantly lower (p< 0.001) than sham group on all days after ischemia.

**Discussion**

In this study, we examined the cochlear oxidative stress and hearing status after bilateral common carotid artery occlusion in rat. 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 cochlea.

The ABR in rat 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 uses for threshold estimation in rats and their threshold could be low as 10-20dB n HL in normal hearing population. The wave II used for determining the hearing sensitivity in rat (Alvarado et al., 2012; Church et al., 2012). It mainly originates from cochlear 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 seems ischemia group had a greater and earlier effect on higher frequency than lower frequency, so the basal portion of 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 basal portion of cochlea 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). Therefore, during various kind of insults to the cochlea, this weaker antioxidant defense ability of the basal part make it more vulnerable to oxidative stress (Sha et al., 2001; Wong & Ryan, 2015).
The ABR thresholds increased at 1st and 4th days and then slightly decreased at 7th after surgery. The results show that ischemia causes hearing loss and small change from 4th day to 7th day may show some recovery or simply represent fluctuation of hearing sensitivity due to endolymphatic hydrops.

The endolymphatic hydrops is consequence of abnormal production or absorption of endolymph in the cochlea. In Electrocochleography, elevated SP/AP amplitude ratio shows endolymphatic hydrops and different studies used recording of SP and AP and calculating the SP/AP ratio in the animals (Franz & Anderson, 2008; van Deelen, Ruding, Veldman, Huizing, & Smoorenburg, 1987). Results showed SP/AP ratios were abnormal and increased after ischemia that indicates endolymphatic hydrops induced by bilateral carotid artery occlusion. High ratio (up to 85%) of cases had an abnormal SP/AP ratio in different days after surgery and comparison of ABR and EcochG results showed cochlear damage. So, our result showed that ischemia as a blood supply abnormality can generate endolymphatic hydrops in cochlea and endolymphatic hydrops could be responsible for hearing loss.

Previous studies revealed that oxidative stress has a central 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, Karimian, et al., 2014; Fanaei, Khayat, et al., 2014)) and NO (play important roles in the pathophysiology of hearing loss(Poirrier et al., 2010)) levels in cochlear tissue.

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

So, bilateral carotid artery occlusion creates an imbalance between oxidant and antioxidant process in cochlea that caused by overproduction of reactive oxygen species (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 mechanism of hearing loss for treatment. These models have shown that hearing loss is accompanied by a cascade of electrophysiological and biochemical changes that contribute to the dysfunction(Gyo, 2013). The most common model to induction hearing loss is transient cochlear ischemia in Mongolian gerbil (Gyo, 2013; Takeda et al., 2009).
In adult Mongolian gerbil, circle of willis is not connect to the vertebral arteries and cochlear ischemia induced by transient occluding bilateral vertebral arteries (Gyo, 2013; Yoshida et al., 2007). In our study we used rat for induction hearing loss through bilateral carotid artery occlusion. Circle of Willis of rat is connect to vertebral arteries(Speetzen, Endres, & Kunz, 2013). So, in this study to brain hypoperfusion induction we occluded common carotid arteries that are main source of blood flow to the brain(Speetzen et al., 2013). This occlusion reduces blood flow to the cochlea and induces ischemia in cochlea. This model have some advantages such as: rat is more available than gerbil, access to carotid arteries is easier than vertebral arteries. In addition, 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 rat.

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Figure 1- the ABR thresholds for two groups: Ischemia (left) and control (right).
Figure 2- the SP and AP for two groups: Ischemia (left) and control (right).
Figure 3. Chochlea NO levels in Sham and Ischemic groups on 1 day before ischemia (0 Day) and 1st, 4th and 7th days after ischemia (AI) (mean±SEM). p<0.05, sham versus Ischemia on 1st day AI, ϕ p<0.001, sham versus Ischemia on 4th day AI.
Figure 4. Chochlea MDA levels in Sham and Ischemia groups on 1 day before ischemia (0 Day) and 1\textsuperscript{st}, 4\textsuperscript{th} and 7\textsuperscript{th} days after ischemia (AI) (mean±SEM). $\phi$ $p<0.001$, sham versus Ischemia on 1\textsuperscript{st}, 4\textsuperscript{th} and 7\textsuperscript{th} days AI.
Figure 5. Chochlea SOD activity in Sham and Ischemia groups on 1 day before ischemia (0 Day) and 1\textsuperscript{st}, 4\textsuperscript{th} and 7\textsuperscript{th} days after ischemia (AI) (mean±SEM). \( \phi \ p<0.001 \), sham versus Ischemia on 1\textsuperscript{st}, 4\textsuperscript{th} and 7\textsuperscript{th} days AI.
Figure 6. Chochlea CAT activity in Sham and Ischemia groups on 1 day before ischemia (0 Day) and 1st, 4th and 7th days after ischemia (AI) (mean±SEM). $\phi$ p<0.001, sham versus Ischemia on 1st, 4th and 7th days AI.