A model portraying experimental loss of hair cell: The use of distortion product otoacoustic emission in the assessment of rat's ear

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Abstract: The use of rats in research academies to study deafness is widespread, meanwhile medicinal methods to eliminate hair cells is also increasing. Thus, aminoglycosides and loop diuretics have grasped more attention. This study aimed at establishing an animal model in which a rapid distortion of the hair cell of cochlea administering amikacin and furosemide and using distortion product otoacoustic emission (DPOAE) the functioning of rat's ear would be assessed. Forty-eight male Sprague-Dawley rats (mean weight 200-250g) were randomly divided into six equal groups. Except the control group the rest received 0.5mg/g, 0.75mg/g, 1mg/g, 1.25mg/g, and 1.5mg/g, of subcutaneous amikacin respectively. 30 minutes later every rat received 0.1mg/g of furosemide intrapritoneally. DPOAE of rats was measured before these injections and 72 hours later. Then tissue sections of the rat's cochlea were prepared. All the cases had a significant decrease in their DPOAE with the frequencies 2KHz-8KHz ($p \leq 0.05$). The most change in DPOAE was in rats which had received 1mg/g – 1.5mg/g amikacin. Histological studies approved distortion of hair cell even the apical turn. To establish a deafness model due losing hair cells, it is possible to use a combination of 1mg/g amikacin and 0.1mg/g furosemide. Besides, to approve deafness DPOAE resulted can be used.

Keywords: Aminoglycoside, furosemide, deafness, hair cell, DPOAE, rat.

INTRODUCTION

The unreversable ototoxic effects of aminoglycosides has long been known. This class of drugs includes antibiotics amikacin, kanamycin, neomycin, dihydrosterptomycin, and gentamycin which hurt vestibular system and cause deafness due to the elimination of hair cells. Some of aminoglicosides e.g. gentamycin and streptomycin mainly hurt vestibular system but some primarily damage cochlea (Guthrie, 2008, Matthew C, 2005, Rizzi and Hirose, 2007, Swan *et al*., 2008). In mammals, deafness due to hair cell loss is forever because regeneration of these cells in these animals does not occur (Breuskin *et al*., 2008, Warchol, 2010, Xu *et al*., 1993). The capability of gene manipulation and mutation similar to those in man which occur in the hearing system of the mouse and the rat is the reason for the choice of the rat as an animal model to study the hearing system. Nowadays, aminoglycosides as ototoxic agents are vastly used in animal studies to assess hair cells dying processes or generation and also studies regarding cochlea implantation are taken into consideration. In many of these studies, the principal aim of injecting aminoglycosides is to destroy hair cells without damaging spiral ganglion cells and supporting cells. However, destroying hair cells in mice or rats by means of aminoglycosides themselves co-occurs some problems; e.g. at low doses, which must frequently be used, destruction is not properly done and high doses come along with damaging spiral ganglion and fatal. Therefore, a combination of aminoglycosides and loop diuretics must be used (Alam *et al*., 1998, Bock *et al*., 1983, Brummett, 1981, Brummett, 1983, Gratacap *et al*., 1985, Matsui *et al*., 2005, Shibata and Raphael, Swan *et al*., 2008, Taylor *et al*., 2008).

The present study aimed at determining the necessary dose of amikacin with a specific amount of furosemide (as a loop diuretic) to destroy hair cells and make an animal deafness model to be employed in studying deafness due to hair cell loss. In this study, the effects of amikacin and furosemide on hair cells were studied making use of distortion product otoacoustic emission (DPOAE) and a light microscope.

MATERIALS AND METHODS

Experimental rats

The rats were obtained from Pasteur institution in Tehran, Iran. Besides, the ethical committee in Bagiyatallah University of Medical Sciences approved the experiments on the animals. First of all, 48 Sprague-Dawley rats weighting 200-250g each aged 3 months were chosen, and their baseline respective DPOAE was recorded to

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measure their baseline hearing threshold. The animals were randomly divided into six groups; the first group was taken as the control one. Group 2, group 3, group 4, group 5, and group 6 received 0.5mg/g, 0.75mg/g,1mg/g, 1.25mg/g, and 1.5mg/g amikacin subcutaneously respectively. After 30 minutes these group members were intraperitonealy injected 0.1mg/g loop diuretic furosemide.

DPOAE measurements

Before testing each rat underwent an otoscopic examination and merely those which had a normal outer ear canal and tympanic membrane were selected. While measuring there were no interfering noises and all measurements were done in a quiet room. Then DPOAEs of the rats were obtained using a standard commercial OAE apparatus cochlear emission analyzer (Otoacoustic: Madsen Capella, Danmark) as described by Yezdan Firat *et al* (2008). In brief, through an inserted ear phone primary tones were introduced into each rat's ear canal. Then, by simultaneously presenting two sinusoids, which differed in frequency (f1 and f2) into the ear, dpoaes were generated and measured at 2f1-f2. Intensities of primary stimuli were set as equilevel at 65 dB (L1=L2). The frequencies were adjusted in such a manner that f2/f1=1.22. DPOAEs were recorded as Dp-gram and, input/output DPOAE functions (I/O function). Dp-gram ranged from 2KHZ to 8KHZ. I/O functions were obtained by increasing the primary tones from 50 to 70 dB sound pressure level (SPL) in 5-dB steps. For each rat, I/O function at frequencies of 6KHz and 8KHz were recorded.

Tissue preparation

The rats were killed 3 days after injection of amikacin and furosemide. Anesthesia was done by means of ketamine and xylazine and the rats were transcardially per fused with normal saline whose pH was 7.4, followed by a fixative which consisted of freshly 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (pH 7.4).Then, the temporal bones of the animals were removed and immersed in the same fixative at 4°C for 4 hours. After this, the bones were decalcified through immersing into 0.1 mol/l ethylenediaminetetraacetic acid (EDTA) the pH of which was 7.4, at 4°C for 24 hours. Dehydration of the specimens was done by means of graded concentration of alcohol embedded in paraffin blocks. Now; the specimens were sectioned into 5µm thick slices. After the sections were put on polylysine coated slice glasses, they were stained with hematoxyline and eosin (H&E) for microscopic examination.

STATISTICAL ANALYSIS

The SPSS program for windows (Release 10.0, 1999, SPSS Inc.) was used to run all the statistical analysis. The shift in DPOAEs of all groups was compared with one another using paired sample T test and one way ANOVA. Finally $P \leq 0.05$ was considered as significant.

RESULTS

After combined amikacin-furosemide administration high and low frequency hearing loss was observed.

DPOAE measurements

DP-gram

Figs. 1a and b (DP-grams) illustrate the effect of combined amikacin-furosemide on DPOAE output across the totality of the tested frequencies (2-8KHz). Mean auditory thresholds which were obtained from the control group and case animals (n=42) before injection (fig. 1a) corresponded to the pre-injection DPOAE outputs whereas fig.1.b shows the combined amikacinfurosemide effect that was measured three days after injection.

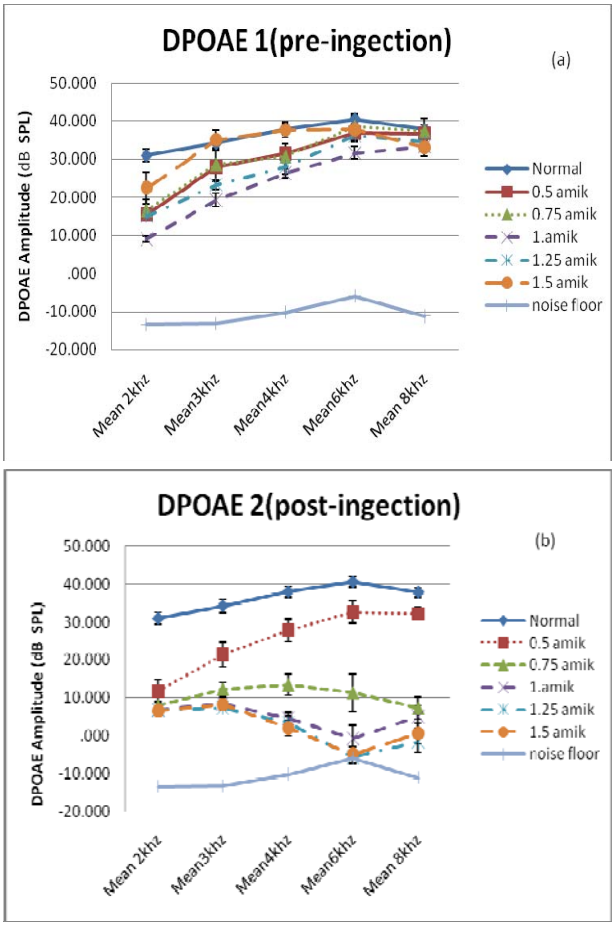

Fig. 1: Pre-injection (a) and 3 days post-injection (b) DP-grams. Each point represents Mean±SEM.

The most variations were seen for the combined doses 1 mg/g-1.5mg/g of amikacinfurosemide so that mean variations of threshold were about 20dB for the frequency 2KHZ and for the frequencies 6khz and 8khz it was about 35dB.The best frequencies sensitive to combined administeration of amikacinfurosemide were 6khr and 8khr.

Input/Output DPOAE function (I/O Function)

fig.2.The growth of DPOAE with increase of stimulus level (DPOAE I/O) prior to and tree days after injecting amikacin-furosemide was measured under the frequencies 6KHz and 8KHz .In all rats significant changes were observed on the growth curves on I/O functions after injection($p \leq 0.05$).

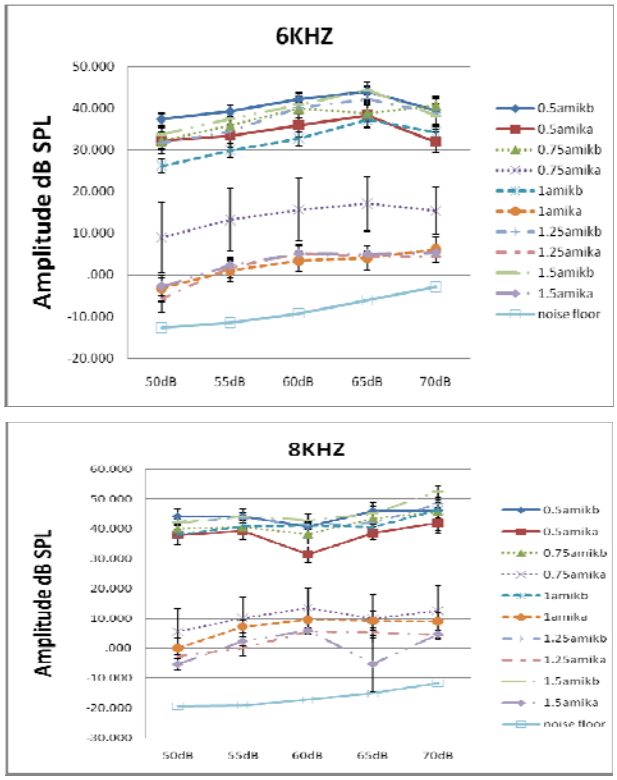

Fig. 2: DPOAE I/O Function at 6KHz and 8KHz in pre-injection (amik-b=before injection) and post-injection (amik-a=after injection). Each group was compared with itself before/b and after/a injection. Each point represents Mean±SEM

Histologic result

Fig. 3. Demonstrates sections from the corti organ and outer hair cells in normal rats and amikacin-furosemide treated ones. The top panel (figs. a, b) shows the hair cells and corti organ in the apical turn of the cochlea of normal rats. But after the administration of amikacin-furosemide nearly all outer hair cells were destroyed and as a result the corti organ lost its natural structure and Dieter cells became disorganized. Basilar membrane was intact and thus the separation between endolymph and perilymph was maintained. In the basal turn of the cochlea the hair cells were extensively damaged and occasionally the entire corti organ was not visible.

Quantification of spiral ganglion cells loss

We tested the hypothesis that the administration of amikacin-furosemide led to a significant alteration in the number of spiral ganglion cells. The number of cells present within each mid modiolar cross section of the cochlea was measured .Cells were counted in normal rats and amikacin-furosemide treated ones (n=5). The results indicate that the amikacin-furosemide treated produced a significant decrease in the density of spiral ganglion cells (especially in 1mg/g-1.5mg/g groups) as compared to normal rats(n=36±1.1 cells/10,000μM^2) (fig. 4).

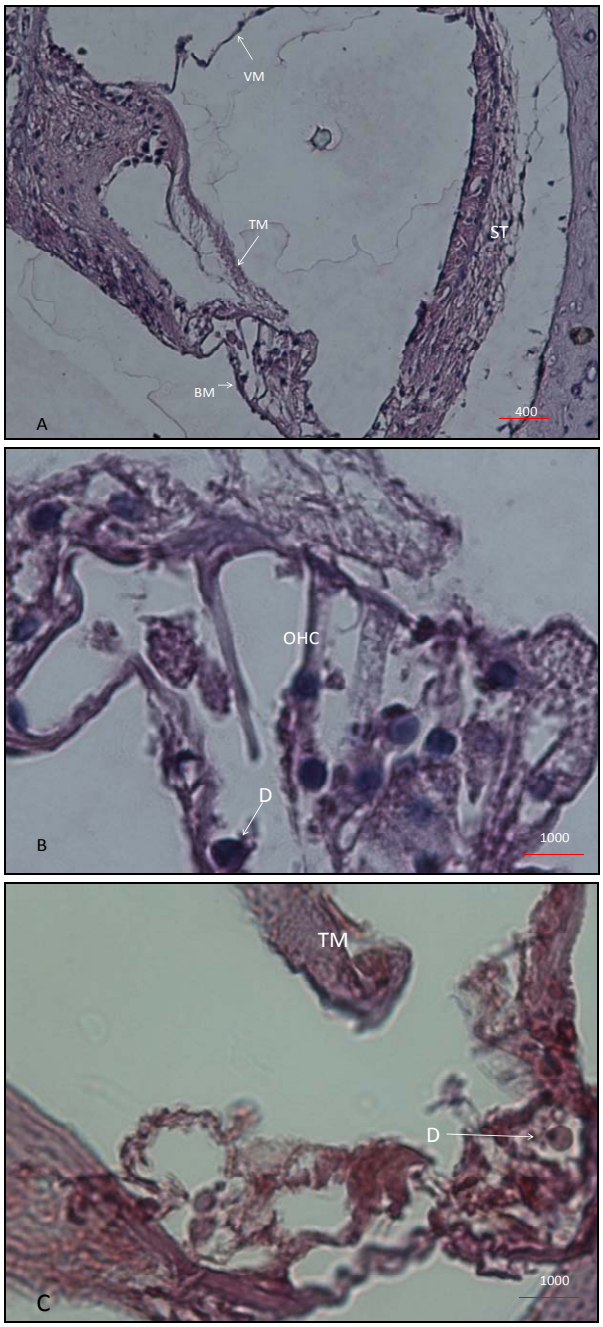

Fig. 3: Hair cell distortion after amikacinfurosemide injection. Control group (a, b) and amikacin-furosemide (1mg/g) group (c). In amikacinfuros-emide group cell organization was not seen and hair cells and most of supporting cells were destroyed. Basilar membrane (BM), Vestibular membrane (VM) Tectorial membrane (TM) Stria vascularis (ST) Dieter's cell or supporting cell (D).

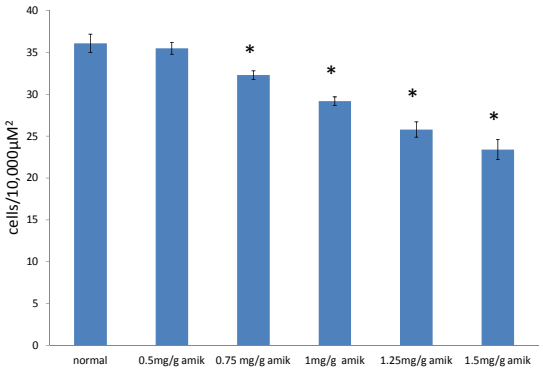

Fig. 4: Quantification of spiral ganglion cell counts in cross- section of the rat's cochlea. Amikacinfurosemide combin-ation produced a significant decrease in the density of spiral ganglion cells. Asterisk=significantly different from normal control ($p \leq 0.05$).

DISCUSSION

There are numerous chemical and physical methods for destruction of hearing cells but often ototoxic drugs are used in this respect. However, for the first time using a combination of an aminoglycoside ototoxic drug and a diuretic loop drug were prescribed by West and his colleagues and then this method was used in various other studies (Guthrie, 2008, Mitchell *et al*., 1997, Versnel *et al*., 2007, Webster and Webster, 1981). In the present study, it was tried to use the necessary dose of amikacin (aminoglycoside drug) and furosemide (loop diuretic), for rapid destruction of rats' hair cell. Then it was decided to monitor the effects of the combination of the above drugs on rats hearing using the assessment measure of DPOAE. In DPOAE gram, the responses were obtained at constant tones (L1=L2=65dB) and at different frequencies. In I/O functions the responses were obtained by increasing the primary tones from 50 dB to 70dB in 5dB steps. An emitted response was accepted if in the DP-gram the ratio of signal to noise amplitude was equal to 6dB or grater. In the later study groups, after amikacin-furosemide injection lower amplitude in all I/O Function and DP-grams were obtained compared to the control group. However, the most decrease in the amplitude was in the doses 1.25mg/g and 1.5mg/g which did not show significant difference with that of 1mg/g ($p \leq 0.05$); the only difference was that in doses higher than 1mg/g in which the mortality of the rats was more (10% in the group with 1.25mg/g amikacin and 15% in the group with 1.5mg/g amikacin). Moreover, the anatomic structures of the corti-organ was seriously damaged and except basilar membrane other structures were destroyed. When the dose used was 1mg/g, the hair cells in the apical turns were destroyed and in addition to the basal turns, other sections of the corti organ were still observable. Other scholars, too, (Hirose and Sato, 2011, Taylor *et al*., 2008) have obtained similar results to ours ; but with mice aged 18-21 days and a combined administration of kanamycin and furosemide. Besides, DPOAEs have been used in many studies as measures to assess the damages to the ear of mice and rats (Hatzopoulos *et al*., 2002, Khvoles *et al*., 1996, Pouyatos *et al*., 2002). Contrary to the findings of the present study, a few of researchers have stated that aminoglycosides can only destroy hair cells in the basal turns (Brummett, 1981, Guthrie, 2008, Henry *et al*., 1981, Wu *et al*., 2001).

In order to explain why the basal turn is damaged more than the apical turn due to this administration and why adding a diuretic drug to the former drug diffuse the damage to the apical turn there are various theories. One theory that proposed is that the concentration gradient of aminoglycoside favors its accumulation in the basal turn. With the addition of a loop diuretic the concentration of aminoglycoside in the perilymph and endolymph increase and could possibly diffuse further into apical turn. Others have proposed that loop diuretic facilitate entry of aminoglycoside into the endolymph while not affecting concentration of the drug in the perilymph (Dai and Steyger, 2008, Huy *et al*., 1986).

The difference between the findings of the present study and those of other researchers may be due to the mouse strain, age of the animal, type and dose of the drugs used. Briefly speaking, it is suggested that a combination of 1mg/g amikacin and furosemide can quickly destroy the hair cells of the rat's cochlea. This damage can be demonstrated through DPOAE. The present study has some limitations, the most important of which are the shortcomings of OAE machine in recording frequencies higher than 8KHz.

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