# Estudo experimental anatômico-funcional da cocleotoxicidade da gentamicina com doses habituais para recém-nascidos****

## Experimental morphological and functional study of gentamicin cochleotoxicity using the regular dose given to neonates

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**Abstract**
Background: gentamicin is an antibiotic that acts in Gram-negative bacilli infections, having as a side effect ototoxicity. Ototoxicity is an iatrogenic disturb provoked by drugs that modify the internal ear, affecting the cochlear and/or vestibular system and causing alterations in two important functions: equilibrium and audition. The main pediatric groups that receive aminoglicosides antibiotics are newborns who present serious infections in Neonate intensive care units. Aim: to verify the occurrence of external cilliary cells (ECC) caused by gentamicin with single dose schemas of 4mg/kg/day and 2,5mg/kg/day every 12 hours, through a morphological - scanning electronic microscopy (SEM) and functional - distortion product otoacoustic emissions (DPOAE) experimental study. Method: 26 albino guinea pigs were evaluated through DPOAE pre and post gentamicin treatment. The guinea pigs were sacrificed in the programmed time after the intramuscular administration of the drugs for anatomic analysis using MEV. Results: the evaluation of the functional state of the ECC indicated preservation of the DPOAE in all of the guinea pigs. The SEM results, after being photographed were analyzed in terms of the number of the ECC in the cochlear basal turn in a specific photographic field. Conclusion: lesions or alterations in the functioning of the ECC of albino guinea-pigs after the use of 4mg/Kg/day and 2,5mg/Kg/day every 12 hours for a period 10 and 14 days were not observed.
**Key Words:** Gentamicin; Electronic Microscopy; Ototoxicity.

**Resumo**
Tema: a gentamicina é um antibiótico que atua nas infecções causadas por bacilos Gram-negativos. Seu efeito colateral mais importante é a ototoxicidade. As ototoxicoses são afecções iatrogênicas provocadas por fármacos que alteram a orelha interna, podendo afetar o sistema coclear e/ou vestibular, alterando duas funções importantes: a audição e o equilíbrio. Os principais grupos pediátricos que recebem antibióticos aminoglicosídeos são recém-nascidos com infecções graves na UTI neonatal. Objetivos: verificar a ocorrência de lesão às células ciliadas externas (CCE) pela gentamicina com os esquemas de dose única de 4mg/Kg/dia e de 2,5mg/Kg/dia a cada 12 horas, por meio de um estudo anatômico por microscopia eletrônica de varredura (MEV) e estudo funcional através das emissões otoacústicas por produto de distorção (OEAPD). Forma de estudo experimental. Método: foram avaliadas 26 cobaias albinas através das EOAPD pré e pós-tratamento com gentamicina. Para a avaliação anatômica por MEV, as cobaias foram sacrificadas em tempo programado após a administração das drogas via intramuscular. Resultados: a avaliação do estado funcional das CCE mostrou preservação das OEAPD em todas as cobaias. Os resultados da MEV, depois de fotografados foram analisados através da contagem do número de CCE da espira basal da cóclea em determinado campo fotográfico. Conclusão: não foram observadas lesões ou alterações no funcionamento das células ciliadas externas mediante a dosagem aplicada em cobaias albinas, de 4mg/Kg/dia (dose única) e 2,5mg/Kg/dia a cada 12 horas, utilizadas por 10 e 14 dias.
**Palavras-Chave:** Gentamicina; Microscopia Eletrônica de Varredura; Ototoxicidade.

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## Introduction

Hearing loss has been considered a severely disabling disorder in virtue of the role of hearing in human communication. One of the causes of hearing loss is the use of ototoxic drugs1.

Gentamicin is a bactericidal antibiotic that acts mainly on infections caused by Gram-negative bacilli. It is the first aminoglycoside of choice for its low cost and for its action on most of resistant aerobic Gram-negative bacilli 2. The most important side effect of gentamicin is ototoxicity, which occurs in 2% of users representing potential severity for auditory and vestibular functions damage 3.

Ototoxicosis is an iatrogenic disease caused by drugs that alter the inner ear. It is diagnosed when there is sensorineural hearing loss greater than 25dB in one or more frequencies on the pure tone audiometry between 250 and 8000 Hz or when vestibular manifestations, such as dizziness or vertigo, occurs 4-5.

The main pediatric group that receives aminoglycoside antibiotics is the one of newborns with severe infections treated in neonatal intensive care units (ICU) 5.

Bilateral hearing loss presents high incidence, occurring in three of every 1,000 live births, and from two to four for every 100 newborns from neonatal ICU 6.

Considering that the hearing impairment caused by gentamicin use causes irreversible damage to the outer hair cells (OHC), it is important to monitor hearing in patients users of this drug in order to determine the initiation and progression of ototoxicity with the possibility of preventing or reducing its severity, and, when installed the hearing loss, to enable hearing rehabilitation with hearing aid adaptation 1, 4.7.

Experimental studies on animals are important to the development of science and new drugs, to improve the understanding of pathophysiological disease mechanisms, to undertake therapeutic trials with new drugs and to study biological markers and techniques to assess prospects for application in humans 8. In the case of gentamicin, studies would enable the acquisition of comprehensive knowledge concerning the possible ototoxic effects on specific aspects obtained by scanning electron microscopy (SEM) OHC images, as well as an analysis of their functional status as measured by distortion product otoacoustic emissions (DPOAE), providing data that may explain the clinical findings of humans exposed to treatment with this antibiotic 8-9-10.

Otoacoustic emissions (OAE), detected on the external auditory canal measure the biomechanical energy feedback in OHC contraction, which amplifies the peak of the traveling wave on the basilar membrane. The OAE have clinical application in assessing hearing integrity - reflecting the OHC functionality - being the only objective and non-invasive means of cochlear investigation11.

The use of gentamicin in neonatal ICU has been recommended in schemes of single dose of 4 mg/kg/day to replace the traditional scheme of 2.5 mg/kg/day every 12 hours 12-13.

The verification of possible gentamicin ototoxic effects in experimental animal models assumes effective importance, as it has been adopted and studied by other authors 9-10, 14.

This study is justified by the importance of research that contributes to a better understanding of this theme allowing health professionals to adopt procedures that aims to maintain hearing integrity for a better quality of life.

The purpose of this study was to verify the occurrence of OHC injury caused by gentamicin with single dose 4mg/Kg/day and 2.5 mg/kg/day every 12 hours schemes, thought anatomical study - via SEM - and functional study - via DPOAE -, testing the following hypotheses:

1. There are anatomical or functional lesions caused by intramuscular application of gentamicin to the cochlea OHC of albino guinea pigs exposed to a dose of 4mg/Kg/day (single dose) during 10 and 14 days.
2. There are anatomical or functional lesions caused by intramuscular application of gentamicin to the cochlea OHC of albino guinea pigs exposed to a dose of 2.5 mg/kg/day every 12 hours during 10 and 14 days.

## Method

Albino guinea pigs with four months of age were chosen as experimental animals on the present study. The reasons for this selection were the easy handling, and the ease of cochlear dissection, manipulation for infusion routes of anesthetic drugs and the intramuscular infusion of experimentation drug 15.

The guidelines on the guide for care and laboratory use of animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, DC. (1996) were followed 16. This study was approved by the Ethics

Committee in Animal Experimentation (CETEA) of FMRP-USP under the protocol 073/2007. The animals were selected through the research of Preyer acoustic reflex and kept in the vivarium of the Experimental Surgery of the FMRP Surgery Department - USP 17.

Twenty-six female albino guinea pigs (Cavia porcellus) weighting between 400 and 500 grams were used. The guinea pigs were subjected to a hearing screening by EAOPD in a sound proof booth and under anesthesia with Ketamine (65 mg / kg). Those which presented present DPOAE were considered for the experiment.

The guinea pigs were divided into five study groups - Group 1: the "Sham" - 2 animals - 4 cochleae - saline for 14 days; Group 2: 6 animals - 12 cochleae - 4mg/Kg/day of gentamicin in a single dose for 10 days; Group 3: 6 animals - 12 cochleae - 4mg/kg/day of gentamicin in a single dose for 14 days; Group 4: 6 animals - 12 cochleae - 2.5 mg/kg/day of gentamicin every 12 hours for 10 days;. Group 5: 6 animals - 12 cochleae - 2.5 mg/kg/day of gentamicin each 12 hours for 14 days.

The DPOAE test was performed before and after treatment - that is moments before the animals were sacrificed - followed by the frequency relationship 2F1 - F2 with ratio F1: F2 = 1.22, resolution 2 points per octave. The equipment used was an ILO 92 CAE System Otodynamics LTD.

The DPOAE were considered as present and absent from 1.5 kHz because the dimensions of the external auditory canal of guinea pig difficult the detection of OAEs below this frequency, obtaining responses that match noise responses.

The high frequency OAE were considered most important for assessing the functionality of the OHC in the cochlea basal turn. For SEM anatomical assessment, the guinea pigs were sacrificed by decapitation in scheduled time after the intramuscularly administration of the drug.

The guinea pigs were anesthetized with ketamine hydrochloride (Ketamine ® 50mg/ml, Laboratory Cristália,) at 40mg/kg and xylazine (Dopaser ® 20mg / ml, Cali Laboratory in Brazil,) at 10mg/kg.

Their temporal bones containing the tympanic bullas were quickly removed and opened for cochlea exposure. A pair of scissors was used for dissection, which was placed in the cervical spine after cutting the skull in the median longitudinal direction, extending to the ears. Then, using the hands and having the external auditory canal as a guide, the bulla was localized with the thumbs and separated from other structures.

The bulla was opened by holding it with one hand and, with a haemostatic clamp; an opening on the posterior air sinus (mastoid) was made, followed by its projection to the top of the rock. Then, positioning the clamp in the external auditory canal and anterior sinus air, in a single movement, all bone part of the leaflet was broken, exposing the cochlea.

The part was fixed by injecting 3% glutaraldehyde through an opening in the apex of the cochlea and the round window, and remained immersed for 4 hours at 4 ° C. It was then washed several times in 0.1 M phosphate buffer solution and subjected to micro dissection for exposure of cochlear turns.

Subsequently, the cochleae were re-fixed in solution of osmium tetroxide at 1% in 0.1 M phosphate buffer for 2 hours at 4 ° C, and washed in 0.1 M phosphate buffer and pH of 7.3.

After dehydration of the structures through ethanol immersions at increasing concentrations of 50, 70, 90 and 95% for about 10 minutes each, the 100% ethanol was used in three baths of 20 minutes each and, on the last immersion, the structures were immersed at room temperature for 12 hours.

The water still present in the material after the dehydration was removed by the process of drying, carried out by the critical point method with carbon dioxide (CO2) fluid. The sample contained in a suitable container was transferred to the drying chamber of the critical point apparatus (BAL-TEC CPD-030 - Critical Point Dryer) where, through successive baths of liquid CO2 at 4 ° C, the ethanol was removed.

Following, the material was subjected to an increase in temperature to 40 ° C in order to move the CO2 from liquid to gaseous state - which happens at 31 ° C.

With the dry material the assembly of the cochleae in metal stubs with conductive carbon paste was conducted.

The biological materials are poor electricity conductors and cannot be observed through SEM. For such, they need to be transformed into electrically conductive materials. A thin layer of gold applied to the evaporator (BAL-TEC SCD 050 - sputter Coater) was used to cover this material.

The microscope Jeol Scanning Electron Microscope - KAL 5200 was used on the visualization of the material.

The results of SEM, after photographed, were analyzed by counting the number of OHC of the

cochlear basal turn on a determined area.

## Results

The apex was disregarded from the anatomical assessment because it is a cochlea region responsible for low frequency sounds and because it shows a pattern of breakdown even in guinea pigs that were not subject to lesion by ototoxic drugs.

The assessment of OHC functional status studied through DPOAE showed preservation of the response in all animals of all groups.

Regarding the dose of gentamicin used, 4mg/Kg/day for 10 consecutive days (group 2), we observed on the SEM the presence of OHC in the basal turn, turn 2 (E2), helix 3 (E3) of the cochlea in all segments (Figure 1).

In the group treated with 4mg/Kg/day for 14 days (group 3), all OHC were present in all segments and in all turns (Figure 2).

In the group treated with gentamicin 2.5 mg/kg/day every 12 hours for 10 days, we observed the presence of all OHC on basal turn without distortion of the cilia. (Figure 3A). The group treated with gentamicin 2.5 mg/kg/day every 12 hours, showed no change in any of the OHC turns after 14 days treatment (Figure 3 B).

## Discussion

The rationale for studying the effects of gentamicin in OHC of albino guinea pigs arose from other studies that affirmed that amongst the aminoglycoside antibiotics, gentamicin is the most used one in newborns for its actions against many species of bacteria 3, 5.18.

The SEM is a method widely used in ototoxicity studies because it allows a detailed study of the normal and altered Corti organ at the inner ear level 19.

In the acute stage, ototoxicity requires attention to the occurrence of cochlear injury that leads to irreversible neurosensorial hearing loss l5, 20. However, the absence of ototoxic effects, such as loss of cochlear function, was observed on the studied gentamicin doses during 10 to 14 days.

In the present study, the use of gentamicin in dosages used in newborn ICU extrapolated to guinea pigs was considered to make possible to assess the security of employment of this medicine, building up as reference for the possible ototoxic effects on therapeutic use and action of drugs in humans, especially in newborns 21.

FIGURE 1. Basal turn of cochlea of a guinea pig treated with gentamicin at 4 mg/kg/day for 10 days - after treatment.

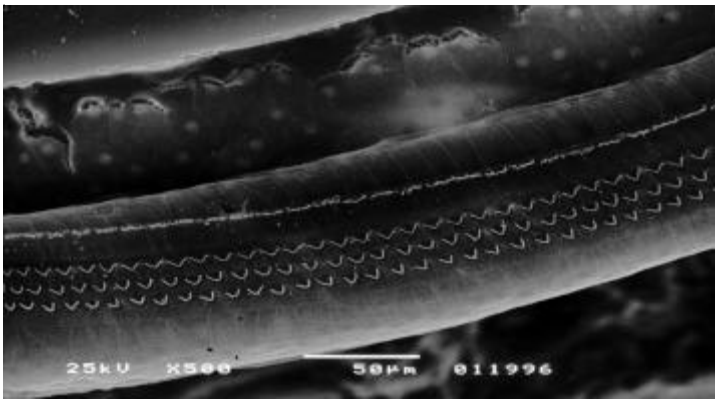



FIGURE 2. Basal turn of cochlea of guinea pigs treated with gentamicin at 4 mg/kg/day for 14 days with presence of OHC in all rows.

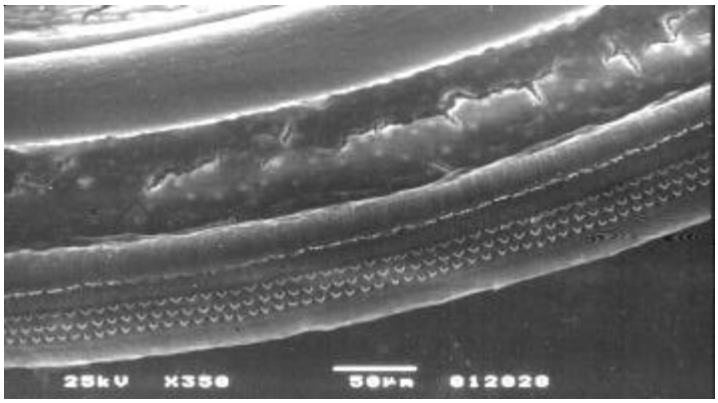
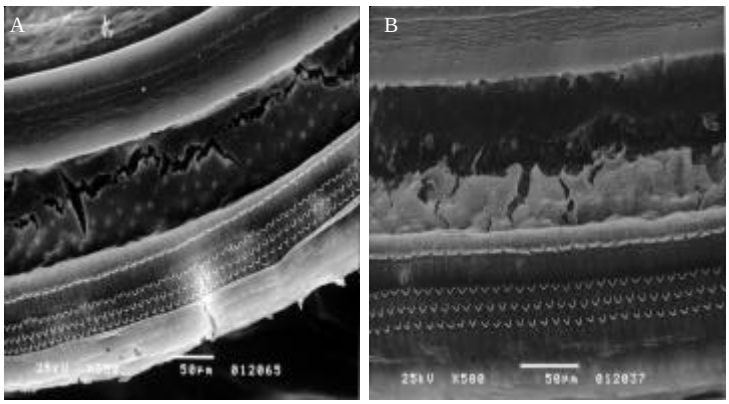



FIGURE 3. (A) SEM of a cochlea of one of the guinea pigs treated with gentamicin 2.5mg/kg/day every 12 hours for 10 consecutive days. (B) basal turn of the cochlea of the group treated with gentamicin 2.5 mg/kg/day every 12 hours for 14 days.



However, when studying the ototoxic effects in animals with experimental techniques to assess prospects for application in humans, it is possible to advance in significant findings, particularly microscopic, not considering other variables of the use of different doses 2-3-8.

The association with other potentially ototoxic drugs, especially loop diuretics (etacrynic acid) potentiates the aminoglycosides ototoxic effects 22-23. It is believed that aminoglycosides act on inner ear cell membranes by increasing its permeability and facilitating the entry and toxicity of these diuretics. In our study, the fact of not having used other drugs associated to gentamicin could justify the absence of OHC lesions.

Inner ear lesions occur predominantly in the basal turn, following to the apical one with greater commitment of the first OHC row, with subsequent extension to inner hair cells (IHC), stria vascularis, spiral ligament and Reissner membrane 5-24. In this experiment, although using aminoglycosides, we found no injury in any of the turns or rows of OHC as observed by the SEM.

Aminoglycosides used in doses above 100mg/kg alter the permeability of the OHC membrane, affecting the synthesis of proteins, DNA, RNA, mitochondrial metabolism, ion transport and synthesis of prostaglandins, mucopolysaccharides, lipids and ganglioside 25.

In an experimental study, high doses of gentamicin 120mg/kg/day applied to albino guinea pigs resulted in hearing losses from 10 to 40dB at 3kHz and from 50 to 70dB at 18kHz four weeks after the drug administration 14. Through SEM, a total loss of OHC in the basal cochlea with preservation of the IHC was detected.

A low dose of gentamicin of 10 mg/kg/day for thirty consecutive days maintains the anatomical structure of the OHC and IHC and their functionality without changing the DPOAE 9-10. There was no histological or functional damage, as measured by DPOAE, caused by the use of gentamicin in the period from 10 to 14 days at doses of 4mg/kg/day and 2.5 mg/kg/day every 12 hours, which is in agreement with the scientific findings of other authors for low doses of gentamicin.

The verification of auditory function in the presence of antibiotics is in line with the existing concern about hearing monitoring as part of the treatment protocol for individuals exposed to ototoxic agents 7. This findings corroborate to studies that point hearing loss as a severely disabling disorder and that the use of ototoxic drugs can cause hearing loss, in accordance to the rationale for this study. In addition, when establishing the security level on the therapeutic use of gentamicin the sensorineural hearing loss may be prevented 1-4.

Confronting the results obtained with SEM to the findings of functional OAE, conclusive data on the absence of cellular lesion on peripheral auditory level were obtained.

Such findings may explain the absence of OHC injury resulting from doses of gentamicin used in neonatal ICU (4 to 5 mg/kg/day for 10 to 14 consecutive days) disregarding other risk factors for neonatal deafness and its concomitant use with other potentially ototoxic drugs.

## Conclusion

No lesions or alterations of the cochlea OHC of albino guinea pigs exposed to doses of 4mg/Kg/day (single dose) and 2.5 mg/kg/day every 12 hours of intramuscular gentamicin used during 10 and 14 days were observed.

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