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Optimal dose of Kanamycin for protection from noise induced hearing loss

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OPTIMAL DOSE OF KANAMYCIN FOR PROTECTION FROM NOISE INDUCED HEARING LOSS

by

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Abstract: Young CBA/J mice were injected with kanamycin under varying schedules then exposed to noise in order to determine the boundary conditions for cochlear protection against noise.

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ABBREVIATIONS

ABR	Auditory Brainstem Response
dB (SPL)	decibels (sound pressure level)
Hz	hertz
i.p.	intraperitoneal
KA	kanamycin
kHz	kilohertz
NIHL	noise-induced hearing loss
NIPTS	noise-induced permanent threshold shift
OHC	outer hair cells
SNHL	sensorineural hearing loss

Introduction

Aminoglycosides are antibiotic drugs used in the treatment of diseases caused by Gram-negative bacteria and tuberculosis. These cost-effective drugs, which include gentamicin, tobramycin, streptomycin, neomycin, and kanamycin are currently applied clinically most frequently in underdeveloped countries, and in the most extreme cases of a life-threatening illness because of their potential ototoxicity. Aminoglycosides are known for causing a loss in hearing sensitivity, vestibular function, or nephrotoxicity to those who are administered the drug (Guthrie, 2008). In the inner ear, these drugs target cochlear outer hair cells (OHCs) and type I vestibular hair cells (Rybak &Whitworth, 2005). This results in a bilateral sensory hearing loss, mostly affecting high frequencies due to damage of the hair cells in the basal turn of the cochlea (Guthrie, 2008).

Mouse models in ototoxin research

To gain a better understanding of the cellular mechanisms of aminoglycoside ototoxicity, it is helpful to study their affects in animal models. Mice, in particular, are increasingly used as a model of human hearing. The mouse cochlea is anatomically and physiologically similar to that of a human. Mice also develop and age quickly, making it convenient to study critical and sensitive periods (Henry & McGinn, 1992). According to Henry (1981), kanamycin's effects are similar in mice and humans with the majority of hair cell loss being in the basal turn of the cochlea. The age of the animal, however, greatly influences sensitivity to ototoxic agents. In an attempt to establish a critical period in mice for kanamycin ototoxicity, Saunders and Chen (1983) injected C57BL/6 inbred mice with 400 mg/kg, intraperitoneally (i.p.), once daily for four

days in groups aged 6-9, 10-13, or 15-18 days, then sacrificed the mice 15 days post-injection for morphologic analysis. Animals aged 10 to 13 days showed complete loss of OHCs throughout the cochlea while mice age 6-9 days only sustained damage to the OHCs in the basal turn.

Different strains of mice have shown different responses to kanamycin treatments. The CBA/J mouse, in particular, has been shown to have greater threshold shifts after treated with kanamycin when compared to other strains, such as the C57BL/6 and NKCC1$^{+/-}$ (Chu et al., 2006). Mice in this study were aged to four weeks in an effort to establish a mouse model of ototoxicity past the "sensitive" period in developing mice. They received daily kanamycin injections (700 mg/kg) subcutaneously for 14 consecutive days. Thresholds continued to worsen 3 weeks post treatment (Chu et al., 2006). Wu and colleagues (2001) used the same dosage of kanamycin as Chen and Saunders (1983) and injected different strains of mice twice daily for 10 consecutive days. They determined that BALBs are affected more sensitive to ototoxicity than the other strains of mice. It should be noted that a greater dosage was necessary in these animals because they were considered adult animals. A kanamycin dose of 400 mg/kg i.p. daily for 10 days was shown to produce threshold shift of 60-70 dB in developing mice (Sha et. al, 2001).

Adult animals are able to tolerate higher dosages of kanamycin without experiencing the same ototoxic effects that occur in their younger counterparts. In a previous study, Henry and colleagues (1981) compared the affects of kanamycin in mice aged 13, 60, and 380 days. They received two daily injections of kanamycin (500 mg/kg, i.p.) for two weeks. Auditory function was assessed two days post treatment with electrocochleograms. Results showed that the pre-weanling mice had dramatic threshold shifts across all frequencies, while the older two groups of mice only had shifts in the mid- and high frequencies (Henry et al., 1981). Histological analysis

revealed an extensive OHC loss across the cochlea, while older mice only showed loss of OHCs in the basal turn. The histological findings supported the electrocochleographic changes after kanamycin.

Early window of heightened noise vulnerability

The pattern of cochlear damage caused by noise-induced hearing loss is also quite similar in mice and humans. Cochlear noise injury is manifested as sensorineural hearing loss (SNHL), mostly affecting the higher frequencies. Like ototoxicity, the consequences of noise exposure are age dependent. An early study by Henry (1983) looked at the susceptibility of CBA/J mice to NIHL throughout their life span. Mice were exposed to 124, 114, or 104 dB SPL of octave-band (12 to 24 kHz) noise for 5 minutes. It was concluded that susceptibility in mice is greatest in a developmental window extending from 16-90 days of age. Ohlemiller, Wright, and Heidbreder (2000) applied a noise dose response paradigm to young and old CBA/CaJ, C57BL/6J and BALB/J mice. In that study, young (1-2 months old) and old (5-7 months old) mice were exposed to 110 dB of 4 to 45 kHz noise for varying durations. Results confirmed that the younger mice are indeed more susceptible to noise. Young CBA/CaJ mice, for example, had a NIPTS after a mere 3.42 minutes of noise exposure, while the older CBA/CaJs required roughly 63 minutes to create the same probability of permanent threshold shifts.

Interactions of simultaneous aminoglycosides and noise

Since the cellular targets of noise and ototoxic drugs are largely the same, it might be expected that their combination would exacerbate hearing loss. This has been demonstrated in

both animals and humans (Dayal et al., 1971; Quante, 1973; Dayal & Barek, 1975; Marques et al., 1975; Hawkins et al., 1975; Ryan & Bone, 1978; Brown et al., 1980 (as cited in Humes, 1984); Brummett, Fox, & Kempton, 1992). Gannon, Tso, and Chung (1979) examined the effect that impulse noise had on guinea pigs that had received a minimal dose of kanamycin (15 mg/kg vs. 50 mg/kg) and concluded that even when kanamycin alone does not cause any noticeable damage to hair cells, it can increase hair cells susceptibility to noise injury. Brummett and colleagues (Brummett, Fox, & Kempton, 1992) injected guinea pigs with subclinical doses of kanamycin and exposed them to 45, 75, 95, or 115 dBA of noise for 7 days. Again it was concluded that subclinical doses of kanamycin paired even with normally harmless levels of noise can cause permanent cochlear damage. Most of these studies used guinea pigs or chinchillas as the animal model.

Recent experiments in young mice, however, have uncovered a protective effect of sub-clinical doses of kanamycin (300 mg/kg) against NIHL. In a recent study CBA/J mice 20 days old were injected every 12 hours for 10 consecutive days, then on day 11 were noise exposed to 30 s of 110 dB SPL broad band noise. While ABRs conducted 10 days post exposure showed substantial threshold shifts (~50 dB) in saline-treated control mice, the experimental mice (both noise and kanamycin) showed statistically normal thresholds (Baum, 2008).

Purpose of the present study

This novel finding created the need to determine the minimal amount of kanamycin that can be administered and still protect from noise injury. Since there is little to no concurring evidence of an ototoxic drug being used in protection from noise-injury, there is a great need for

understanding this phenomenon. The boundaries of this particular event must be defined. How few kanamycin doses can be given and still prevent NIHL? The purpose of this study was to find just how infrequent injections of kanamycin can be administered and protection from noise exposure is still evident. This was done by injecting kanamycin in young CBA/J mice at varying intervals (once daily, once every other day, and once every third day) for a span of 10 days.

MATERIALS AND METHODS

Animals

A total of 24 CBA/J mice of either gender were used in this study. All mice were housed in the Mechanisms of Cochlear Injury Laboratory at Washington University School of Medicine for 10 days while receiving treatment, and for the remainder of time were housed in the Central Institute for the Deaf Animal Colony. All procedures were approved by the Animal Studies Committee at Washington University School of Medicine. Animals were inspected for signs of otitis media. Any animals with middle ear infection were excluded from the data analysis.

Kanamycin

Mice were assigned to one of seven different treatment groups: kanamycin daily only, saline daily plus noise, kanamycin daily plus noise, kanamycin daily plus longer duration of noise, saline daily plus longer duration of noise, kanamycin every other day plus noise, and kanamycin every third day plus noise (see Table 1 below). Mice that received kanamycin were injected subcutaneously with a 0.9% commercial saline solution containing 63.93 mg/ml of

kanamycin to yield 300 mg/kg per dose. Injections were given between the hours of 7-9 AM. No mice died as result of the drug treatment.

Treatment Group	Number of Animals
Kanamycin 1/day (no noise)	2
Saline 1/day + 30 s noise	4
Kanamycin 1/day + 30 s noise	6
Kanamycin every other day + 30 s noise	6
Kanamycin every 3rd day + 30 s noise	3
Saline 1/day + 1.88 min noise	1
Kanamycin 1/day + 1.88 min noise	2

Table 1: Number of animals per treatment group

Noise Exposure

The majority of the mice assigned to a noise treatment group were exposed to 30 seconds of 110 dB SPL broadband noise (4-45 kHz). This level and duration is the result of early noise-dosing results in the previous study. In keeping with prior studies, the initial hypothesis in the foundational study Baum (2008) was that subclinical kanamycin would act synergistically to produce substantial injury. Thus the goal was to identify noise exposure durations (while fixing overall intensity) that produced little or no NIPTS. Pilot exposures in young CBA/J, however, showed that exposures as brief as 0.5 minutes resulted in 100% of the mice exhibiting severe hearing loss. An exposure duration of 4.0 min was originally selected for that study based upon research conducted by Ohlemiller, Wright and Heidbreder (2000). That study showed that 3.42

minutes was sufficient enough in causing a NIPTS in 6 week old CBA/CaJ mice. Because of the dramatic hearing loss created in pilot experiments, it was determined that a noise-dose response experiment needed to be conduced. Rice, Gagnon, and Ohlemiller (2009) compared 6 week-old CBA/J and CBA/CaJ mice to find the threshold of duration in which NIPTS would be seen for each strain separately. The conclusions of this study were that 0.90 minutes of noise was sufficient enough to cause 90% of the young CBA/Js to have a NIPTS, while 4.05 minutes was the length of duration that caused a similar loss in the CBA/CaJs. It was decided that for this study, 30 seconds of noise would be reliably produce a NIPTS in young CBA/J mice. It should be noted that this duration of noise produces more hearing loss in 30 day old CBA/J mice than suggested by Figure 1, presumably because the extent of noise vulnerability decreases between 30 days and 6 wks of age.

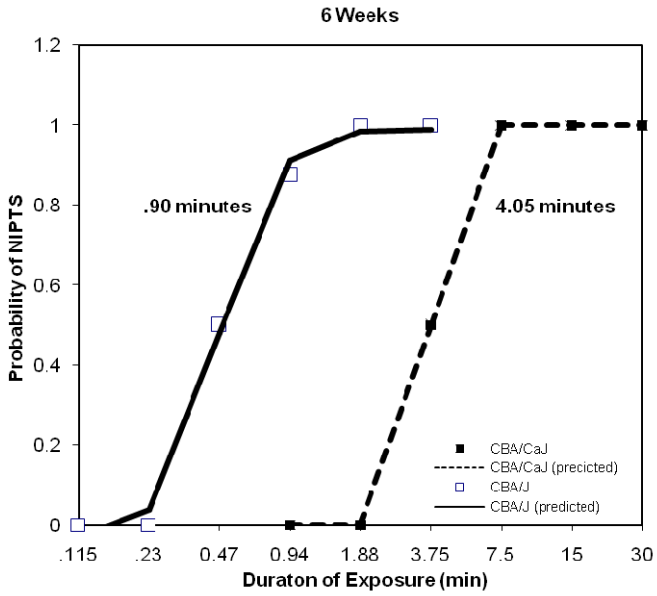

Figure 1: The proportion of young CBA/J and CBA/CaJ mice meeting the criterion for NIPTS. The minimum NIPTS exposure for CBA/J (0.9 min) was clearly different from that in CBA/CaJ (4.05 min)

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Mice were placed in groups of two in a 21x21x11 cm wire cage surrounded by four speakers set at 0, 90, 180, and 360 degrees azimuth. This apparatus was housed within a single-walled, foam-treated sound booth. In order to ensure uniform exposure, the cage was rotated at

0.013 Hz throughout the exposure duration. The noise was generated using General Radio 1310 generators and filtered with Krohn-Hite 3550 filters. Food and water were not accessible during the time of exposure. Mice in a noise treatment group were exposed 15 minutes after final injections. The rationale for this was previous evidence indicating that peak serum levels are reached approximately 15 minutes after administering kanamycin (cited in Wu et al., 2001).

ABR Recordings

Auditory brainstem recordings were obtained ten days post treatment, or approximately 40 days post gestation. Animals in the kanamycin only group (no noise exposure) underwent ABR testing at 15 minutes post injection on day 10 (that is, at the time when noise exposure would occur), as well as 40 days post gestation. Animals were anesthetized using a ketamine and xylazine solution (80/15 mg/kg, i.p). Subdermal needle electrodes were placed in the vertex, back, and behind the right pinna. The animal's body temperature was kept at a constant 37.0 °C through the use of an isothermal pad and monitored via rectal probe. Mice were positioned with their head's placed 7 cm from the speaker. The base of the left ear was clamped with a small clip so that only the right side was stimulated. Stimuli were presented 1000 times through the right speaker in 5 millisecond tonebursts at the following frequencies: 5, 10, 20, 28.3, and 40 kHz. Stimulus presentation and data acquisition utilized Tucker Davis Technologies System II hardware and software (BioSig 32). Thresholds were defined as the lowest sound level (varied in 5 dB steps) that produced Wave I of the ABR.

After ABR thresholds were obtained for each frequency, mice were overdosed using sodium pentobarbital (60 mg/kg i.p.) and transcardially perfused with 2.0% paraformaldehide

solution. The cochleae were harvested and placed in fixative. Cochleae were later decalcified using an EDTA sodium solution, stained with Osmium, dehydrated using Acetone, and finally embedded in Epon-Araldite.

RESULTS

A two-way analysis of variance (ANOVA) was performed to in order to determine if there was any statistical significance in threshold differences between groups. Among the seven different treatment groups, only two were found to have statistically significant differences in thresholds. There was a clear difference between the ABR thresholds in animals who received kanamycin daily and 30 seconds of noise and those animals that received the same dosage of saline daily and noise (Figure 2). Animals that received saline daily for 10 days had thresholds that were 30 to 40 dB greater than those animals in the kanamycin daily group.

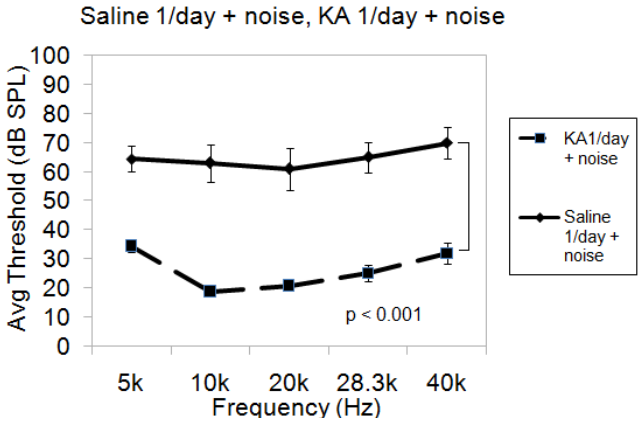

Figure 2: Average thresholds for saline 1/day + 30 s noise and kanamycin 1/day + 30 s noise

Animals who received kanamycin every other day also showed statistically different thresholds when compared to the saline daily + noise group (Figure 3). Majority of those within the kanamycin every other day group saw at least partial, if not complete, protection from 30 seconds of noise. There was little variation in between thresholds at all frequencies in all of the groups that received doses of kanamycin. Animals that were injected daily with kanamycin and not exposed to noise showed no threshold shifts across all tested frequencies when compared to previous thresholds obtained at 30 days post-gestation directly following drug treatment.

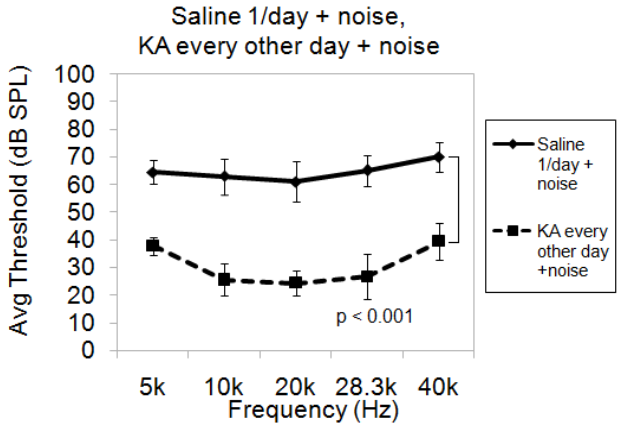

Figure 3: Saline 1/day + 30 s noise, KA every other day + 30 s noise

There were two animals who received daily injections of kanamycin for 10 days then were exposed to noise for 1.88 min. One of the animals showed complete protection from even this duration of noise, while the other had substantially elevated thresholds. A larger number would help determine the effectiveness of protection that kanamycin has for longer durations of noise exposures.

DISCUSSION

The purpose of this study was to determine the minimum applications of kanamycin that can protect young CBA/J mice from NIHL. The findings of this study help confirm surprising

interactions previously reported. Baum (2008) concluded that mice receiving twice daily doses of kanamycin plus noise exposure showed no significant average shifts in threshold compared to control groups. The present results argue for the existence of a robust and easily established 'protected state', such that even injections of kanamycin given every third day for ten days are sufficient to protect from NIHL.

Subclinical kanamycin as a preconditioning stressor

Threshold preservation against noise by kanamycin may represent a form of preconditioning. Preconditioning, as defined by Gagnon and colleagues (2007), refers to "the ability of a non-damaging or minimally damaging stressor to confer protection against the effects of a later and more injurious stressor". These investigators found that hypoxia prevents the exacerbation of NIHL in CBA mice when exposed to 90 minutes of broadband noise. Yoshida and colleagues (1999) determined that heat stress preconditioning can also protect mice from NIHL. An increase in heat shock proteins within the CBA/CaJ mouse cochlea created protection from 100 dB of octave band noise. Other cellular processes that may underlie preconditioning are increased levels of glucocorticoid stress hormones and improved blood flow (Wang & Liberman, 2002).

It is possible that the stress of handling mice alone is somewhat protective against noise exposure. That is why one treatment group received saline daily prior to noise exposure. Since the mice in that group had significant threshold shifts this factor could be ruled out in our results. Also, we included a treatment group that received only kanamycin daily with no noise exposure. Animals in that group showed no threshold shifts due to kanamycin alone, therefore this dose of

kanamycin does not cause any hearing loss after 10 consecutive days of administration. More recent findings have shown that a single dose of kanamycin, when administered 15 minutes prior to noise, is not enough to protect from NIHL (Baum, 2008). These findings imply that the mere presence of kanamycin is not adequate to mediate protection. Chronic treatment with kanamycin may be required to up-regulate protective mechanisms in the cochlea that prevent NIHL. The minimum number of kanamycin injections that is protective was not addressed in this present study.

There is little support of the protective nature of aminoglycosides in preventing NIHL in the literature. This could be due to the fact that most of the studies that looked at the synergistic effects between kanamycin and noise were done with adult animals. The protection may be seen only in the mouse and not in guinea pigs or chinchillas, which were the model of animals utilized in majority previous studies. Doses of kanamycin also varied widely among these studies as well.

Clinical implications and future experiments

This study confirms the previous finding that kanamycin, when paired with noise in young CBA/J mice, can protect against NIHL Identification of 'boundary conditions' for establishing the postulated protected state is important, as it should promote the discovery of the underlying cellular mechanisms. Surprisingly, the present experiments actually produced little evidence of limits to the protection afforded by kanamycin. More experimental conditions must therefore be tested. Among the remaining unknowns is the question of *how much noise* can be protected against. One group in the present study indicated that protection by kanamycin is not limited to 30 s exposures. However, this group contained two animals, which is not a large

enough sample to determine the effectiveness of kanamycin protection.

Further studies should address the generality of the present results both with regard to mouse strain and type of ototoxin that can elicit protection. That young CBA/J mice are somehow unique in this regard cannot be ruled out, given the evidence presented here that these mice are phenomenally vulnerable to noise. Investigations of this type may help establish whether there are specific genes that determine whether kanamycin protects–or worsens-noise injury in any individual.

It is truly paradoxical that a compound widely agreed to be highly toxic the inner ear as kanamycin can be protective. The findings of this study bring about the possibility of using pharmaceuticals in the prevention of auditory damage. There is a great need to find protective agents for those individuals who are exposed to recreational and occupational noise. It is important to find the underlying protective mechanisms that kanamycin induces to prevent NIHL. Further more, if the mechanisms of protection induced by kanamycin are discovered, alternative ways of up-regulating these pathways could be used to prevent cochlear damage and hearing loss from noise.

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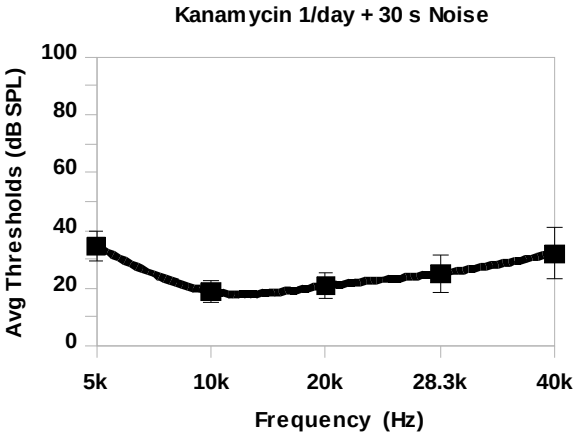
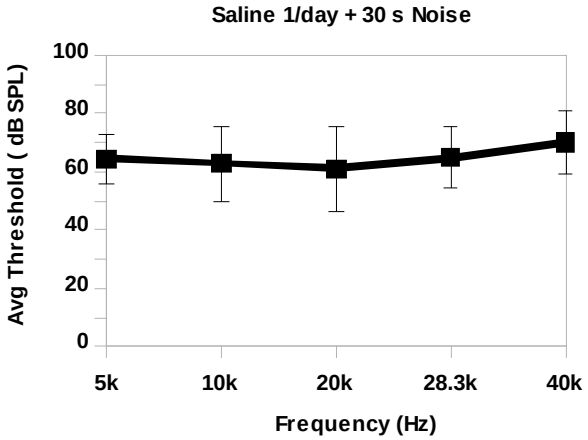
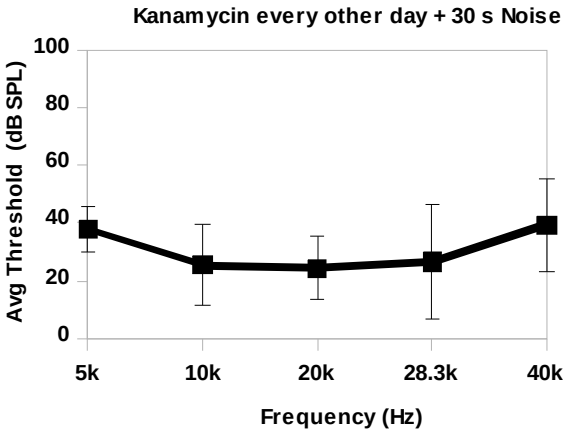
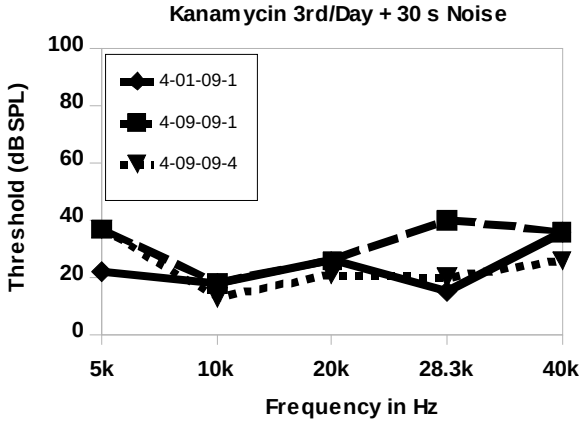
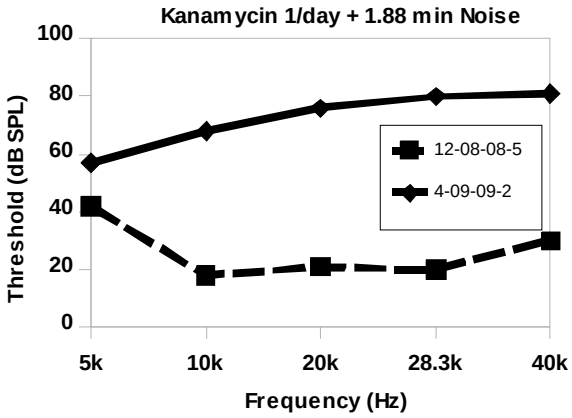
APPENDIX A: Mean thresholds by treatment group

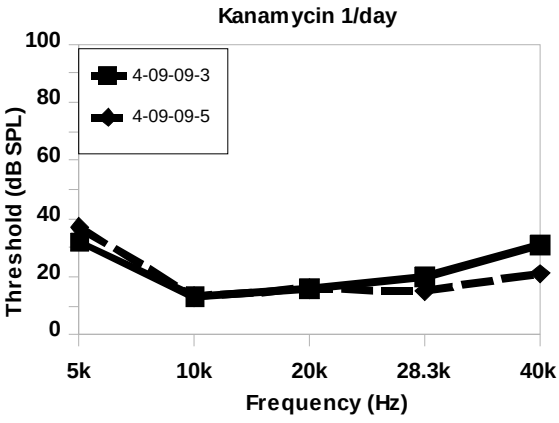












APPENDIX B: Daily Injections/Body Weight Data Form

Todays Date:			Factor =	
ID	Treatment	BW	Inj. Volume	

APPENDIX C: ABR Data Log Form

ABR / CAP Data Log

sheet version date 8/3/04

ID Number _____

Animal type: mouse / rat / gerbil

Strain: **Genotype** (tentative / confirmed): **Sac Date** _____

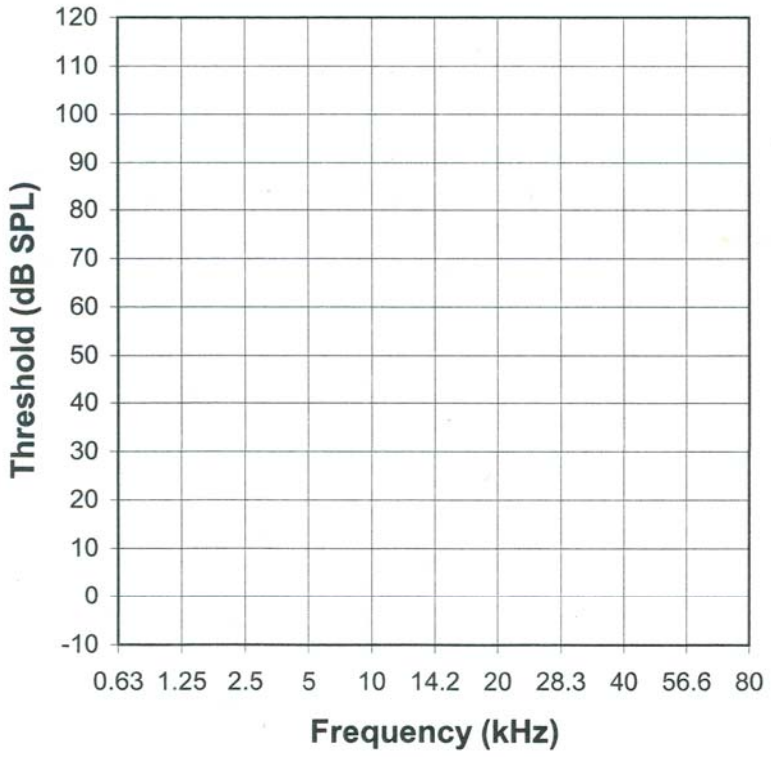
Sex: **DOB:** **Age:**

Identifying marks: L R **Project:**

Collaborating PI:

ABR Conditions: 5 ms tone, 1000 reps, 20/sec, 100-10,000 Hz, x100,000, D/A 30 kHz
Anesthetic: 80/15 mg/kg (Ket/Xyl) / other: **Rise Time:** 1.0 ms / other:
Ear: Right / Left **Speaker Distance:** 7 cm / 10 cm
Ground: Back/Other: **Reference:** Vertex/Other:

CAP Conditions: 5 ms tone, 100 reps, 3/sec, 30-3,000 Hz, x100,000, D/A 30 kHz
Anesthetic: Pentobarb (60 mg/kg) / other: **Rise Time:** 1.0 ms / other:
Round window of: Right / Left **Speaker Distance:** 7 cm / 10 cm
Ground: Hindleg / Other: **Reference:** Neck musculature/Other:



Date / Time: _____
Tester (Initials):
Condition:

Date / Time: _____
Tester (Initials):
Condition:

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Date / Time: _____
Tester (Initials):
Condition:

Notes: