의학석사 학위논문

Cochlear uptake of fluorescent gentamicin in neonatal mice

신생 생쥐에서 형광 태그된 젠타마이신의 와우내 분포 연구

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문 성 중

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이 논문을 의학과 석사 학위논문으로 제출함

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Cochlear uptake of fluorescent gentamicin in neonatal mice

by Sung-Joong Moon

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학위논문 원문제공 서비스에 대한 동의서

본인의 학위논문에 대하여 서울대학교가 아래와 같이 학위논문 제공하는 것에 동의합니다.

1. 동의사항

① 본인의 논문을 보존이나 인터넷 등을 통한 온라인 서비스 목적으로 복제할 경우 저작물의 내용을 변경하지 않는 범위 내에서의 복제를 허용합니다.

② 본인의 논문을 디지털화하여 인터넷 등 정보통신망을 통한 논문의 일부 또는 전부의 복제.배포 및 전송 시 무료로 제공하는 것에 동의합니다.

2. 개인(저작자)의 의무

본 논문의 저작권을 타인에게 양도하거나 또는 출판을 허락하는 등 동의 내용을 변경하고자 할 때는 소속대학(원)에 공개의 유보 또는 해지를 즉시 통보하겠습니다.

3. 서울대학교의 의무

① 서울대학교는 본 논문을 외부에 제공할 경우 저작권 보호장치(DRM)를 사용하여야 합니다.

② 서울대학교는 본 논문에 대한 공개의 유보나 해지 신청 시 즉시 처리해야 합니다.

논문제목 : 신생 백서에서 형광 태그된 젠타마이신의 와우내 분포 연구

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Abstract

Background: Ototoxicity in neonates and premature newborns has not been well defined if prematurity is a potential risk factor of aminoglycoside ototoxicity. Unlike the functionally mature cochlea of human newborns, the murine cochlea becomes functionally mature around 2 weeks after birth. This dynamic postnatal cochlear maturation in the mouse provides a unique opportunity to study how ototoxic reagents permeate the developing blood-labyrinth barrier to reach sensory hair cells. This study was conducted to investigate if cochlear uptake of aminoglycoside is increased in neonatal mice after systemic administration of fluorescent gentamicin (gentamicin-Texas Red, GTTR).

Materials and Methods: C57Bl/6 mice were intraperitoneally injected with GTTR at postnatal 7, 21, 35 days (P7, P21, P35). Harvested cochlear and kidney tissues were either whole-mounted or cryosectioned for microscopic exam. The intensities of GTTR in the cochlear lateral wall, cochlear hair cells and renal proximal tubular cells were quantified and compared.

Results: GTTR fluorescence in the hair cells and the lateral wall (including marginal, intermediate and basal cells of the stria vascularis, and fibrocytes of the spiral ligament) was significantly higher at P7

than at P35 ($p<0.01$). Animals treated with Texas Red only showed negligible Texas Red fluorescence at every time point. Renal proximal tubules did not show significant differences in fluorescence intensity between different time points.

Conclusion: Prior to complete maturation of the cochlear blood-labyrinth barrier, GTTR readily permeates into strial cells, fibrocytes and sensory hair cells.

Keywords: Ototoxicity, Gentamicin, Hearing Loss, Prematurity, Neonate, Mouse

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Introduction

Aminoglycoside antibiotics are essential for prophylaxis or treatment of bacterial sepsis, especially in prematurely born babies, but are cytotoxic to inner ear hair cells causing hearing loss and balance problems. Though several risk factors have been known to increase aminoglycoside ototoxicity, ototoxicity in neonates and premature newborns have not been well defined[1] and reported incidence is ranged wide as 0-47%.[2,3]

The mechanism of aminoglycoside ototoxicity is quite complex and influenced by many factors, nevertheless ototoxicity usually occurs in a dose-dependent manner.[4] The routes by which systemically administered aminoglycosides enter the cochlear tissues and hair cells are not clealy understood. A series of reports suggest that cochlear hair cells primarily take up aminoglycosides across their apical membranes, i.e. from endolymph in vivo.[5-8] Indirect evidence suggests that systemic aminoglycosides are trafficked from the strial capillaries across the stria vascularis into endolymph.[5,9] Strial trafficking of aminoglycosides appears to be regulated at the strial endothelial cell membranes and at the marginal cell-intermediate/endothelial cell boundary,[9] which together constitute functional barriers of the cochlea (blood-labyrinth barrier, BLB), and is similar to the blood-brain barrier (BBB) that separates the central nervous system and cerebrospinal

fluid from the systemic vasculature. The disrupted barrier may change permeability, which can modulate cochlear uptake of some substance, i.e. gentamicin.[10]

Despite alleged side effects of aminoglycosides, it is still very effectively used for prophylactic treatment of infection in prematurely born babies. But prematurity in the inner ear and blood labyrinth barrier may increase the chance of gentamicin trafficking into the inner ear. Thus, it is important to determine if cochlear uptake of aminoglycoside is increased in prematurely born babies in a controlled manner. Unlike the functionally mature cochlea of human newborns, the murine cochlea becomes functionally mature around 2 weeks after birth. In mice, tight junctions between adjacent marginal cells, and between adjacent endothelial cells, within the stria vascularis are established during this postnatal period, with consequent enrichment of endolymph with potassium. This dynamic postnatal cochlear maturation in the mouse provides a unique opportunity to study how ototoxic reagents permeate the developing blood-labyrinth barrier to reach young sensory hair cells.

In this study, we tested if cochlear uptake of aminoglycoside is increased in neonatal mice after systemic administration of fluorescent gentamicin (gentamicin-Texas Red, GTTR).

Materials and Methods

Preparation of gentamicin-Texas Red (GTTR) conjugate

Gentamicin sulfate (in 100mM K_2CO_3, pH=10) and succinimidyl esters of Texas Red (Invitrogen, CA; 10 mg/ml in dimethyl formamide) were agitated together for several days at 4°C. A high ratio of free gentamicin(GT) to Texas Red esters ensures that only one Texas Red molecule is conjugated to any individual GT conjugate.[11] We used reversed phase chromatography using C-18 columns (Grace, Deerfield, IL, USA) to purify the conjugate from unconjugated gentamicin, and contamination by unreacted Texas Red.[12]

Experimental groups

C57BL/6 mice (experimental and control groups - a pair of 3 groups: 1, 3, 5 weeks of age, n=6, 3, 6 respectively in experimental group and 2, 2, 2 in control group) were used. Each experimental group (P7, P21 and P35 indicate the postnatal ages) by age was intraperitoneally injected with fluorescently-conjugated gentamicin (2 mg/kg of GTTR). At 60 min after GTTR injection, mice were anesthetized and euthanized.

To determine if unconjugated Texas Red was taken up in the cochlear and kidney tissue, the mice of each control group were injected by an intraperitoneal route of the molar equivalent of Texas

Red.

All experimental protocols complied with the Guidelines of the National Institute of Health and the Declaration of Helsinki, and were approved by Seoul National University Bundang Hospital Institutional Animal Care and Use Committee (IACUC). IACUC number is BA1207-108/053-01.

Murine tissue preparation

To euthanize was executed by transcardiac perfusion with DPBS 1ml, and followed by with 4% paraformaldehyde for fixation. Following fixation, the bony labyrinth and kidney were obtained and postfixed in 4% paraformaldehyde solution for 15min. The lateral wall was excised, and cochlear coils containing the organ of Corti were isolated and whole-mounted. Sections of kidney were cut on a microtome with a thickness of 4 μm. Prepared tissues were permeabilized with fixative containing 0.5% Triton X-100 for 45 min, rinsed, labeled with Alexa-488-conjugated phalloidin to localize filamentous actin, and postfixed with 4% paraformaldehyde for 15 min.[5]

Imaging

Tissues from the most apical half-turn of the cochlea and a quarter turn of the basal coil adjacent to the hook region were mounted on slides, immersed in VECTASHIELD® Mounting Medium (H-1000; vector

laboratories Inc., Calif., USA), cover slipped and examined using a Carl Zeiss LSM 510META confocal system. All specimens from the cochlea of the red channel were imaged at the same intensity and gain setting, including control tissues.[9] The settings were adjusted for imaging kidney sections.

Immunohistochemistry

For acquiring the wide view of structures, some cochlea and saccule tissues were frozen sectioned. Fixation was executed by immersing tissues into 4% paraformaldehyde at 4°C for 24 hours. Using 0.135M EDTA solution, decalcification was followed for 3days. After washing three times by 1X PBS, OCT embedding was done. Sections of tissues were cut on a microtome to a thickness of 5μm. Followed by drying in 37°C incubation, then the immunostaining was done.

Prepared tissues were blocked in blocking buffer(1X PBS/ 1% BSA/ 0.1% Triton X-100) for 60 minutes. After aspirating blocking solution, apply diluted primary antibody (1:250 anti-calretinin antibody/ MAB1568;Millipore). Followed by incubating overnight at 4°C, then incubate specimen in fluorochrome-conjugated secondary antibody (1:200 Alexa-488 conjugated gout anti-mouse antibody/ A11017;Molecule Probe) diluted in antibody dilution buffer for 1-2 hours at room temperature in dark. After rinsing in PBS, coverslip slides with VECTASHIELD® Mounting Medium.

Image analysis

Focal planes representing the marginal cell layer, intrastrial tissues, basal cell layer, stria ligament, and hair cells were localized by Alexa-488-conjugated palloidin-labeled (green) images obtained during sequential imaging. Images of each region of interest (ROI) from the red channel were manually segmented for pixel intensity determination using ImageJ®. To normalize data between experimental sets, the mean intensity was standardized against the control specimen (intensity of the marginal cell intensity in the organ of Corti) and plotted.

Statistical Analyses

Data were expressed as mean ± SEM (standard error of the mean). The Kruskal-Wallis test generated by SPSS version 18.0 (SPSS, Chicago, IL) were used to determine the difference in the GTTR intensity. A value of $P < 0.05$ was considered significant. And post-hoc tests were done by Mann-Whitney tests and Bonferroni correction. When Bonferooni correction was applied, all uptake differences were reported at 0.025 level of significance, where 0.025 equals 0.05/2, and 2 means the number of Mann-Whitney tests conducted in post-hoc tests (P7 vs P35 and P21 vs P35).

Results

Increased GTTR uptake in neonatal mice

In P7 mice, cochlear uptake of GTTR is noticeably increased in the cochlear lateral wall as well as in the hair cell region. However, cochlear uptake of GTTR is weaker and limited in the lateral wall of the cochlea in P21 and P35 mice (Fig. 1).

Unconjugated TR (TR only) is not taken up in neonatal mice

There is no TR fluorescence in marginal or renal proximal tubule cells when TR only was given in neonatal mice (Fig. 2).

Quantitative analysis of GTTR uptake in the cochlear lateral wall

Quantitative image analysis of GTTR fluorescence was conducted. GTTR fluorescence intensity in each ROI (marginal cells, intrastrial tissues, basal cells and strial ligaments) was statistically significantly higher in P7 than in P35 (Table 1, Fig. 3, 4).

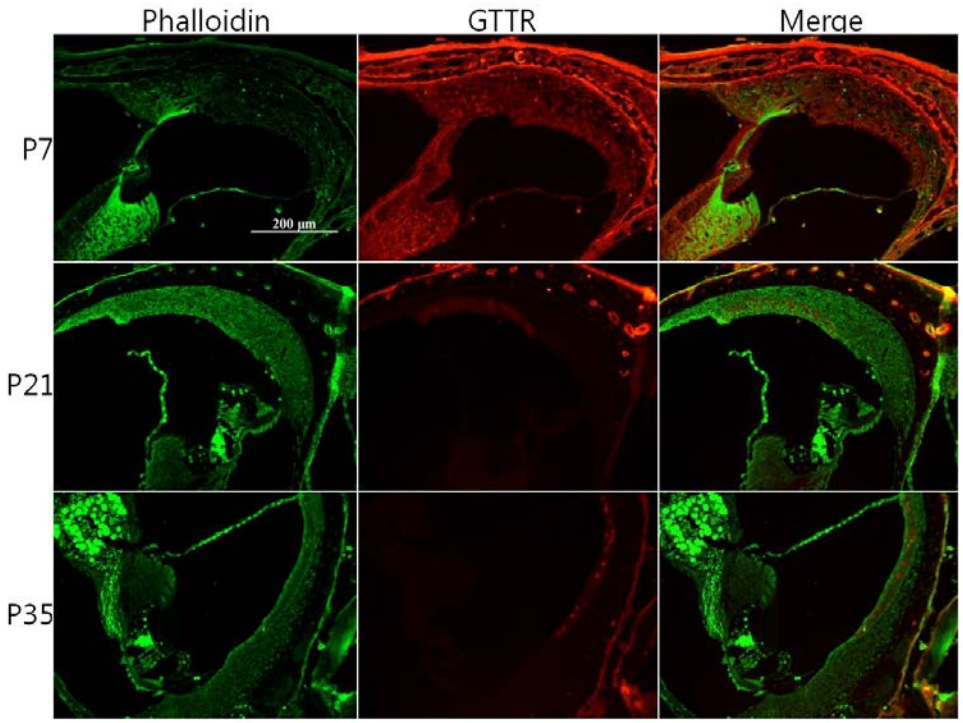

Figure 2. Cochlear uptake of GTTR according to age. GTTR uptake (red fluorescence) is increased in P7 mice, where P7, P21 and P35 indicate the postnatal ages. In P7 mice, cochlear uptake of GTTR is increased in the cochlear lateral wall as well as in the hair cell region. Note that cochlear uptake of GTTR is weaker and limited in the lateral wall of the cochlea in P21 and P35 mice.

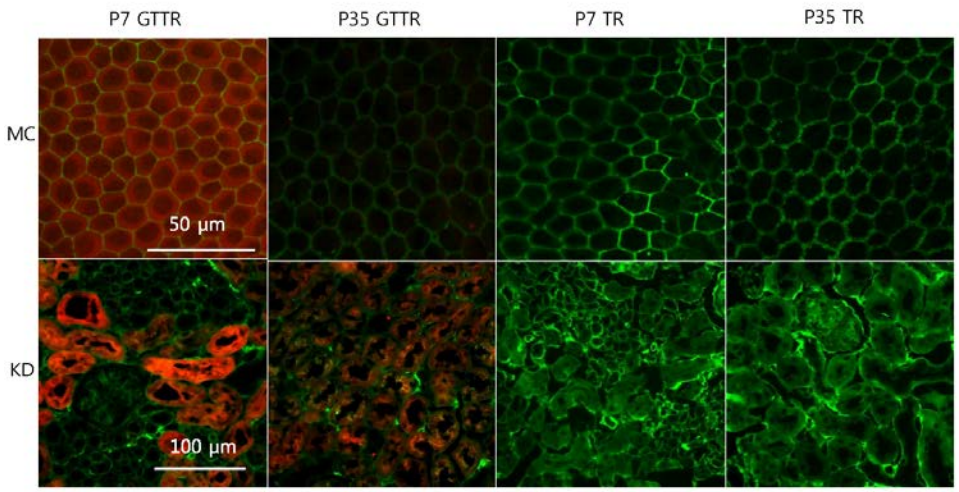

Figure 2. The comparisons of TR uptake depending on the conjugation of gentamicin in marginal cell layers and kidney. In the marginal cells, neonatal mice show strong uptake of GTTR compared to P35 mice, while uptakes of unconjugated TR are not seen. In renal proximal tubules, both P7 and P35 show no differences in GTTR uptake pattern. And uptakes of unconjugated TR are also not seen.

Table 1. Statistical results about GTTR uptake differences in cochlear apicomiddle and basal turn ROIs according to age

	Apicomiddle turn		Basal turn	
	H(2)	**P-value**	**H(2)**	**P-value**
MC	6.866	0.032*	9.458	0.009*
IC	6.240	0.044*	10.150	0.006*
BC	5.950	0.051	10.350	0.006*
SL	10.381	0.005*	10.225	0.006*
HC	10.522	0.006*	11.025	0.004*

MC: marginal cell layer, IC: intrastrial tissue, BC: basal cell layer, SL: spiral ligament, HC: hair cells, * $P<.05$

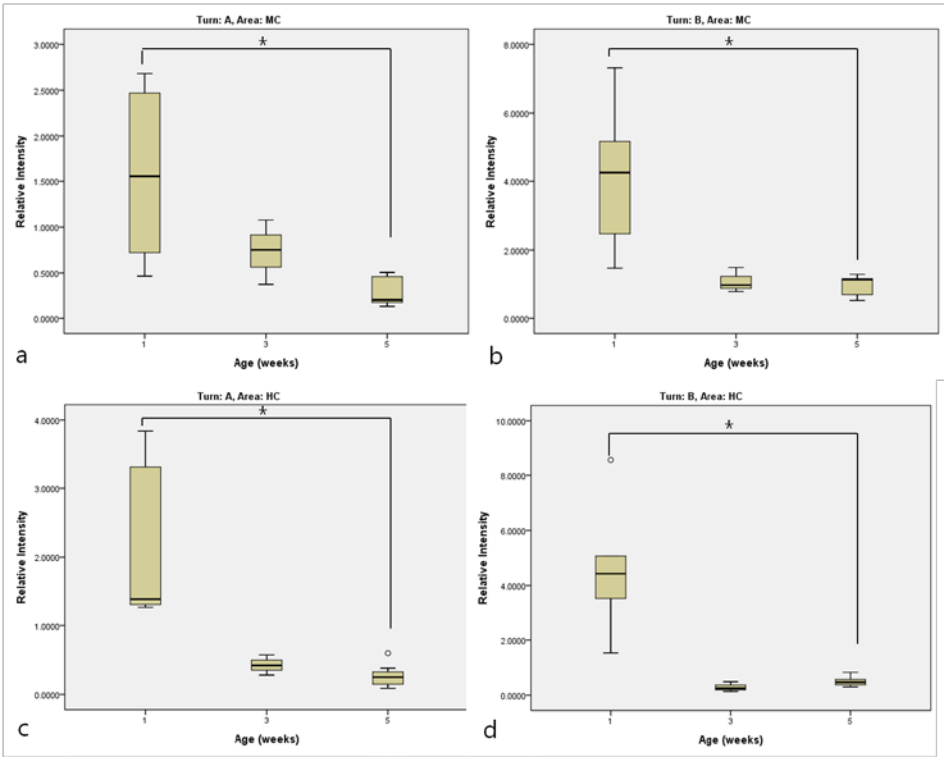

Figure 3. Relative intensity of MC and HC GTTR uptake in cochlear apicomiddle and basal turn. GTTR fluorescence intensity in cochlear lateral wall and hair cell was significantly higher in neonate than in P35. **a** apical turn, MC. **b** basal turn, MC. **c** apical turn, HC. **d** basal turn, HC. MC: marginal cell layer, HC: hair cells, * $P<.05$

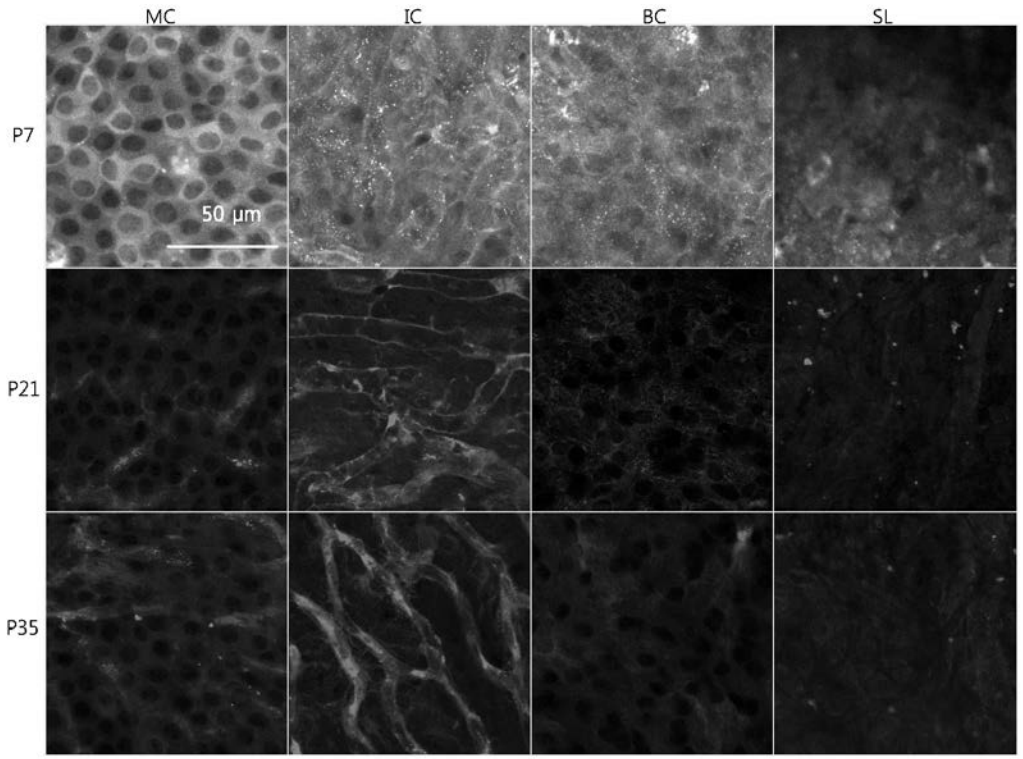

Figure 4. GTTR uptake in the cochlear lateral wall. Tissue layers of the stria vascularis and the spiral ligament show higher uptake GTTR in P7 mice compared to P21 and P35. MC: marginal cell layer, IC: intrastrial tissues, BC: basal cell layer, SL: spiral ligament

Quantitative analysis of GTTR uptake in the outer hair cells

GTTR fluorescence intensity in another ROI (outer hair cells) was statistically significantly higher in P7 than in P35 (Table 1, Fig. 3, 5).

GTTR uptake in renal proximal tubule

Experiment was conducted to determine whether GTTR uptake in renal proximal tubule was affected in neonate or not (Fig. 6). In renal proximal tubular cells, GTTR uptake is not different in neonatal mice with another age groups (P21, P35).

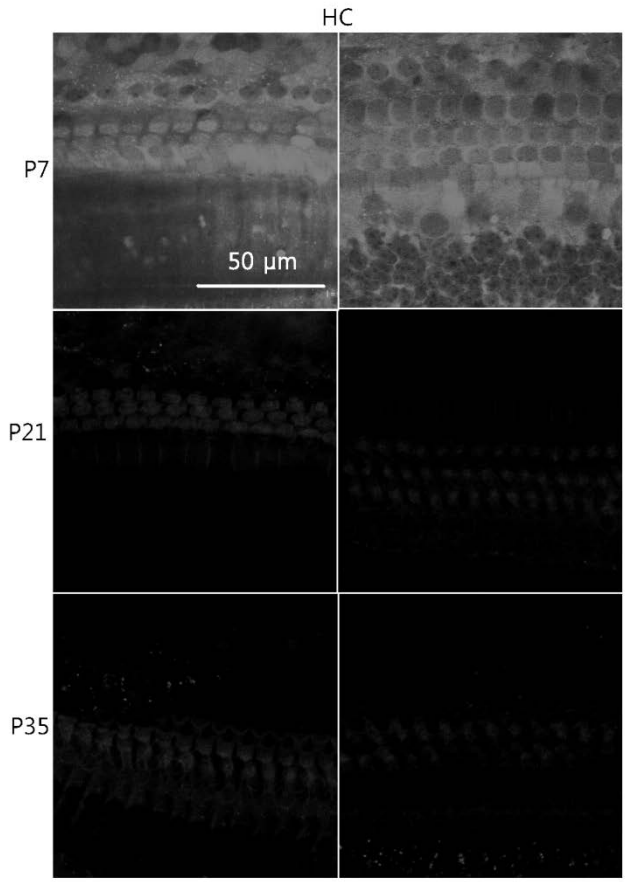

Figure 5. GTTR uptake in the cochlear hair cells. GTTR uptake in the outer hair cells is marked increased in in P7 mice compared to P21 and P35. HC: hair cells

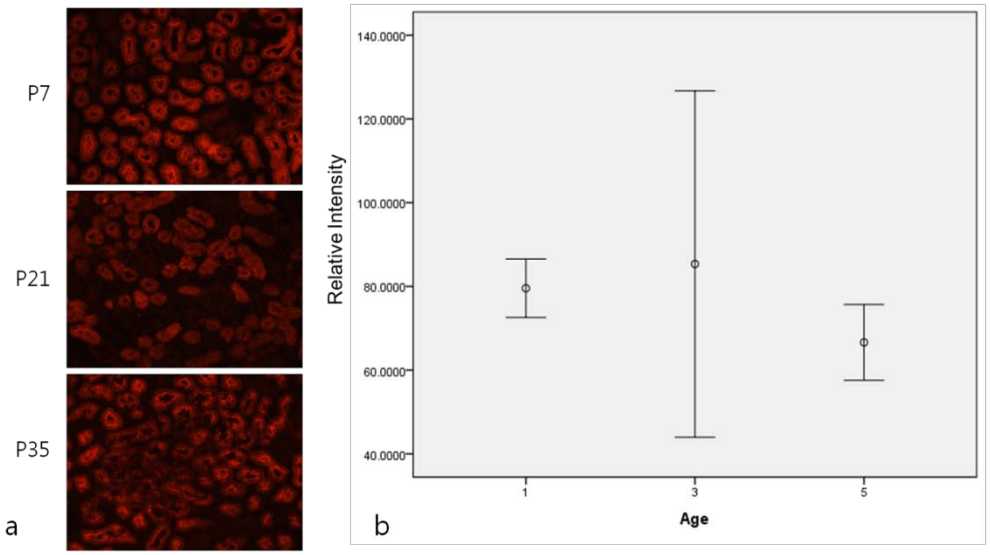

Figure 6. GTTR uptake in renal proximal tubule.

a Similar GTTR fluorescence at different age groups.

b Comparison of fluorescent intensity according to postnatal age (p>.05)

Discussion

The present study demonstrates that strial and hair cell uptakes of fluorescent gentamicin are increased in neonatal mice, while such augmentation is not present in renal proximal tubules. These results suggest that increased cochlear uptake of gentamicin is due to cochlear prematurity, not secondary to renal dysfunction.

Unconjugated TR didn't show uptake in cochlea and kidney tissues. In GTTR uptake, possible confusion due to individual TR uptake can be excluded, where tagged gentamicin is needed to organ uptake.

The mouse[13] is excellent animal model in which to examine the pharmacological effects of ototoxic agents during critical developmental periods, because the onset and maturation of hearing develops postnatally.[14,15] A sensitive developmental period for aminoglycoside has been reported for mice,[16] that is P7 to P10.

In aminoglycoside induced ototoxicity, it is thought that the increased drug concentration in endolymph and the hair cell susceptibility are mainly important as the causative factors. We selected the experimental point of the increased drug concentration in endolymph. The study about the hair cell susceptibility will need the other experimental model.

And the study methods of practicing ototoxicity phenomena can be divided into two ones, which are anatomical and functional. This study belongs to the anatomical one. Additionally ultrastructural investigation with electron microscopy[17] or others are also the kinds of the anatomical methods. The visualization of anatomy clarifies the entire understanding and accuracy of the individual result. In the functional methods, there are many other experiments, i.e. DPOAE (Distortion-product otoacoustic emission),[18] ABR (auditory brainstem response),[19-22] electrocochleography (isoelectric auditory nerve-evoked potential thresholds from 2,000 to 64,000 Hz),[23] thresholds of the scalp-recorded compound action potential of the auditory nerve[14] etc. These functional methods can elude the invasiveness of the process and reflect the importance in actual circumstances.

Uptake of aminoglycoside is increased after systemic administration of fluorescent gentamicin in premature cochlea of neonatal mice. Prior to complete maturation of the cochlear blood-labyrinth barrier, GTTR readily permeates into strial cells, fibrocytes and sensory hair cells. If these results in mice can be applied to human, systemic administration of gentamicin in premature neonate may elevate the cochlear uptake of gentamicin and the possibility of ototoxicity. So it is important to monitor cochlear toxicity when aminoglycoside is prescribed in prematurely born babies.

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국문 초록

서론: 아미노글리코사이드는 그람음성균에 효과적인 항생제로, 특히 미숙아에서 패혈증의 예방과 치료목적으로 유용하게 처방되고 있다. 그러나 아미노글리코사이드는 신독성과 함께 이독성의 가능성이 있어 사용시 이러한 합병증을 모니터하며 사용해야 한다. 신생아에서 성인에 비해 아미노글리코사이드 이독성의 발생이 증가한다고 알려져 있지는 않으나 미숙아에서는 기관이 완성되기 전이라 약물에 대한 감수성이 더 높을 가능성도 배제할 수 없다.

신생 생쥐는 생후 2주경에 내이가 완성되기 때문에 미숙아에서의 이독성 약물의 이동기전에 대한 연구를 용이하게 하는 동물모델이 된다. 본 연구에서는 생후 1, 3, 5주의 생쥐에 형광물질을 태그한 젠타마이신을 전신투여하고 내이분포를 비교해보아 미숙아에서의 이독성 증가가능성을 동물모델로 검증해보고자 하였다.

대상 및 연구 방법: 신생 C57Bl/6 백서에 형광물질이 덧붙은 젠타마이신 (GTTR)을 생후 7일, 21일, 35일째 복강내 주사하였다. 얻어진 내이와 신장 조직들을 일부는 완전고정 후 그리고 일부는 동결절편 후에 공초점 현미경으로 관찰하였다. 모든 조직에서 형광의 세기와 분포는 동일한 현미경 조건에서 얻었다. 와우조직과 신장조직에서 형광의 세기정도를 정량화하여 비교하였다.

결과: 유모세포와 와우의 외측벽에서 측정한 GTTR 형광의 세기정도는 생후 7일째 쥐에서 생후 35일째 쥐에서 보다 통계적으로 유의하게 높게 나왔다($p<0.01$). 색소(텍사스 레드)만 주사한 경우에 모든 경우에서 형광의 세기는 실험의 모든 시점에서 거의 전무하였다. 신장의 근위부 세뇨관에서는 실험군과 대조군의 형광 세기정도가 유의한 차이를 보여주지 않았다.

결론: 신생 생쥐에서 아미노글라이코사이드의 와우조직과 유모세포로의 유입은 유의하게 증가하였다.

주요어: 이독성, 젠타마이신, 난청, 신생아, 미숙아, 생쥐

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