Ann Otol Rhinol Laryngol 113:2004
REPRINTED FROM *ANNALS OF OTOLOGY, RHINOLOGY & LARYNGOLOGY*
January 2004 Volume 113 Number 1

TOLERABILITY OF *N*-CHLOROTAURINE IN THE GUINEA PIG MIDDLE EAR: A PILOT STUDY USING AN IMPROVED APPLICATION SYSTEM

ANDREAS NEHER, MD
INNSBRUCK, AUSTRIA

MARKUS NAGL, MD
INNSBRUCK, AUSTRIA

DIANE PRIESKORN
ANN ARBOR, MICHIGAN

ALICE MITCHELL
ANN ARBOR, MICHIGAN

NADINE BROWN, MS
ANN ARBOR, MICHIGAN

ANNELIESE SCHROTT-FISCHER, PHD
INNSBRUCK, AUSTRIA

JOSEF M. MILLER, PHD
ANN ARBOR, MICHIGAN

The tissue tolerance of *N*-chlorotaurine (NCT), a mild endogenous antimicrobial oxidant, has been investigated by application to the guinea pig middle ear. The animals were implanted with a novel cannula system that allows chronic external drug delivery to the round window niche. In the first part of the study, 3 animals each received 100 µL of 0.1% NCT (5.5 mmol/L) and 1% NCT, respectively, in aqueous solution twice daily for 8 days. In the second part, NCT was dissolved in phosphate-buffered saline solution to 300 milliosmolar (isotonic), and 27 µL was injected in 3 additional animals twice daily for 7 days. The guinea pigs injected with 100 µL of NCT developed immediate dizziness and nystagmus and did not thrive. Other reactions included mucosal thickening in the middle ear, rupture of the tympanic membrane, and blood and gelatinous material in the cochlea accompanied by hair cell loss and a 10- to 90-dB elevation of the hearing threshold as determined by auditory brain stem responses. The effects seemed to be dose-dependent, but the rate of variability was high across animals. In contrast, the guinea pigs treated with 27 µL of isotonic NCT showed no signs of discomfort, no or only moderate thickening of the middle ear mucosa, no shift of the hearing threshold, and no hair cell loss. Positive control animals injected with 10% neomycin sulfate developed extensive hair cell loss. Provided that the membranes of the inner ear are intact and that low single-dose volumes are used to avoid increased middle ear pressure, isotonic NCT seems to be well tolerated in the tympanic cavity. The new drug delivery system proved to be advantageous for ototoxicity studies.

KEY WORDS — application system, guinea pig, middle ear, *N*-chlorotaurine, tolerability.

INTRODUCTION

Infection of the external auditory canal by bacteria or fungi is a frequent disease triggered by moisture and trauma.[1] Usually, topical treatment with antibiotics or acidifying agents accompanied by cleaning of the external ear canal is effective.[1] Sometimes, edematous obstruction of the canal limits visualization of the tympanic membrane so that involvement of the middle ear and possible perforation of the tympanic membrane cannot be judged.[2] In these cases, externally applied medications may reach the middle ear. Therefore, a disinfectant that is effective against all of the causative pathogens and well tolerated not only in the external but also in the middle ear would be of clinical advantage.

N-chlorotaurine (NCT; ClHN-CH_2-CH_2-SO_3H) is a broad-spectrum antimicrobial agent with bactericidal and fungicidal activity.[3-5] As the main representative of long-lived oxidants produced by activated granulocytes and monocytes,[6] it is thought to participate in destruction of pathogens.[4] On the other hand, it may significantly attenuate the inflammatory process by inhibition of proinflammatory cytokines.[7] Special features of NCT, as compared to other *N*-chloro amino derivatives, are availability as a crystalline sodium salt, solubility in aqueous solutions, and outstanding stability.[8]

Because of these properties, NCT has been proposed as a new antimicrobial agent in human medicine. Clinical studies have demonstrated excellent tolerance of the 1% NCT aqueous solution in the human eye and urinary bladder.[9,10] In otology, NCT (1% and 10%) applied to the middle ear of mice by a single injection through the tympanic membrane caused only a temporary increase in auditory brain stem response (ABR) threshold, probably due to conductive loss resulting from middle ear fluid and tympanic membrane perforation.[11] No signs of adverse effects

From the Department of Otorhinolaryngology (Neher) and the Inner Ear Research Laboratory (Schrott-Fischer), University Hospital of Innsbruck, and the Institute of Hygiene and Social Medicine, Leopold-Franzens-University of Innsbruck (Nagl), Innsbruck, Austria, the Kresge Hearing Research Institute, University of Michigan, Ann Arbor, Michigan (Prieskorn, Mitchell, Brown, Miller), and the Center for Hearing and Communication, Karolinska Institute, Stockholm, Sweden (Miller). This work was supported by the Austrian Science Fund (grant P15240) and the Jubilee Research Fund of the Austrian National Bank (grant 8366). This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH *Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan, Ann Arbor, Michigan.

CORRESPONDENCE — Andreas Neher, MD, Dept of Otorhinolaryngology, University Hospital of Innsbruck, Anichstr 35, Innsbruck, Austria.

could be found by histologic assessment of the inner ear, and no side effects were seen when a concentration of 0.1% was used.

The aim of the present pilot study was to investigate NCT tolerance after repeated application to the middle ear, in an improved model using guinea pigs implanted with a cannula system for chronic external drug delivery.

MATERIALS AND METHODS

Reagents. Pure NCT as a crystalline sodium salt (molecular weight, 181.57 g/mol, kindly prepared by Waldemar Gottardi, Institute of Hygiene and Social Medicine, Innsbruck, Austria) was dissolved in sterile double-distilled water (first part of the study, see Study Design) or in phosphate-buffered saline solution (300 milliosmolar, second part of the study) to concentrations of 0.1% (5.5 mmol/L) and 1% (55 mmol/L). Neomycin (Sigma Chemical Co, St Louis, Missouri) was prepared as a 10% solution in sterile water. The solutions were stored at 2°C during the study. Aliquots were warmed to 37°C immediately before injection into the animals.

Animals. NIH-strain pigmented guinea pigs of both genders were obtained from Elm Hill Breeding Laboratory (Chelmsford, Massachusetts). The animals used in this study were maintained by the Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, Michigan, which is registered with the US Department of Agriculture and fully accredited by the American Association for Accreditation of Laboratory Animal Care International (AAALAC). The study protocol was reviewed and approved by the University Committee for the Use and Care of Animals. Only animals that appeared healthy and with normal hearing, as determined by the ABR, were used. The guinea pigs were anesthetized by intramuscular injection of 10 mg/kg xylazine hydrochloride (Lloyd Laboratories, Shenandoah, Iowa) and 40 mg/kg ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, Iowa) before ABR measurement and surgery.

Study Design (Survey). The guinea pigs received a baseline hearing assessment and then were implanted with a drug delivery system to the middle ear that was accessible via an injection cap on top of the animals' heads. Dosing began the day following surgery, which was considered study day 0. All subjects, randomly selected from each group, were injected via their cannulas twice a day (900 and 1600 hours).

In the first part of the study, 3 animals each were injected with 100 μL of 0.1% or 1% aqueous NCT solution for 8 consecutive days. In the second part, 27 μL of each solution in phosphate-buffered saline solution was applied to 3 animals each for 7 days. Three additional animals that served as a positive control group received 10% neomycin. Hearing assessment was repeated on day 9 in the first part of the study and on day 8 in the second part. Body weights were monitored, and the animals were observed for clinical signs of systemic toxicity. Within 48 hours following drug treatment in the first part of the study, and after 96 hours in the second part, the animals were euthanized and decapitated, the middle ear was assessed, and the cochlea was removed and prepared for inner ear histology.

Drug Delivery System. The drug delivery system was developed in the Cochlear Signaling and Tissue Engineering Laboratory at Kresge Hearing Research Institute. The system was constructed from a 5.8-cm length of polyurethane tubing (Micro-Renathane tubing MRE 025, Braintree Scientific, Inc, Braintree, Massachusetts) and the plastic sheath of a 24-gauge Angiocath (Becton Dickinson Vascular Access, Sandy, Utah) cut at a 45° angle approximately 8 mm from the hub. The tubing was soaked in toluene to enlarge its diameter, allowing it to be slid over the sheath. As the toluene evaporated, the tubing returned to its original size, firmly secured to the sheath. A Luer lock injection adaptor (MX492, Medex, Inc, Hilliard, Ohio) was attached to the Angiocath to allow for serial substance administration. The cannulas were sterilized with ethylene oxide.

Surgery. The surgery was performed under appropriate anesthetic, with aseptic technique. On study day minus 1, the animals underwent unilateral implantation. The dorsal surface of the skull was exposed. With the bregma (the intersection of the sagittal and coronal suture lines) as a reference, small self-tapping stainless steel screws were placed in holes drilled 1 cm posterior (at vertex) and another 1 cm anterior to the bregma; 2 additional screws were placed 0.5 to 0.6 cm laterally, and slightly caudal to the vertex, in the parietal regions. The screws anchored the implant to the skull. The vertex screw also served as the epidural recording site for the ABR assessment.

The left bulla was exposed behind the auricle, and a small defect was made to access the middle ear. Under an operating stereoscope, the cannula was advanced approximately 2 mm into the middle ear cavity. The cannula was secured, and the entire defect was covered with carboxylate cement (Durelon, ESPE-Premier Sales Corp, Norristown, Pennsylvania). After the cement hardened, the Luer lock injection cap was attached to the Angiocath end of the cannula. The cannula was looped around the self-tapped screws

TABLE 1. CHANGE IN BODY WEIGHT OF GUINEA PIGS FROM BEFORE DOSING TO AFTER END OF DOSING

First Study (100-μL Single Dose)						*Second Study (27-μL Single Dose)*					
0.1% NCT (n = 3)			*1% NCT (n = 3)*			*0.1% NCT (n = 3)*			*1% NCT (n = 3)*		
Day 0	*Day 9*	*Change*	*Day 0*	*Day 9*	*Change*	*Day 0*	*Day 8*	*Change*	*Day 0*	*Day 8*	*Change*
532	587	+55	473	536	+63	354	393	+39	329	364	+35
470	527	+57	494	514	+20	326	364	+38	307	347	+40
444	482	+38	436	443	+7	351	405	+54	344	360	+16

Data are weights and changes in grams. Three rows for each study group represent 3 different animals.
NCT — *N*-chlorotaurine.

on the dorsal head. To prevent separation of the cannula, 3-0 surgical steel (Ethicon, Inc, Somerville, New Jersey) was wrapped tightly around the hub of the Angiocath, and the steel was brought down, one wire on each side, and wrapped around the appropriate parietal screw. The injection cap was mounted between the vertex screw and the screw contralateral to the implanted ear, at a 30° to 45° angle from the skull. The screws and port were secured to the head with methyl methacrylate. The incision was sutured, and the skin edges around the methacrylate headset were sealed with a thin layer of methyl methacrylate. The contralateral ear was untreated.

Electrophysiology. The auditory sensitivity of the guinea pigs was evaluated under xylazine (10 mg/kg) and ketamine (40 mg/kg) anesthesia. In a soundproof booth, the animals were placed on a heating pad and sound stimuli were delivered via an encased, shielded Beyer transducer through a tube into the ear canal. Tonal acoustic stimuli bursts at 15 ms (rise-fall time, 1 ms) were presented at frequencies of 2.0, 8.0, and 16.0 kHz. Responses were recorded from subcutaneous needle electrodes, filtered (300 Hz to 3 kHz), and amplified (gain of 100,000), and 1,024 epochs were averaged with a Tucker-Davis Technology acquisition system. The threshold was defined as the lowest intensity at which a clear, reproducible neural response was observed. The measurements were performed unilaterally (left ear) before treatment to confirm normal hearing, and on study day 8 before termination. The animals were not treated on the day of their last ABR test, because excessive fluid in the middle ear may elevate thresholds. The neomycin-treated positive control group did not undergo pure tone ABR testing. They underwent a baseline click ABR test (alternating polarity, 160 μs duration, 50 pps, 1,024 epochs, bandpass-filtered 30 Hz to 3 kHz at a gain of 100,000) to confirm normal hearing before implantation.

Euthanasia, Gross Pathology, and Histologic Assessment. On study day 8 and 9, respectively, after ABR testing, the animals were deeply anesthetized and decapitated and the left cochleas were removed. The middle ears were assessed for abnormalities. The cochleas were locally perfused with fixative (4% paraformaldehyde in phosphate buffer) through a hole in the apex and immersed in fixative overnight.

The next day, the cochleas were rinsed in phosphate-buffered saline solution. The otic capsule, tectorial membrane, and Reissner's membrane were removed. Each cochlea was stained with fluorescent-labeled phalloidin, followed by buffer rinse, then microdissected and mounted on slides for hair cell assessment with a Leitz photomicroscope under epifluorescent illumination. A cytocochleogram was generated, mapping the presence or absence of hair cells by location along the cochlear spiral.[12]

RESULTS

STUDY 1: INJECTION VOLUME OF 100 μL

Clinical Findings. The majority of the animals appeared stressed during the dosing. All but 1 animal vocalized, and most times they shook their heads or struggled. The dosing caused various degrees of nystagmus, and 2 animals from the 1% NCT group and 1 from the 0.1% group were excessively dizzy (ranging from 1 to 20 minutes after dosing) as indicated by their inability to stand in their cage after dosing. Other signs of dizziness were nystagmus in all 6 animals, varying from slight to severe, and occasional head bobbing and cage circling. All of the high-dose group and 1 animal from the low-dose group exhibited head tilts on occasion. On the last day of dosing, fluid was noticed in the ear canal of 2 animals treated with 1% NCT after dosing, indicating a ruptured tympanic membrane, which was confirmed at sacrifice by gross pathology. These 2 animals did not thrive, as indicated by their minimal increases in body weight of 7 and 20 g, respectively, as compared to 38 to 63 g for the other ones (Table 1).

Electrophysiology. The threshold shifts (decibels) between baseline (before experimental manipulation) and day 8 and 9, respectively, are shown in Table 2. There were some cases in which ABR thresholds were greater than the limits of our acquisition system (100 dB sound pressure level) and therefore resulted in a "greater than" threshold shift from baseline. According to the highest possible value, the mean threshold shifts at 2, 8, and 16 kHz were 51.7, 35.7, and 40.7 dB, respectively, in the 0.1% NCT group. For the 1%

TABLE 2. SHIFT IN AUDITORY BRAIN STEM RESPONSE THRESHOLD OF GUINEA PIGS FROM BEFORE DOSING TO AFTER END OF DOSING

	First Study (100-µL Single Dose)						*Second Study (27-µL Single Dose)*					
	0.1% NCT (n = 3)			*1% NCT (n = 3)*			*0.1% NCT (n = 3)*			*1% NCT (n = 3)*		
	Day 0	*Day 9*	*Shift*	*Day 0*	*Day 9*	*Shift*	*Day 0*	*Day 8*	*Shift*	*Day 0*	*Day 8*	*Shift*
2 kHz	20	>100	>80	15	70	55	42	48	6	49	38	−11
	25	55	30	13	>100	>87	46	50	4	41	30	−11
	20	65	45	28	>100	>72	50	35	−15	43	40	−3
8 kHz	48	>100	>52	35	80	45	22	20	−2	36	35	−1
	20	40	20	20	75	55	32	35	3	40	28	−12
	15	50	35	10	>100	>90	32	28	−4	31	30	−1
16 kHz	18	90	72	15	73	58	23	20	−3	18	18	0
	25	65	40	10	90	80	32	20	−12	23	15	−8
	5	15	10	20	>100	>80	14	15	1	15	20	+5

Data are thresholds and shifts in decibels. Threshold shifts were significant only in first study ($p < .01$). Three rows for each study group represent 3 different animals.

NCT group, the corresponding values were 71.3, 63.3, and 72.7 dB.

Gross Pathology. Under the dissection microscope, all 6 animals showed mucosal thickening and clear or yellow-brown fluid in the middle ear. The tympanic membrane was perforated in 2 of 3 animals in the 1% NCT group and in 1 animal of the 0.1% group. There was blood in the apical cochlear turns in the animals treated with 1% NCT and in 1 of those treated with 0.1% NCT. For 2 animals in the 1% NCT group and 1 in the 0.1% NCT group, gelatinous material was present in the basal scala tympani, near the round window.

Histology. Cytocochleograms for the low-dose group showed a normal hair cell complement in 2 of 3 animals. The third animal had severe hair cell loss. The high-dose group showed no hair cells present in 2 of 3 animals, whereas the third had significant outer hair cell loss only in the basal turn.

STUDY 2: INJECTION VOLUME OF 27 µL

Clinical Findings. In strong contrast to the experiments performed with an injection volume of 100 µL, guinea pigs treated with 27 µL showed no signs of discomfort or toxicity during or after the injection, except for one occasion in the neomycin group in which the guinea pig struggled during dosing and displayed a jerking motion, similar to gagging, for less than 1 minute after dosing. One animal in the 1% NCT group gained 16 g, and 1 animal in the neomycin group gained 27 g. The others showed a normal weight gain of 35 to 68 g from day 1 through day 8 of the study (Table 1).

Electrophysiology. The threshold shifts (decibels) between the baseline (before experimental manipulation) and day 8 are shown in Table 2. In all animals exposed to NCT, there was no hearing loss over time, regardless of concentration. The mean threshold shifts at 2, 8, and 16 kHz were −1.7, −2.7, and −4.7 dB, respectively, for 0.1% NCT, and −5.3, −4.7, and −1.0 dB, respectively, for 1% NCT.

Gross Pathology. The middle ear mucosa was moderately thickened in 1 high-dose animal. No changes were observed in the other guinea pigs.

Histology. Two of the ears treated with 1.0% NCT were stained with rhodamine phalloidin, slide-mounted, and grossly checked from apex through base for hair cell loss. Both ears appeared normal, with only occasional missing ("scarred") outer hair cells. Because the ears of the high-dose subjects appeared normal, we did not assess those of the low-dose subjects. The neomycin-treated positive control animals showed extensive hair cell loss in the 2 basalmost cochlear turns (Table 3).

DISCUSSION

The new drug delivery system used in this study has some special advantages. Given the surgical anatomy of the middle ear of the guinea pig, it is an ideal preparation for studies of inner ear toxicity. It is relatively easy to position a cannula into the middle ear such that it terminates immediately adjacent to the

TABLE 3. LOSS OF OUTER AND INNER HAIR CELLS AFTER 7 DAYS' TREATMENT WITH 10% NEOMYCIN

	Animal No.	*Outer*	*Inner*
Apex and third turn	1-3	0%	0%
Second turn	1	20%	0%
	2	0%	0%
	3	100%	100%
Base	1	50%-100%	10%
	2	90%-100%	0%
	3	100%	100%

round window. This is readily connected to the fixation site on the top of the skull, which permits easy access to the port. Drug delivery is ensured to reliably bathe the surface of the round window and the middle ear mucosa. The fixture is well tolerated, and if it is prepared and maintained under appropriate aseptic conditions, long-term dosing without the complications of infection can be expected. Moreover, given the utility of the surface preparation to evaluate inner ear damage, it is possible to identify a single sensory cell loss as a result of experimental manipulation. This provides a uniquely sensitive measure of toxicity of agents to the inner ear.

N-chlorotaurine is one of the least toxic oxidants known, and a benefit of its production by human leukocytes from taurine and hypochlorous acid is that it consumes the latter to avoid tissue damage during inflammation.[7] Our findings indicate that it is also tolerated after application to the middle ear, but only under the right circumstances. The changes seen in the group of guinea pigs treated with 100 µL may be assumed to be caused by access of NCT to the inner ear. Direct contact of NCT solution with the inner ear epithelium probably caused the severe pathophysiological changes by oxidation of the hair cells despite its generally low toxicity. Possibly, the damage was potentiated by decrease of the osmotic pressure due to the hypotonic solution applied in the first part of the study. Osmotically active substances in the middle ear can alter the inner ear fluid osmolality, since the round window membrane is permeable to water.[13]

The round window membrane likely represents a limited permeability barrier for NCT. Indeed, low–molecular weight substances such as antibiotics (eg, aminoglycosides, ciprofloxacin, cefmetazole[14-17]) and trimethylphenylammonium pass through the window,[18] whereas high–molecular weight compounds significantly penetrate only a round window damaged by noxious agents.[19] However, we think that diffusion was not the main route of NCT to the inner ear in our study. First, NCT as a sodium salt of an amino acid is more hydrophilic than the above-mentioned substances and will not penetrate the 3 layers of the round window easily.[20] This belief is supported by a previous report that the membrane is impermeable for sodium chloride, even for a saturated solution.[21] Second, diffusion does not explain the variability of effects seen, particularly in the histopathology, since we know from experience and observation in these animals that the exposure of the round window membrane to the substance was uniform across the preparations. Third, dizziness occurred immediately during the dosing. Fourth, in the case of rapid diffusion of NCT to the inner ear, the adverse effects should have occurred also in the second part of the study, in which 27 µL was applied. The sensitivity of the model was sufficient, as we were able to detect toxicity by application of 27 µL of neomycin, which is known to penetrate the round window rapidly in guinea pigs and cats.[14,16] Because we did not wash out the NCT after dosing and because NCT is a long-lived oxidant even in the presence of organic materials,[3] the contact time in the round window niche can be assumed to be sufficient to reveal a significant diffusion leading to inner ear damage.

Because of these considerations, we interpret the adverse effects to indicate that the high volume of 100 µL created a significant pressure change in the middle ear, resulting in rupture of the round window membrane. This idea would also be consistent with the observed tympanic membrane ruptures. In guinea pigs, application of pressure to the round window has been reported to cause a functional loss or irreversible changes of the cochlea and rupture of the round window membrane.[22,23] In our own past studies[24] (also unpublished observations), saline-treated control animals have not been routinely disturbed by our dosing method; however, nystagmus has been noted in some animals, and the incidence seems to decrease as the volume administered is decreased. The rupture pressure of the round window is very different among species: 0.031 atm in cats, 1.7 to >2.4 atm in cattle, and 4 to 7 atm in humans.[25,26] Hence, it is presently impossible to draw general conclusions for safe application conditions.

There were no changes in the middle ear mucosa except for a minimal irritation, which is in accordance with the findings of our previous investigation using a mouse model in which we injected NCT solution through the tympanic membrane.[11] A reversible increase in ABR thresholds in mice treated once with 5 µL of 1% or 10% NCT was caused by local irritation around the artificial perforation of the tympanic membrane, and not by the drug's effect on the inner ear.[11] Moreover, the solutions used were all aqueous and varied from 5.5 (0.1%) to 55 (1%) and 550 mmol/L (10%), so osmolality does not seem to play a major role in inner ear toxicity in the mouse model. Although we cannot exclude that there are significant differences in the permeability for NCT between the round window membranes of mice and guinea pigs, the results of the mouse model confirm our assumption that the cochlear disturbances in the guinea pig were caused by overpressure rather than by diffusion.

Therefore, the volume administered is a critical parameter to avoid toxic effects. This is true also for the middle ear, especially for the development of fibrous tissue, which can contribute to elevation of

thresholds. The absolute amount of NCT applied in single doses could be critical for middle ear adverse effects, whereas perforation of the round window membrane is likely necessary for inner ear damage.

In conclusion, repeated application of small volumes of isotonic 0.1% or 1.0% NCT to the middle ear proved to be harmless in the guinea pig model, provided that the window membranes were intact, prohibiting direct contact of NCT with the inner ear epithelia. This study confirms our assumption that NCT is relatively safe for application to the external auditory canal for treatment of otitis externa. Whether small single doses are tolerated in the infected middle ear and are suitable for treatment of middle ear inflammation cannot be answered presently. It will be a future challenge to investigate the safety and efficacy of NCT in an infected animal model.

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