N-Chlorotaurine, a Novel Endogenous Antimicrobial Agent

Tolerability Testing in a Mouse Model

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Objective: To investigate the tolerability of N-chlorotaurine, a new antimicrobial agent, by application to the middle ear in a mouse model.

Methods: Five BALB/c mice were each injected through the tympanic membrane with 5 µL of 0.1%, 1.0%, and 10% N-chlorotaurine and compared with animals in which 0.9% isotonic sodium chloride solution, 0.2% gentamicin sulfate, and 0.25% trimethylin chloride were instilled. Auditory brainstem responses to clicks were evaluated repeatedly between 4 and 75 days after injection, and histologic investigations of the inner ear were performed subsequently. Three additional groups of mice were injected with isotonic sodium chloride solution, 1.0% N-chlorotaurine, and 0.25% trimethyltin, and brainstem responses to tone bursts of 8, 16, and 32 kHz were tested. In addition, the middle ear was examined histologically.

Results: Mice treated with isotonic sodium chloride solution, 0.1% N-chlorotaurine, and 0.2% gentamicin sulfate did not show changes in response threshold. Treatment with 1.0% and 10% N-chlorotaurine caused a reversible increase in auditory brainstem response threshold by 20 dB 4 days after application because of local irritation around the perforation of the tympanic membrane. In contrast, 0.25% trimethyltin showed a permanent elevation of auditory brainstem response threshold of 10 to 15 dB and a scattered loss of outer hair cells predominantly in the apical turn. No alterations of the inner ear were observed in the other treatment groups. The mucous membrane of the middle ear remained unaffected in all test groups.

Conclusion: Application of N-chlorotaurine to the middle ear is well tolerated without adverse effects and may be a useful new endogenous antimicrobial agent for local treatment of otologic infections.

MATERIALS AND METHODS

REAGENTS

Pure NCT as a crystalline sodium salt (molecular weight, 181.52 g/mol) was dissolved in sterile distilled water (pH 8.1) to concentrations of 0.1%, 1.0%, and 10%. Gentamicin sulfate (80-mg/mL aqueous solution; Tyrol Pharma, Vienna, Austria) was diluted to 2 mg/mL (0.2%) in distilled water. Triethylmethyl chloride (TMT) (Sigma-Aldrich Corp, St Louis, Mo) was dissolved in isotonic sodium chloride solution to a concentration of 0.25%. For anesthesia, ketamine hydrochloride (10-mg/mL aqueous solution; Parke-Davis, Berlin, Germany) and xylazine hydrochloride (20-mg/mL aqueous solution; Graeub Inc, Bern, Switzerland) were used. Xylazine was 10-fold diluted in distilled water to 2 mg/mL.

ANIMALS AND TOLERABILITY TESTS

Male 6- to 8-week-old BALB/c mice (22-29 g) with otoscopically normal findings, particularly unaffected tympanic membranes, were used. Animal tests were performed according to the principles of animal care and approved by the Austrian Federal Government of Science and Research. Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (10 mg/100 g of body weight=1 mL/100 g of the 10-mg/mL stock) and xylazine hydrochloride (1 mg/100 g of body weight=0.5 mL/100 g of the 2-mg/mL stock).

Immediately after these measurements, mice were divided into 6 groups with different treatment: group 1 (control animals) was treated with 0.9% isotonic sodium chloride solution; groups 2 to 4 (test groups) with 0.1%, 1.0%, and 10% NCT, respectively; and groups 5 and 6 (positive control groups) with the ototoxicants 0.2% gentamicin sulfate and 0.25% TMT, respectively (n=5 per group). Five microliters of each solution was injected through the posteroinferior quadrant of the tympanic membrane with a special syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). This procedure was performed on both ears, leading to a saturation of the middle ear bullae.

RESULTS

AUDITORY BRAINSTEM RESPONSES

In unselected BALB/c mice exposed to clicks, the ABR threshold ranged between 30- and 50-dB sound pressure level before treatment (starting point, 0 dB; Figure 1).

The control group treated with isotonic sodium chloride solution showed no change in ABR threshold (within 5 dB) at 4 to 75 days later. The groups treated with NCT showed no elevation of ABR threshold at the 0.1% concentration, whereas injection of 1% and 10% NCT caused an elevation of ABR threshold of about 20 dB after 4 days. This increase of ABR threshold returned to the starting point at day 14 for 1% NCT and at day 21 for 10% NCT. Mice treated with 0.2% gentamicin and 2 additional mice treated with 8.0% gentamicin maintained normal thresholds, whereas injection of 0.25% TMT led to a significant permanent elevation of ABR threshold of 10 to 15 dB. Additional ABR tests performed with tone bursts of 8 and 16 kHz evoked auditory potentials at minimum sound pressure levels between 40 and 50 dB, while thresholds for ABRs at 32-kHz bursts were markedly elevated (70-80 dB) in BALB/c mice. Subsequent to challenge with
isotonic sodium chloride solution or 1% NCT, threshold at all frequencies was slightly higher on day 7 and almost completely normal again on day 14. Treatment with 0.25% TMT, however, caused a marked and prolonged increase of the threshold of 10 to 20 dB for all frequencies tested.

VISIBLE MORPHOLOGIC CHANGES

Visual inspection of the external auditory canal and the tympanic membrane disclosed incrustation of the artificial perforation connected with tymefaction of the surrounding tympanic membrane in all mice, being more pronounced in animals treated with 1% and 10% NCT in a dose-dependent manner. Perforations healed and tymefaction disappeared 1 to 2 weeks after exposure in animals treated with isotonic sodium chloride solution. The respective time was 2 weeks for 1% NCT and 3 weeks for 10% NCT. Treatment with 0.1% NCT, 0.2% and 8.0% gentamicin, and 0.23% TMT did not cause any visible changes different from those with isotonic sodium chloride solution or delayed healing.

There was no clinical evidence of vestibular hypofunction, since the behavior of the animals did not change. In particular, no head tilt, dizziness, or circling was observed when the animals awakened from anesthesia.

HISTOLOGIC FINDINGS

No alterations of the inner ear were observed in mice exposed to isotonic sodium chloride solution and in mice treated with 0.1%, 1%, and 10% NCT (Figure 2 and Figure 3A). The inner ears of these mice showed normal inner and outer hair cells and no reduction in hair cells. By contrast, in animals treated with TMT, we found a scattered loss of outer hair cells of 30% in the apical turn, 30% in the middle turn, and 25% in the basal turn (Figure 3B and Figure 4). At the level of the spiral ganglion, no alteration was observed. In the mouse model, where we applied gentamicin, we could not identify visible histologic changes at the light microscopic level. Concerning effects on the middle ear mucosa, no alterations were detected on histologic evaluation. The epithelium appeared unaffected; there was no edema or fibrosis of the submucosa and no ossification.

COMMENT

In accord with previous investigations in the rabbit and human eye, as well as in the human urinary tract, NCT was tolerated without long-term adverse effects by application to the middle ear in our model. A concentration of 0.1% proved to be free of toxic effects, while 1% and, to a greater extent, 10% caused some local irritation around the artificial perforation of the tympanic membrane. This alteration, which probably affects the vibration of the membrane, may explain the temporary increase in ABR threshold. Since 1% NCT has been well tolerated and proved to be sufficiently active against bacteria.
both in vitro and in vivo.11,13 This concentration will be preferred for further clinical trials. Slight irritative effects were masked by inflammatory symptoms when NCT was applied in patients with infectious conjunctivitis.12

The present study also confirmed the therapeutic safety of 1% NCT application, because even 10% NCT did not cause severe or permanent changes of ABR threshold for click stimulation. This finding was further substantiated by the samples tested with frequency-specific ABR. The efficiency of the test design was verified by the fact that the positive control group treated with TMT showed a permanent increase of ABR threshold as well as scattered loss of outer hair cells along all turns of the cochlea. In contrast, NCT did not produce any alteration in different turns. The absence of toxic effects in the inner ear may be explained by the hydrophilic character of NCT.10 It cannot penetrate membranes by simple diffusion, but only by active transport mechanisms,17 so that high concentrations are unlikely to occur within the cochlea after application to the middle ear.

Trimethyltin, a substance known to induce a rapid increase of ABR thresholds after intraperitoneal injection in guinea pigs,18,19 led to extensive destruction of hair cells after a single application to the middle ear of BALB/c mice. Both methods of exposure result in a hearing loss at a wide range of frequencies, when the same absolute dose of 0.5 mg/kg is used, indicating significant penetration of TMT into the cochlea. Therefore, TMT serves as a sufficient positive control in our experimental design. By contrast, single application of gentamicin did not cause an elevation of the ABR threshold, even when a concentration of 80 mg/mL was used. This finding confirms the observation by Nordemar and Anniko20 that only repeated exposure to aminoglycosides leads to a permanent destruction of hair cells, although Janas et al21 found damage to cochlear hair cells in the chicken after a single high dose of gentamicin, followed by regeneration within 5 weeks.

According to our experience with transtympanic application of different substances, daily challenge with this method is not feasible because of the artificial damage of the tympanic membrane causing conductive hearing loss. On the other hand, a single instillation of certain disinfectants (alcohol, chlorhexidine, quaternary ammonium compounds) to the middle ear was sufficient to demonstrate cochlear damage beyond all doubt.22,23 Moreover, in these studies, the agent was washed out again with isotonic sodium chloride solution after 10 to 60 minutes, which was not the case in the present study. N-chlorotaurine has been shown to retain oxidative capacity within human body fluids and inflammation samples for several hours,10,16 so that it also can be assumed to be active in the middle ear for such a period. Because of these facts, a considerable incubation time of the test agents can be assumed in our mouse model, which closely matches the condition of clinical application planned. Although NCT is primarily considered to be used for application to the external auditory canal, it is important to note that the middle ear mucosa remained completely unaffected by the substance.

Concerning clinical application, treatment of bacterial otitis externa appears to be an interesting possibility. The generally established therapy with instillation of a local antibiotic in the outer ear is not always sufficient, particularly when resistant strains of bacteria are causing the infection. For such cases, application of NCT could be a promising alternative that can be used even when a perforation of the tympanic membrane cannot be ruled out.

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