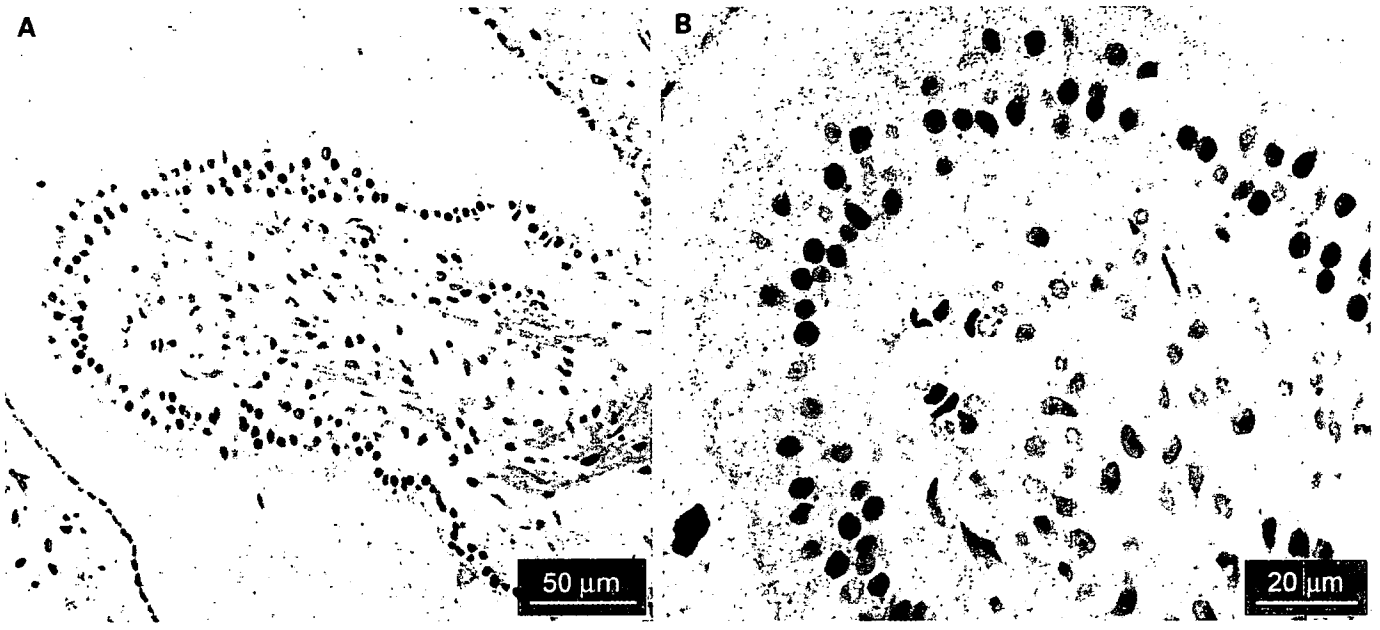

Fig. 4. Immunohistochemical findings of the crista of the horizontal semicircular canal of control animals. **A** CGRP-immunoreactive fibers were located in the connective tissue layer. Bar = 50 μm. **B** A number of CGRP-immunoreactive fibers were located in the connective tissue layer. Bar = 20 μm.

On the other hand, we found that the number of CGRP-immunoreactive fibers passing through the basement membrane increased and that a number of them ramified in order to produce numerous CGRP-immunoreactive terminals within the neurosensory epithelia in the TTX-administered animals 7 days after TTX discontinuation. These increased immunoreactivities were particularly pronounced in the area surrounding the sensory hair cells. We speculate that the increase in CGRP immunoreactivity should be expressed in the vestibular efferent nerve. The vestibular efferent system has both excitatory and inhibitory functions [28–30]. Very little information is available on the functional role of CGRP in the vestibular periphery of higher vertebrates. Adams et al. [31] reported that in frogs, CGRP has an excitatory role in the lateral line organ. On the other hand, Bailey and Sewell [32] reported that CGRP is an inhibitory efferent neurotransmitter that suppresses the responses of the lateral line organ to mechanical displacement. If CGRP has an excitatory effect, the increase in CGRP immunoreactivity in our study might be induced due to an increase in the rate of the release of neurotransmitters from hair cells under the condition of continuously inhibited nerve activity. Previous studies report that a major neurotransmitter of the vestibular efferent synapses is acetylcholine [33, 34] and that CGRP may be one of the many factors of neural origin that stimulate the synthesis of acetylcholine receptors. In this study, we did not investigate the immunohistochemical evaluation of other neurotransmitters or the ultrastructural studies of utricular maculae or horizontal semicircular canals. Further inves-

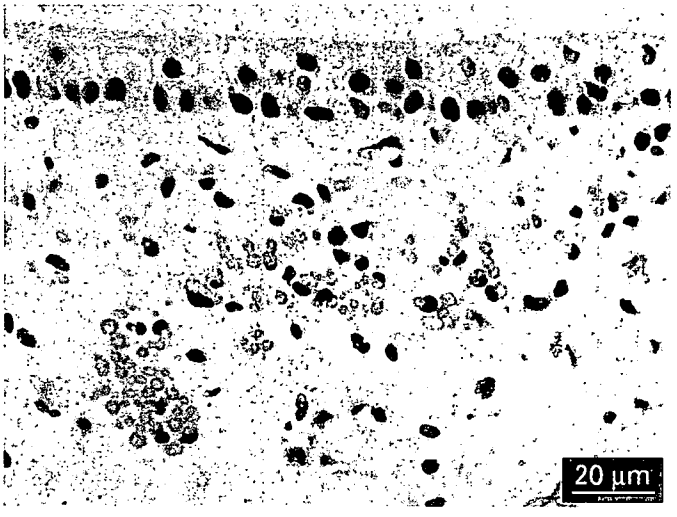

Fig. 5. Immunohistochemical findings of the utricular macula of control animals. A number of CGRP-immunoreactive fibers were located in the connective tissue layer. Bar = 20 μm.

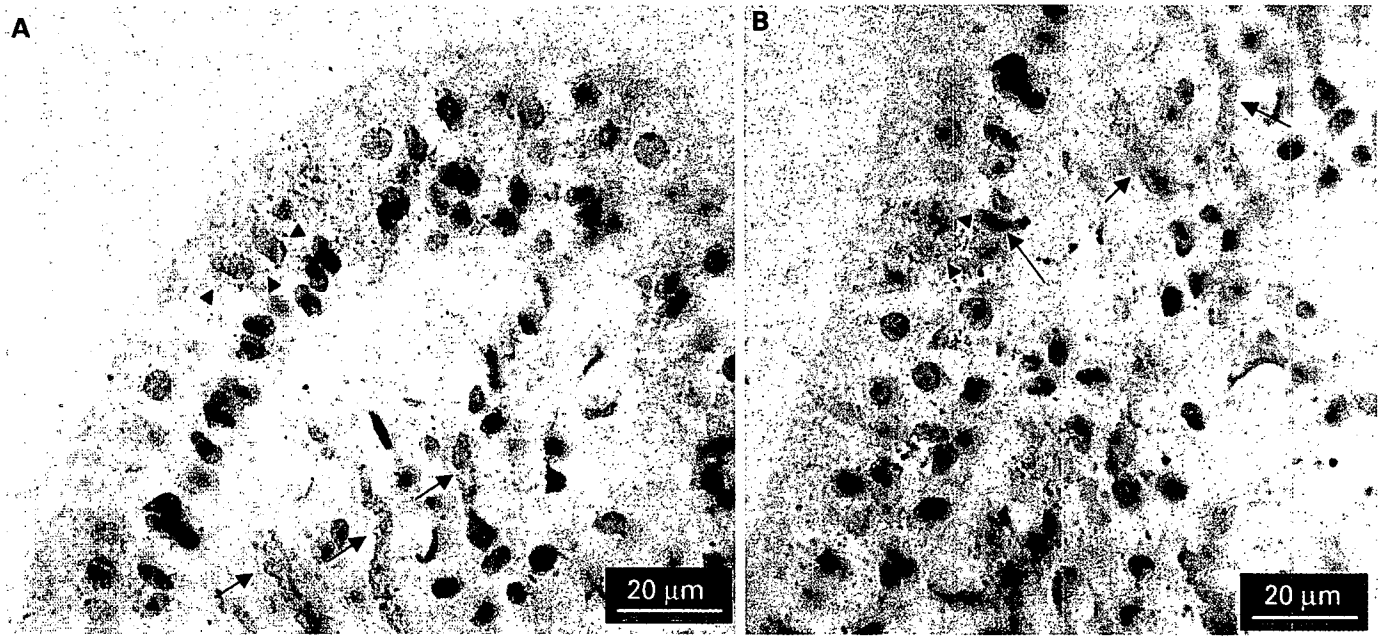

Fig. 6. Immunohistochemical findings of the crista of the horizontal semicircular canal of TTX-treated animals. **A** A number of CGRP-immunoreactive fibers located in the connective tissue layer beneath the sensory epithelium were observed to have increased (arrows). **B** CGRP-immunoreactive terminals within the neurosensory epithelia of the crista were observed to have increased (arrowheads). Bar = 20 μm.

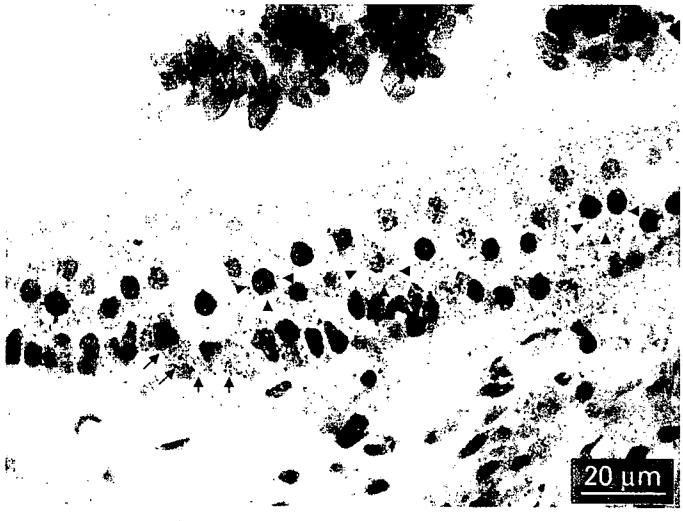

Fig. 7. Immunohistochemical findings of the utricular macula of TTX-treated animals. CGRP-immunoreactive fiber passing through the basement membrane was observed (arrows). CGRP-immunoreactive terminals within the sensory epithelium were observed to have remarkably increased (arrowheads). These increased immunoreactivities were especially marked in the area surrounding the sensory hair cells. Bar = 20 μm.

tigation is needed to better understand the functional role of CGRP in this model.

In this study, neither did we examine the onset of the increase in CGRP immunoreactivity nor the possibility of CGRP immunoreactivity reducing to the pretreatment level later than 7 days after TTX discontinuation. Thus far, the effect of reversible blockage of vestibular input on the efferent system has not been examined. However, with an animal model of vestibular compensation prepared by administering streptomycin into the rat vestibular organ in order to destroy the unilateral vestibular function, Chi et al. [35] reported that the number of CGRP- immunoreactive neurons and the level of CGRP immunoreactivity increased in the efferent vestibular system during the vestibular disorder and that they decreased with vestibular compensation. They concluded that the activity of CGRP in the efferent vestibular system plays a regular role in accelerating vestibular compensation. According to their report, the increase in CGRP immunoreactivity in this model might occur later than 24 h after TTX administration and might reduce later than 7 days after TTX discontinuation because the VOR was completely eliminated at 24 h after TTX initiation and the VOR gain returned to the preoperative level within 120 h after TTX discontinuation. Our model is reversible. In the bilateral

vestibular disorder model, the changes in CGRP immunoreactivity may be different from those in the model with irreversible and unilateral vestibular disorder. Additional studies are required to confirm this hypothesis.

Conclusion

An animal model of reversible bilateral vestibular disorders was established and an obvious increase in the number of CGRP-immunoreactive fibers within the neurosensory epithelia of the maculae and cristae was observed in this model. This model can be used to conduct numerous investigations into the plasticity of the vestibular nervous system.

Acknowledgment

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References

1 Zheng Y, Darlington CL, Smith PF: Bilateral labyrinthectomy causes long-term deficit in object recognition in rat. Neuroreport 2004;15: 1913–1916.
2 Saxon DW: Asymmetric Fos labeling in lobule X of the cerebellum following transtympanic tetrodotoxin (TTX) in the rat. Neurosci Lett 2003;339:57–61.
3 Saxon DW, White G: Episodic blockade of cranial nerve VIII provokes asymmetric changes in lobule X of the rat. Brain Res 2004;997:165–175.
4 Narahashi T, Moore JW, Scott WR: Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. J Gen Physiol 1964;47: 965–974.
5 Narahashi T, Haas HG, Therrien EF: Saxitoxin and tetrodotoxin: comparison of nerve blocking mechanism. Science 1967;147:1441–1442.
6 Katsuki Y, Yanagisawa K, Kanzaki J: Tetraethylammonium and tetrodotoxin: effects on cochlear potentials. Science 1966;151:1544–1545.
7 Pasic TR, Rubel EW: Rapid changes in cochlear nucleus cell size following blockade of auditory nerve electrical activity in gerbils. J Comp Neurol 1989;283:474–480.
8 Born DE, Rubel EW: Afferent influences on brain stem auditory nuclei of the chicken: presynaptic action potentials regulate protein synthesis in nucleus magnocellularis neurons. J Neurosci 1988;8:901–919.
9 Weisleder P, Rubel EW: Reversible blockade of vestibular evoked activity in the chick. Hear Res 1990;47:257–261.
10 Saxon DW, Anderson JH, Beitz AJ: Transtympanic tetrodotoxin alters the VOR and Fos labeling in the vestibular complex. Neuroreport 2001;12:3051–3055.
11 Saxon DW: Distribution of Fos labeling in the inferior olive following transient blockade of the VIIIth cranial nerve. Brain Res 2003;966: 134–149.
12 Dechesne C, Raymond J, Sans A: The efferent vestibular system in the cat: a horseradish peroxidase and fluorescent retrograde tracers study. Neuroscience 1984;11:893–901.
13 Gacek RR, Lyon M: The localization of vestibular efferent neurons in the kitten with horseradish peroxidase. Acta Otolaryngol 1974;77:92–101.
14 Warr WB: Olivocochlear and vestibular efferent neurons of the feline brain stem: their location, morphology and number determined by retrograde axonal transport and acetylcholinesterase histochemistry. J Comp Neurol 1975; 161:159–181.
15 Wackym PA: Ultrastructural organization of calcitonin gene-related peptide immunoreactive efferent axons and terminals in the vestibular periphery. Am J Otol 1993;14:41–50.
16 Dememes D, Broca C: Calcitonin gene-related peptide immunoreactivity in the rat efferent vestibular system during development. Brain Res Dev Brain Res 1998;108:59–67.
17 Dememes D, Dechesne CJ, Venteo S, Gaven F, Raymond J: Development of the rat efferent vestibular system on the ground and in microgravity. Brain Res Dev Brain Res 2001;128: 35–44.
18 Shimogori H, Yamashita H: Efficacy of intracochlear administration of betamethasone on peripheral vestibular disorder in the guinea pig. Neurosci Lett 2000;294:21–24.
19 Shimogori H, Yamashita H: Rapid correction of vestibular imbalance by intracochlear administration of ATP in a guinea pig model of unilateral peripheral vestibular disorder. Neurosci Lett 2001;315:69–72.
20 Sugahara K, Shimogori H, Yamashita H: The role of acidic fibroblast growth factor in recovery of acoustic trauma. Neuroreport 2001;12: 3299–3302.
21 Horiike O, Shimogori H, Ikeda T, Yamashita H: Protective effect of edaravone against streptomycin-induced vestibulotoxicity in the guinea pig. Eur J Pharmacol 2003;464:75–78.
22 Horiike O, Ikeda T, Shimogori H, Sugahara K, Hashimoto M, Okuda T, Yamashita H: Development of eye movement image analysis technique in conscious guinea pig (in Japanese, abstract in English). Equilib Res 2002;61:28–33.
23 Ross MD: Morphological changes in rat vestibular system following weightlessness. J Vestib Res 1993;3:241–251.
24 Ross MD: A spaceflight study of synaptic plasticity in adult rat vestibular maculas. Acta Otolaryngol Suppl 1994;516:1–14.
25 Tanaka M, Takeda N, Senba E, Tohyama M, Kubo T, Matsunaga T: Localization of calcitonin gene-related peptide in the vestibular endorgans in the rat: an immunohistochemical study. Brain Res 1988;447:175–177.
26 Tanaka M, Takeda N, Senba E, Tohyama M, Kubo T, Matsunaga T: Localization, origin and fine structure of calcitonin gene-related peptide-containing fibers in the vestibular endorgans of the rat. Brain Res 1989;504:31–35.
27 Ohno K, Takeda N, Tanaka-Tsuji M, Matsunaga T: Calcitonin gene-related peptide in the efferent system of the inner ear: a review. Acta Otolaryngol Suppl 1993;501:16–20.
28 Goldberg JM, Fernandez C: Efferent vestibular system in the squirrel monkey: anatomical location and influence on afferent activity. J Neurophysiol 1980;43:986–1025.
29 Highstein SM, Baker R: Action of the efferent vestibular system on primary afferents in the toadfish, *Opsanus tau*. J Neurophysiol 1985; 54:370–384.
30 Rossi ML, Prigioni I, Valli P, Casella C: Activation of the efferent system in the isolated frog labyrinth: effects on the afferent EPSPs and spike discharge recorded from single fibres of the posterior nerve. Brain Res 1980;185:125–137.
31 Adams JC, Mroz EA, Sewell WF: A possible neurotransmitter role for CGRP in a hair-cell sensory organ. Brain Res 1987;419:347–351.
32 Bailey GP, Sewell WF: Calcitonin gene-related peptide suppresses hair cell response to mechanical stimulation in the Xenopus lateral line organ. J Neurosci 2000;20:5163–5169.
33 Guth PS, Norris CH: The hair cell acetylcholine receptors: a synthesis. Hear Res 1996;98: 1–8.
34 Iurato S, Luciano L, Pannese E, Reale E: Efferent vestibular fibers in mammals: morphological and histochemical aspects. Prog Brain Res 1972;37:429–443.
35 Chi F, Wang Z, Li K: Calcitonin gene-related peptide changes in efferent vestibular system during vestibular compensation (in Chinese, abstract in English). Zhonghua Er Bi Yan Hou Ke Za Zhi 1999;34:11–13.

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Research Report

The systemic application of diazepam facilitates the reacquisition of a well-balanced vestibular function in a unilateral vestibular re-input model with intracochlear tetrodotoxin infusion using an osmotic pump

Kenji Takeno, Hiroaki Shimogori, Tsuyoshi Takemoto, Kuniyoshi Tanaka, Takefumi Mikuriya, Hiroshi Orita, Hiroshi Yamashita *

Department of Otolaryngology, Yamaguchi University School of Medicine, Minamikogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan

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TTX, tetrodotoxin
VOR, vestibuloocular reflex
DP, directional preponderance of the nystagmus
MVN, medial vestibular nucleus
GABA, γ-aminobutyric acid

ABSTRACT

Diazepam is a popular medicine used in the treatment of acute vertigo. In the past, many studies investigating the effect of diazepam in peripheral vestibular destruction have been reported. However, no previous study has yet investigated the effect of diazepam on a model with a transient and reversible vestibular function similar to recurrent vertigo as seen in Meniere's disease. We thus made a peripheral vestibular re-input model by the unilateral intracochlear administration of tetrodotoxin (TTX) using an osmotic pump and then examined the influence of diazepam on the vestibular system in this model. Hartley white guinea pigs were intracochlearly administered with TTX on the right side for 3 days by an osmotic pump. Animals were divided into three groups, TTX alone (control group (n = 7)), TTX and an intraperitoneal diazepam injection once a day for 3 days (diazepam group (n = 6)) and vehicle injection (vehicle group (n = 6)). A caloric response and vestibuloocular reflex (VOR) were observed at 7 and 14 days after completing 3 days of TTX administration. Seven days after vestibular re-input, a directional preponderance of the nystagmus (DP) to the TTX-treated side was observed in the control and vehicle groups on VOR examination. DP was not observed in the diazepam group on any examined day. The R/L time ratio of caloric response showed no statistical difference between three groups on any examined day. These results suggest that diazepam may thus be useful for patients in an acute stage of peripheral vestibular vertigo by decreasing their vertiginous symptoms.

1. Introduction

Following peripheral vestibular destruction, body trunk deviation to the lesioned side and spontaneous nystagmus to the intact side are observed, however, these symptoms tend to gradually decrease over time. This phenomenon is well known as "vestibular compensation", namely the plasticity of the central nervous system. Many studies on vestibular compensation have been previously reported (Smith and Darlington, 1991; Curthoys and Halmagyi, 1995; Dieringer, 1995). However,

* *Corresponding author.* Fax: +81 22 836 22 2280.
E-mail address: hiro-shi@yamaguchi-u.ac.jp (H. Yamashita).

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peripheral vestibular disorder tends to be clinically transient rather than permanent (Brandt, 1999).

In the past, several studies have reported using TTX on the inner ear for the transient blockage of the peripheral vestibular or cochlear input (Weisleder and Rubel, 1990; Brown et al., 1993; Praetorius et al., 2001; Saxon, 2003). The aim of the present study was thus to make a transient peripheral vestibular input blockage model by tetrodotoxin (TTX) administration directly to the inner ear by an osmotic pump and to investigate the effect of diazepam which was commonly used for acute vertiginous patients in clinical (Brandt, 1999; Cesarani et al., 2004) using this model.

2. Results

2.1. VOR gain

2.1.1. Control group

At 3 days after operation, vestibuloocular reflex (VOR) was observed to have almost vanished. The VOR gains on first, second and third rotation to the TTX-treated side were 0.045 ± 0.061, 0.023 ± 0.060, 0.017 ± 0.045, respectively. The VOR gain on each rotation to the intact side decreased, and a statistical difference between preoperation and 3 days after operation was observed (preoperation vs. 3 days after operation; 0.476 ± 0.108 vs. 0.164 ± 0.105; $P = 0.0027$, 0.485 ± 0.081 vs. 0.184 ± 0.121; $P = 0.0027$, 0.452 ± 0.090 vs. 0.174 ± 0.134; $P = 0.0040$) (Fig. 1).

At 7 days after the vestibular re-input, there was no statistical difference in the VOR gains between right and left at first rotation (0.320 ± 0.144 vs. 0.416 ± 0.146; $P = 0.2248$). However, a significant difference was seen in the VOR gains at second and third rotation between the right and left side (0.242 ± 0.136 vs. 0.497 ± 0.164; $P = 0.0127$ and 0.171 ± 0.108 vs. 0.556 ± 0.123; $P = 0.0017$) (Fig. 1). The third rotation showed a greater discrepancy than the second rotation. In addition, the postrotatory nystagmus toward the lesioned side was observed (Fig. 3).

At 14 days after vestibular re-input, the VOR gain on each rotation recovered to the preoperative value, and no statistical difference was seen in the VOR gains between preoperation and 14 days after vestibular re-input or between the right and left side on each rotation (preoperation vs. 14 days after vestibular re-input; 0.507 ± 0.055 vs. 0.526 ± 0.093; $P = 0.5672$, 0.480 ± 0.065 vs. 0.509 ± 0.144; $P = 0.7494$, 0.475 ± 0.078 vs. 0.491 ± 0.117; $P = 0.8480$ on the TTX-treated side; 0.476 ± 0.108 vs. 0.472 ± 0.123; $P = 0.7494$, 0.485 ± 0.081 vs. 0.450 ± 0.121; $P = 0.5653$, 0.452 ± 0.090 vs. 0.402 ± 0.124; $P = 0.3379$ on the intact side) (Fig. 1). In addition, no postrotatory nystagmus was observed.

2.1.2. Diazepam and vehicle group

In the vehicle group, no statistical difference was seen in the VOR gains of all time courses between the control and the vehicle group.

In the diazepam group, at 3 days after operation, the VOR gains on each rotation to both sides were greater than in the vehicle group, but no statistical difference was seen in the VOR gains between the right and left side on each rotation (vehicle vs. diazepam group; 0.000 ± 0.000 vs. 0.104 ± 0.121; $P = 0.0614$, 0.015 ± 0.037 vs. 0.102 ± 0.097; $P = 0.0688$, 0.018 ± 0.044 vs. 0.078 ± 0.115; $P = 0.2589$ on the TTX-treated side; 0.200 ± 0.081 vs. 0.221 ± 0.081; $P = 0.6642$, 0.198 ± 0.050 vs. 0.227 ± 0.100; $P = 0.5376$, 0.165 ± 0.056 vs. 0.215 ± 0.120; $P = 0.3788$ on the intact side) (Fig. 2).

At 7 days after vestibular re-input, no statistical difference was seen in the VOR gains between the right and left side on each rotation (0.476 ± 0.205 vs. 0.441 ± 0.242; $P = 0.6310$, 0.538 ± 0.181 vs. 0.401 ± 0.193; $P = 0.3358$, 0.493 ± 0.147 vs. 0.369 ± 0.151; $P = 0.2002$) (Fig. 2). These results were different from those of the other groups. In addition, the number of postrotatory nystagmus was statically smaller than that of the vehicle group (3.167 ± 2.137 vs. 0.667 ± 1.211; $P = 0.0318$) (Fig. 3).

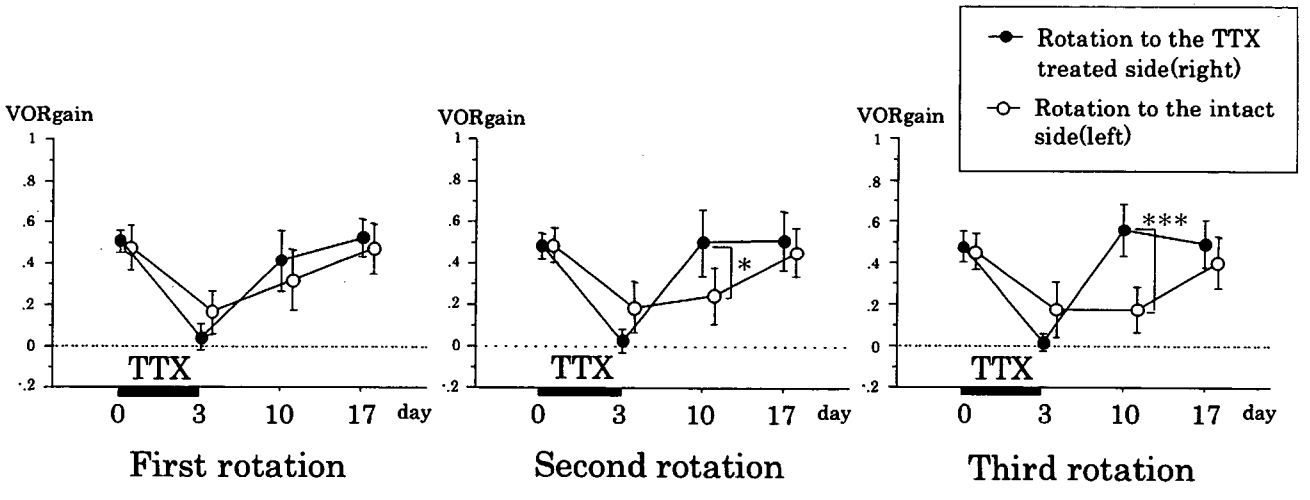

Fig. 1 – The VOR gain of each rotation in the control group. The VOR gain mean ± SD is plotted. At 7 days after vestibular re-input, there was no statistical difference in the VOR gains between right and left at first rotation. But there were significant differences in the VOR gains at second and third rotations between right and left. Third rotation showed greater discrepancy than second rotation. *$P < 0.05$, ***$P < 0.005$. TTX: tetrodotoxin, VOR: vestibuloocular reflex.

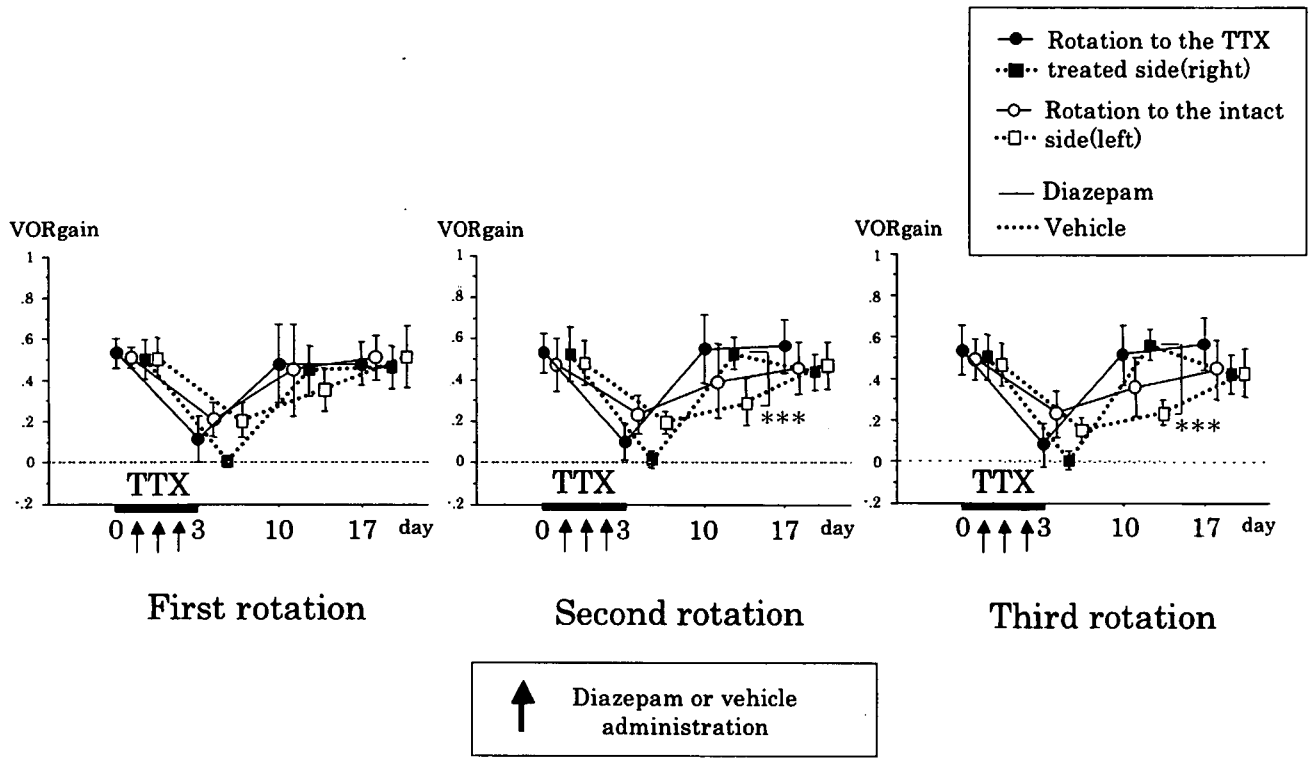

Fig. 2 – The VOR gains of each rotation in the diazepam and vehicle group. The VOR gain mean ± SD is plotted. In vehicle group, at 7 days after vestibular re-input, as well as the control group, there were significant differences in the VOR gains at second and third rotations between right and left. Third rotation showed greater discrepancy than second rotation. In the diazepam group, at 7 days after vestibular re-input, there was no statistical difference in the VOR gains between right and left on each rotation. ***$P < 0.005$. TTX: tetrodotoxin, VOR: vestibuloocular reflex.

At 14 days after vestibular re-input, the VOR gains of each rotation recovered to the preoperative value as well as the other groups, and no statistical difference was seen in the VOR gains between the right and left side on each rotation (0.492 ± 0.108 vs. 0.492 ± 0.110; P = 0.9999, 0.545 ± 0.128 vs. 0.456 ± 0.132; P = 0.3776, 0.541 ± 0.115 vs. 0.460 ± 0.142; P = 0.2623) (Fig. 2).

2.2. *Caloric test*

2.2.1. *Control group*

At 3 days after operation, canal paralysis (CP) on the TTX-treated side was observed. In addition, the duration time of the nystagmus on the intact side was shorter than that of preoperation.

At 7 days after vestibular re-input, the caloric response showed a gradual recovery, but statistical difference of the R/L ratio remained between the preoperative findings and those at 7 days after vestibular re-input (1.013 ± 0.089 vs. 0.481 ± 0.202; P = 0.0026) (Fig. 4).

At 14 days after vestibular re-input, the R/L ratio improved remarkably, and no statistical difference was seen between the preoperative levels and those at 14 days after vestibular re-input (1.013 ± 0.089 vs. 0.918 ± 0.299; P = 0.0845) (Fig. 4).

2.2.2. *Diazepam and vehicle group*

In the vehicle group, no statistical difference was seen in the R/L ratio at any time courses between the control and the vehicle group.

In the diazepam group, at 3 days after operation, CP on the TTX-treated side was observed as well as in the other groups. However, the duration time of the nystagmus on the intact side tended to be longer than that of the control.

At 7 days after vestibular re-input, the caloric response showed a gradual recovery the same as in the other groups (vehicle vs. diazepam, 0.387 ± 0.091 vs. 0.569 ± 0.235; P = 0.1062) (Fig. 4), but a statistical difference in the R/L ratio remained between the preoperative levels and those at 7 days after vestibular re-input (0.993 ± 0.046 vs. 0.569 ± 0.235; P = 0.0129) (Fig. 4).

At 14 days after vestibular re-input, the R/L ratio improved remarkably the same as in the control group. As a result, no statistical difference was seen between the preoperative levels and those at 14 days after vestibular re-input (0.993 ± 0.046 vs. 0.953 ± 0.306; P = 0.5218) (Fig. 4). In addition, no statistical difference was seen between the vehicle group and the diazepam group (1.097 ± 0.327 vs. 0.953 ± 0.306; P = 0.4509) (Fig. 4).

3. Discussion

TTX is a voltage-dependent sodium channel blocker which is able to block the action potential of neurons without causing any histological damage. In the past, several studies have reported using TTX on the inner ear for the transient blockage of peripheral vestibular or cochlear input (Weisleder and Rubel, 1990; Brown et al., 1993; Praetorius et al., 2001; Saxon, 2003). We previously reported that, according to our method,

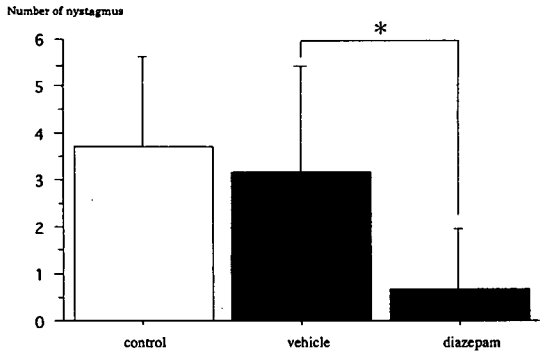

Fig. 3 – The postrotatory nystagmus of each group after 7 days from vestibular re-input. Number of nystagmus beats as the mean ± SD is plotted. No statistical difference showed the number of postrotatory nystagmus between the vehicle group and the control group. However, the number of postrotatory nystagmus in the diazepam group was statistically smaller than that in the diazepam group. *$P < 0.05$.

no functional or histological damage occurred in the inner ear by the direct intracochlear administration of drugs using an osmotic pump in guinea pigs (Shimogori et al., 1999; Shimogori and Yamashita, 2000a,b, 2001). In the present study, we used the TTX characteristics and our osmotic pump implantation technique to study vestibular compensation on the process of the functional recovery of the vestibular periphery.

In a guinea pig, at 48 h after a unilateral labyrinthectomy, the sensitivity of the contralesional vestibular neurons to horizontal rotation stimulation decreased to a half of control (Ris and Godaux, 1998). We confirmed that no spontaneous nystagmus was observed in any animals from each group within 60 h from the operation. This result is assumed to be one of the vestibular compensation processes that is caused by cerebellar inhibition to the intact side. Our results thus suggested the process of vestibular compensation between TTX administration to unilateral inner ear and unilateral labyrinthectomy to be similar.

At 14 days after vestibular re-input, the vestibular function recovered to preoperative levels, and no statistical difference was seen between the right and left side, thus indicating that our model was a reversible peripheral vestibular damage model. The VOR asymmetry by a unilateral labyrinthectomy has been shown to be permanent (Maioli and Precht, 1985; Fetter and Zee, 1988). It is a characteristic of our model that VOR asymmetry, which once occurred, later vanished.

Concerning the inhibitory system to the vestibular nucleus, two pathways are well known, namely the commissural and cerebellum. Both pathways have been said to be GABAergic (Prechet et al., 1973; Kitahara et al., 1997). Several previous studies have suggested that an adaptive regulation of the functional efficacy of GABA receptor could play a role as an important mechanism in the control of excitability of the medial vestibular nucleus (MVN) neurons (Eleore et al., 2005). Based on another study, following a unilateral labyrinthectomy in the rat, down-regulation of GABA receptor in the ipsilesional medial vestibular nucleus (MVN) neurons and up-regulation of GABA receptor in the contralesional MVN neurons were observed (Yamanaka et al., 2000). These results could be due to changes in the affinity and/or efficacy rather than changes in total protein expression (Gliddon et al., 2005). Moreover, the cerebellar inhibition to the contralesional MVN neurons continued for 1 week and then disappeared at 2 weeks (Kitahara et al., 1995, 1997).

In this study, the results at 7 days after vestibular re-input suggested that the cerebellar inhibition to the MVN neurons on the intact side might remain. The accumulation of sinusoidal rotation stimulation might cause an increase in the inhibition to the intact side of MVN neurons and a decrease in the inhibition to the TTX-treated side of MVN neurons. As a result, the directional preponderance of nystagmus (DP) to the TTX-treated side may thus be formed.

We often clinically use the head-shaking test to observe the presence of any after shaking nystagmus as semicircular canal stimulus was done as one of the vestibular function tests (Takahashi et al., 1990; Katsarkas et al., 2000; Guidetti et al., 2002; Pérez Vazquez et al., 2004). When vestibular function asymmetry existed, semicircular canal stimulus by shaking the head accumulated on the vestibular commissure (Holstein et al., 1999). Head-shaking nystagmus, namely nystagmus toward the contralesional side, was observed after shaking the head by discharging the accumulated velocity that is known to be a velocity storage mechanism (Raphan et al., 1979; Hain et al., 1987). The nystagmus toward the lesioned side which occurs after the head-shaking test was observed in the recovery period of peripheral vestibular disorder (Matsuzaki and Kamei, 1995). The direction of head-shaking nystagmus correlated with that of postrotatory nystagmus (Takahashi et al., 1990). In this model, the DP observed during vestibular recovery may be related to the velocity storage mechanism.

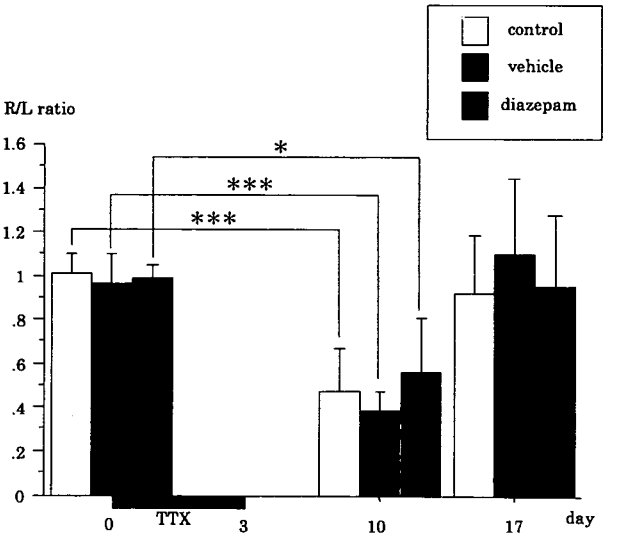

Fig. 4 – The R/L ratios of each group in caloric test. The R/L ratio mean ± SD is plotted. At 7 days after vestibular re-input, caloric response showed a gradual recovery on all groups. Statistical difference of the R/L ratio remained between preoperation and at 7 days after vestibular re-input on all groups. At 14 days after vestibular re-input, the R/L ratio improved remarkably in both groups. No statistical difference was observed between all groups on any examined day. *$P < 0.05$, ***$P < 0.005$. TTX: tetrodotoxin.

In the past, there have been many studies about the effect of diazepam for vestibular compensation in the peripheral vestibular destruction model. These studies insisted that diazepam reduced the static symptoms in the acute stage of peripheral vestibular damage, but it did not impair vestibular compensation process (Martin et al., 1996).

In vestibular research, Hutchinson et al. examined the neuronal activity in the MVN neuron using diazepam 2 mg/kg i.p. injection in the guinea pig (Hutchinson et al., 1995). In another study, once daily 6 mg/kg i.p. injection of diazepam for 5 days did not produce any significant tolerance (Smith and Darlington, 1994). We referred to these studies, so we used a daily 2 mg/kg i.p. injection of diazepam for 3 days.

It is known that diazepam inhibits neuronal activity in the MVN neuron by conjugating $GABA_A$ receptors in the MVN neurons. At acute stage of peripheral unilateral vestibular disorder, diazepam decreases vestibular asymmetry. Our results suggested that diazepam reduces the inhibition to the intact side MVN neurons induced by a vestibular compensation mechanism when vestibular re-input was going on.

A caloric test is an effective clinical examination of peripheral vestibular function (Saadat et al., 1995; Allum and Ledin, 1999; Enticott et al., 2003). Observations of the DP by rotation stimulus have also been reported to be effective for investigating the progress of rehabilitation in chronic vertiginous patients (Szturm et al., 1994; Black et al., 2001; Enticott et al., 2005).

At 7 days after vestibular re-input, DP to the TTX-treated side was observed in the sinusoidal rotation, but the duration time in the caloric response on the TTX-treated side statistically decreased in comparison to that observed preoperatively. These results look conflicting.

In our model, in the process of the vestibular function recovery, it was suggested that the central nervous system affected the VOR gain strongly rather than the caloric response. In the findings of the caloric test, at 7 days after vestibular re-input, the diazepam group showed less asymmetry of the caloric response than the vehicle group, but no statistical difference was seen between the two groups. Thus, indicating that, when we try to evaluate vestibular asymmetry, the duration time in the caloric stimulus may thus be less sensitive than the VOR gain in the sinusoidal rotation stimulus.

Inhibiting the asymmetry of the VOR at an acute stage of peripheral vestibular disorder leads to a decrease in the vertiginous symptoms of the dynamic equilibrium.

Our results show direct evidence that diazepam facilitates the reacquisition of a well-balanced vestibular function in the acute stage of a peripheral vestibular disorder, thus indicating the diazepam administration commonly used for patients with acute peripheral vestibular disorders to be clinically effective.

4. Experimental procedures

4.1. Animals

Nineteen male Hartley guinea pigs with normal Preyer's reflexes and tympanic membranes were used in this study. The animals were divided into three groups. Seven of the nineteen animals received TTX alone (control group). Six of the nineteen animals received diazepam 2 mg/kg (0.4 ml/kg) i.p. once a day during TTX administration (diazepam group). Six of the remaining animals received vehicle (0.4 ml/kg) i.p. once a day during TTX administration (vehicle group). The experimental protocol was approved by the Committee for Ethics for Animal Experiments of the Yamaguchi University School of Medicine. All experiments were carried out under the Guidelines for Animal Experiments of the Yamaguchi University School of Medicine and the Law and Notification of the Government of Japan.

4.2. Medicine

Diazepam (Cercine, Takeda pharma, Osaka, Japan) was administered i.p. to all animals. The vehicle consisted in 48.5% deionized water, 40% propylene glycol, 5% sodium benzoate, 5% benzoic acid and 1.5% benzyl alcohol.

4.3. Osmotic pump implantation

Before osmotic pump implantation, we observed the VOR and caloric response and used animals without a right and left discrepancy of the vestibular function for this study.

Under ketamine hydrochloride (16 mg/kg, i.p.)–xylazine (16 mg/kg, i.p.) anesthesia, 1.5 ml of lidocaine HCl was injected into the right postauricular region of each guinea pig, and the mastoid bulla was opened by a postauricular incision to allow for the visualization of the round window under a surgical microscopy. A tiny hole was made adjacent to the round window with an ultrasonic mini cutter (USW-335, Honda Electronics co., Aichi, Japan). A catheter filled with TTX (0.03 μM, dissolved in water, Sigma Chemical Co., St. Louis, MO, USA) and connected to an osmotic pump (Model 2002, Alza, Palo Alto, CA, USA) was then inserted. The pump was placed under the skin on the back. After the wound was washed with saline, a small amount of piperacillin sodium was introduced. After wound closure, piperacillin sodium at a dose of 50 mg/kg was injected i.m. and oxytetracycline HCl ointment was applied to the wound. During the operation and for 3 h following the operation, each animal was kept warm using electric blanket. In our previous study, the vestibular function after the implantation of an osmotic pump and the infusion of saline was within the preoperative range (Shimogori et al., 1999; Shimogori and Yamashita, 2000a,b, 2001).

4.4. Evaluation of the vestibular function

At 3 days after operation, after determining the caloric response and VOR observations, the TTX administration was stopped by clamping the catheter. The caloric response and VOR were observed at 7 and 14 days after the 3-day TTX administration was finished (7 and 14 days after vestibular re-input).

VOR was observed using our method (Horiike et al., 2003). In brief, for the purpose of immobilizing the guinea pig, a cage designed to hold the animal still during experiments was mounted on top of a turntable apparatus (Daiichi Medical, Tokyo, Japan). The animal's head was fixed firmly with both

auricles held between sponge-covered plates that held both acoustic meati horizontally such that the midpoint of a straight line joining the lateral semicircular canals was located on the rotation axis of the turntable. We set up an infrared CCD camera (Nagashima Medical, Tokyo, Japan) perpendicular to the sagittal plane of the guinea pig's head and in a plane parallel to the rotational plane of the turntable apparatus. By opening an aperture on the left side of the head cage, eye movements of guinea pigs were videotaped (mini DV format, Canon, Tokyo, Japan) in the dark with the infrared CCD camera. We stored the video images on a computer (Power Mac G4, Apple Computer, CA, USA). Each image was converted to an image file using QuickTime 4.0 optional (Apple Computer). For the automatic analysis of guinea pig eye movement, we created a macro for use with the National Institutes of Health (NIH) Image analysis software (http://rsb.info.nih.gov/nih-image/). Our macro is available at http://www.cc.yamaguchi-u.ac.jp/~ent/gankyu3d/ikeda.html. After capturing the eye movement on the computer with this macro, we removed any unnecessary portions from the images and set the threshold to provide for clear outlines of the pupil. The X–Y center of the pupil was analyzed, and the horizontal and vertical components of eye movements were calculated. We calculated the slow-phase velocities, found the maximum slow-phase velocity and calculated the horizontal vestibuloocular reflex gain by dividing the maximum slow-phase velocity by the peak angular velocity.

We measured the gains with sinusoidal rotation at 0.1 Hz and a peak angular velocity of 60°/s for three rotations. We calculated the VOR gain of first, second and three rotation by dividing the maximal slow-phase velocity of each direction by the peak angular velocity (60°/s).

A caloric test was done with our method (Shimogori and Yamashita, 2004). In brief, the caloric test was performed by irrigation of the external auditory meatus with 5 ml of ice water for 10 s in the dark. Nystagmus was recorded on videotape using an infrared CCD camera, and the caloric response time was measured. We calculated the R/L ratio as the TTX-treated side response time (right) to the untreated side response time (left).

4.5. *Statistical analysis*

Data are expressed as the mean ± SD. The differences in the VOR gain and the L/R ratio and the number of postrotatory nystagmus were analyzed using the StatView version 4.5 J software package for Macintosh (Abacus Concepts, Berkley, CA, USA). The differences in these values among the groups with the same time course were assayed using the unpaired t test, and the differences in these values among differential time course in same group were assayed using the paired t test. A value of $P < 0.05$ was considered to be as statistically significant.

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REFERENCES

Allum, J.H., Ledin, T., 1999. Recovery of vestibulo-ocular reflex-function in subjects with an acute unilateral peripheral vestibular deficit. J. Vestib. Res. 9, 135–144.

Black, F., Gianna-Poulin, C., Pesznecker, S.C., 2001. Recovery from vestibular ototoxicity. Otol. Neurotol. 22, 662–671.

Brandt, T., 1999. Vertigo: Its Multisensory Syndromes, 2nd ed. Springer-Verlag London Limited.

Brown, J.N., Miller, J.M., Altschule, R.A., Nuttall, A.L., 1993. Osmotic pump implant for chronic infusion of drugs into the inner ear. Hear. Res. 70, 167–172.

Cesarani, A., Alpini, D., Monti, B., Raponi, G., 2004. The treatment of acute vertigo. Neurol. Sci. 24, 26–30.

Curthoys, I.S., Halmagyi, G.M., 1995. Vestibular compensation: a review of the oculomotor, neural, and clinical consequences of unilateral vestibular loss. J. Vestib. Res. 5, 67–107.

Dieringer, N., 1995. "Vestibular compensation": neural plasticity and its relations to functional recovery after labyrinthine lesions in frogs and other vertebrates. Prog. Neurobiol. 46, 97–129.

Eleore, L., Vassias, I., Bernat, I., Vidal, P.P., de Waele, C., 2005. An in situ hybridization and immunofluorescence study of $GABA_A$ and $GABA_B$ receptors in the vestibular nuclei of the intact and unilaterally labyrinthectomized rat. Exp. Brain Res. 160, 166–179.

Enticott, J.C., Dowell, R.C., O'Leary, S.J., 2003. A comparison of the monothermal and bithermal caloric tests. J. Vestib. Res. 13, 113–119.

Enticott, J.C., O'Leary, S.J., Briggs, J.S., 2005. Effects of vestibulo-ocular reflex exercises on vestibular compensation after vestibular schwannoma surgery. Otol. Neurotol. 26, 265–269.

Fetter, M., Zee, D.S., 1988. Recovery from unilateral labyrinthectomy in rhesus monkey. J. Neurophysiol. 59, 370–393.

Gliddon, C.M., Darlington, C.L., Smith, P.F., 2005. $GABA_A$ receptor subunit expression in the guinea pig vestibular nucleus complex during the development of vestibular compensation. Exp. Brain Res. 166, 71–77.

Guidetti, G., Monzani, D., Civiero, N., 2002. Head-shaking nystagmus in the follow-up of patients with vestibular diseases. Clin. Otolaryngol. 27, 124–128.

Hain, T.C., Fetter, M., Zee, D.S., 1987. Head-shaking nystagmus in patients with unilateral peripheral vestibular lesions. Am. J. Otolaryngol. 8, 36–47.

Holstein, G.R., Martinelli, G.P., Wearne, S., Cohen, B., 1999. Ultrastructure of vestibular commissural neurons related to velocity storage in the monkey. Neuroscience 93, 155–170.

Horiike, O., Shimogori, H., Ikeda, T., Yamashita, H., 2003. Protective effect of edaravone against streptomycin-induced vestibulotoxicity in the guinea pig. Eur. J. Pharmacol. 464, 75–78.

Hutchinson, M.A., Darlington, C.L., Smith, P.F., 1995. The effects of long-term, low-dose diazepam treatment on the guinea pig righting reflex and medial vestibular nucleus neuronal activity. Pharmacol. Biochem. Behav. 50, 665–669.

Katsarkas, A., Smith, H., Galiana, H., 2000. Head-shaking nystagmus(HSN): the theoretical explanation and the experimental proof. Acta Oto-Laryngol. 120, 177–181.

Kitahara, T., Takeda, N., Saika, T., Kubo, T., Kiyama, H., 1995. Effect of MK801 on Fos expression in the rat brainstem after unilateral labyrinthectomy. Brain Res. 700, 182–190.

Kitahara, T., Takeda, N., Saika, T., Kubo, T., Kiyama, H., 1997. Role of the flocculus in the development of vestibular compensation: immunohistochemical studies with retrograde tracing and flocculectomy using fos expression as a maker in the rat brainstem. Neuroscience 76, 571–580.

Maioli, C., Precht, W., 1985. ON the role of vestibulo-ocular reflex plasticity in recovery after unilateral peripheral vestibular lesions. Exp. Brain Res. 59, 267–272.

Martin, J., Gilchrist, D.P., Smith, P.F., Darlington, C.L., 1996. Early diazepam treatment following unilateral labyrinthectomy does not impair vestibular compensation of spontaneous nystagmus in guinea pig. J. Vestib. Res. 6, 135–139.

Matsuzaki, M., Kamei, T., 1995. Stage-assessment of the progress of continuous vertigo of peripheral origin by means of spontaneous and head-shaking nystagmus findings. Acta Oto-Laryngol., Suppl. 519, 188–190.

Pérez Vazquez, P., Rodriguez Prado, N., Sequeiros Santiago, G., Llorente Pendas, J.L., Gomez Martinez, J.R., Suarez Nieto, C., 2004. Functional significance of peripheral head-shaking nystagmus. Laryngoscope 114, 1078–1084.

Praetorius, M., Limberger, A., Muller, M., Lehner, R., Schick, B., Zenner, H.P., Plinkert, P., Knipper, M., 2001. A novel microperfusion system for the long-term local supply of drugs to the inner ear: implantation and function in the rat model. Audiol. Neuro-Otol. 6, 250–258.

Prechet, W., Schwindt, P.C., Baker, R., 1973. Removal of vestibular commissural inhibition by antagonists of GABA and glycine. Brain Res. 62, 222–226.

Raphan, T., Matsuo, V., Cohen, B., 1979. Velocity storage in the vestibulo-ocular reflex arc (VOR). Exp. Brain Res. 35, 229–248.

Ris, L., Godaux, E., 1998. Neuronal activity in the vestibular nuclei after contralateral or bilateral labyrinthectomy in the alert guinea pig. J. Neurophysiol. 80, 2352–2367.

Saadat, D., O'Leary, D.P., Pulec, J.L., Kitano, H., 1995. Comparison of vestibular autorotation and caloric testing. Otolaryngol.—Head Neck Surg. 113, 215–222.

Saxon, D.W., 2003. Asymmetric Fos labeling in lobule X of the cerebellum following transtympanic tetrodotoxin (TTX) in the rat. Neurosci. Lett. 339, 57–61.

Shimogori, H., Yamashita, H., 2000a. Effectiveness and utility of chemical labyrinthectomy with streptomycin using osmotic pump. ORL, J. Oto-Rhino-Laryngol. Relate Spec. 62, 60–62.

Shimogori, H., Yamashita, H., 2000b. Efficacy of intracochlear administration of betamethasone on peripheral vestibular disorder in the guinea pig. Neurosci. Lett. 294, 21–24.

Shimogori, H., Yamashita, H., 2001. Rapid correction of vestibular imbalance by intracochlear administration of ATP in a guinea pig model of unilateral peripheral vestibular disorder. Neurosci. Lett. 315, 69–72.

Shimogori, H., Yamashita, H., 2004. Peripheral vestibular disorder induced by (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA). Neurosci. Lett. 371, 69–72.

Shimogori, H., Yamashita, H., Watanabe, T., Nakamura, S., 1999. A role of glucocorticoid receptors in the guinea pig vestibular system. Brain Res. 851, 258–260.

Smith, P.F., Darlington, C.L., 1991. Neurochemical mechanisms of recovery from peripheral vestibular lesions (vestibular compensation). Brain Res. Brain Res. Rev. 16, 117–133.

Smith, P.F., Darlington, C.L., 1994. Rapid tolerance to the depressive effects of diazepam on guinea pig motor control using divided doses. Pharmacol. Biochem. Behav. 48, 535–538.

Szturm, T., Ireland, D.J., Lessing-Turner, M., 1994. Comparison of different exercise programs in the rehabilitation of patients with chronic peripheral vestibular dysfunction. J. Vestib. Res. 4, 461–479.

Takahashi, S., Fetter, M., Koenig, B., Dichgans, J., 1990. The clinical significance of head-shaking nystagmus in the dizzy patient. Acta Oto-Laryngol. 109, 8–14.

Yamanaka, T., Him, A., Cameron, A.S., Dutia, M.B., 2000. Rapid compensatory changes in GABA receptor efficacy in rat vestibular neurons after unilateral labyrinthectomy. J. Physiol. 523, 413–424.

Weisleder, P., Rubel, E.W., 1990. Reversible blockade of vestibular evoked activity in the chick. Hear. Res. 47, 257–261.

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エダラボン局所投与法の内耳への影響

折田　浩志、下郡　博明、竹野　研二、御厨　剛史、山下　裕司
山口大学大学院医学系研究科耳鼻咽喉科学分野

A study for the influence of inner ear application of edaravone

Hiroshi Orita, Hiroaki Shimogori, Kenji Takeno, Takefumi Mikuriya, Hiroshi Yamashita
Department of Otolaryngology, Yamaguchi University School of Medicine

The aim of study was to evaluate the safety of edaravone applied into the inner ear of the guinea pig, as assessed physiologically and morphologically.

Edaravone (6 mg/ml)-soaked Gelform pieces were put on the round window membrane of guinea pigs in the right ear. Before and 7 days after treatment, each animal was studied auditory brainstem response (ABR) and caloric test. After physiological examination, ampulla of the lateral semicircular canal, utricle and cochlea were investigated morphologically. No significant ABR threshold shift was observed in the animals between before and 7 days after treatment. No significant difference was found in the caloric response time between the right and left side 7 days after treatment. Seven days after treatment, no obvious morphological change in the vestibular and cochlear endorgans was observed in all animals.

These results suggested that the topical application of edaravone to the inner ear induces no obvious tissue damage physiologically and morphologically.

Key words：edaravone, topical application therapy, caloric test, ABR

はじめに

近年、内耳への直接薬物投与法や、薬物輸送システムが注目を集めている[1)~3)]。内耳局所への投与法の利点としては、薬物の全身に与える影響を最小限にして、かつ効果発現するための十分量が投与できる可能性があることである。さらには、内耳・血管関門を通過しにくい薬物も内耳へ作用させることが可能である。

エダラボン（ラジカット®）は、脳梗塞急性期に用いられているフリーラジカルスカベンジャーである。近年、種々の内耳障害にはラジカルの関与が示唆されており[4)]、ラジカル除去をターゲットとした治療法は、当然その有効性が期待され、我々も過去にいくつかの基礎的研究を報告した[5)~7)]。ストレプトマイシンで一側内耳を障害したモデルに対しエダラボンの全身投与、あるいはエダラボンをゼルフォームに浸して正円窓膜上に留置する局所投与で比較したところ、局所投与が障害後の自発眼振や頭部偏奇を有意に抑制したことを報告した[6)]。このことから、エダラボンをゼルフォームに浸して正円窓膜上に留置する方法で、エダラボンが経外リンパ的に内耳に作用することはほぼ間違いないと考えられ、エダラボンは内耳局所投与治療法の有力な候補となりうる可能性を考えた。

今回我々は、エダラボンの正常内耳への影響を確認するために、エダラボンの局所投与自体が内耳に及ぼす影響を機能的、形態的に評価したので報告する。

方　法

プライエル反射正常、鼓膜正常のハートレイ系白色モルモットの雄6匹を用いた。術前に前庭機能の評価のために、カロリックテストを施行した。カロリックテストは少量氷水注入法（5 ml/10 sec）にて行った。当科で作成した固定器を用いて、頭部が水平面に比し50度挙上される体位で固定した。暗所開眼下で検査を行い、眼球運動を赤外線CCDカメラでビデオテープに録画して、

眼振持続時間を計測した。処置側の眼振持続時間を健常側の眼振持続時間で割った比を算出した。術前の聴覚系の検査として、キシラジン（16mg/kg）、ケタミン（16mg/kg）の腹腔内投与による全身麻酔下にABRを施行した。刺激音は2 kHz、4 kHz、8 kHzの8 msecのトーンバーストを用いた。rise and fall timeは2 msec、durationは4 msecとした。この条件は、波形の明瞭さから当教室で以前より行っているものである。刺激は10cmのチューブをイヤホンに接続し外耳道に挿入して行った。ABR記録装置は、シグナルプロセッサ（Synax1100, NEC Co., Tokyo, Japan）を用いた。10dBステップで測定し、I波、あるいはV波が消失した時点で閾値とした。術前のカロリックテスト、ABRで左右差のない動物を実験に用いた。

キシラジン（16mg/kg）、ケタミン（16mg/kg）の腹腔内投与による全身麻酔下に、右耳後部に1％キシロカイン®を局注し、切開の後側頭骨前面を露出した。手術用顕微鏡下に乳突骨包を開放し、正円窓膜を明視下においた。正円窓膜上に、2×2×2 mmのゼルフォーム片にエダラボンを浸して留置した。エダラボンは手術直前に6 mg/mlに調整したもの（pH 7）を用いた。エダラボンの濃度は、過去の基礎実験では3 mg/mlのものが用いられており、我々もこれまでは同濃度で実験を行ってきたが、安全性を検討するに当たり、より高濃度での内耳への影響を観察するために、6 mg/mlとした。調整したエダラボン溶液は、対生食浸透圧比が1、室温で空気中曝露にて24時間安定であることがわかっている（三菱ウェルファーマ株式会社、personal communication）。エダラボンを浸したゼルフォームを正円窓膜上に留置したまま創部にペントシリン®粉末を散布した後閉創し、テラマイシン®軟膏を塗布した。40mg/kgでペントシリン®を筋注後、3時間電気毛布で保温した。なお、術中は直腸温で37度となるようモニタリングした。

術後1週間目に、術前と同様に、カロリックテスト、ABRを施行した。カロリックテストは術後の左右の反応時間を、ABRは術前後の反応閾値の変化を比較検討した。統計学的検討にはWilcoxonの符号付順位検定を用い、p値が0.05未満を有意と判定した。統計処理に関する値は、すべて平均値±標準偏差で示した。組織学的検討は、Duanらの方法に準じた[8]。検査終了後、ネンブタール深麻酔下に1％パラホルムアルデヒド、2.5％グルタールアルデヒドで外リンパ潅流を行い、断頭後側頭骨を摘出した。実体顕微鏡下に膜迷路摘出の後、0.1Mカコジル酸ナトリウムで洗浄、EDTAで脱灰し、1％オスミウムで1時間染色した。洗浄後、アルコール系列で脱水を行い、プラスチック包埋した。2 μmの切片を作成し、光学顕微鏡下に油浸で観察した。

結　果

カロリックテストでは、術前、術後も反応時間の左右の比には有意な影響は認められなかった（術前：1.072±0.328、術後：0.986±0.150）。図1に、当科でコントロールとして用いている正常モルモット61匹のカロリックテストのデータ（0.994±0.141）と、今回の術後結果とをあわせて示した。コントロールとエダラボン投与群間には統計学的に有意差は認めなかった。ABRの反応閾値は、術前は、2 kHz：43.0±10.954、4 kHz：35.333±15.055、8 kHz：13.333±17.224、術後は、2 kHz：39.667±16.330、4 kHz：42.0±15.492、8 kHz：20.0±18.708であった。術後と術前で、統計学的には各周波数別に有意差は認めなかった（図2）。組織学的所見を図3に示した。前庭器については、外側半規管、卵形嚢を観察した。有毛細胞を含め、感覚上皮はおおむねその形態は保たれていた。蝸牛に関しては、Corti器において、内・外有毛細胞、支持細胞を含め形態には問題を認めなかった。

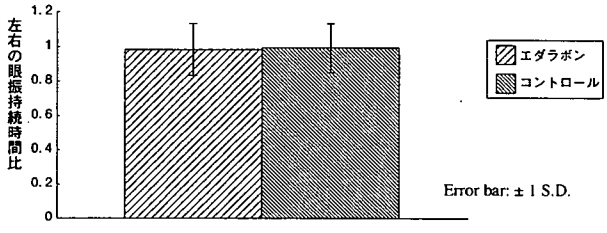

図1

術後に施行したカロリックテストでの手術側（右）の眼振持続時間を無処置側（左）の眼振持続時間で割った値を示す。コントロールデータと比較しても差を認めない。

考　察

経正円窓膜的に局所投与を行う際に必ず問題となるのが、投与物質の正円窓膜透過性である。国内でも行われているメニエール病に対するゲンタマイシン鼓室内投与、最近欧米で報告が散見される急性感音難聴に対するステロイド鼓室内投与、これらは薬物が主として経正円窓膜的に内耳に移行することを期待して行われている[9),10)]。正円窓膜の透過性は、分子量174.2のエダラボンと比較してかなり大きい分子量960のトレパンブルーも通過することが確認されている[11)]。薬剤では、ネオマ

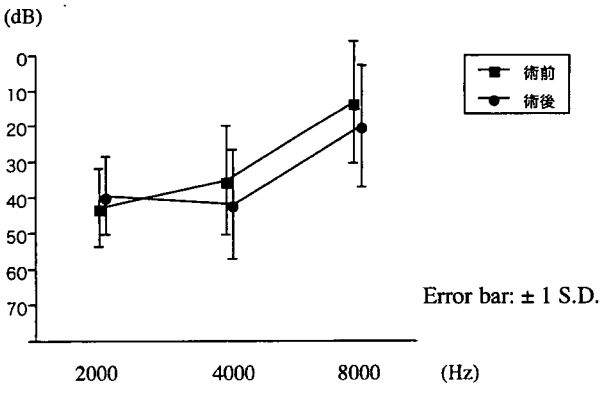

図2

手術側における術前後のABR閾値の変化を示す。測定した3周波数での閾値の変動に，統計学的に有意差は認められない。

イシン、セフメタゾールが通過することが報告されている[12),13)]。セフメタゾールはセファマイシン系抗生物質であり、分子量493.51とエダラボンより大きく、かつ対生食浸透圧比は1とエダラボンと同等である。これらのことからも、エダラボンが正円窓膜を通過する可能性は十分にある。さらに、AMPA局所投与により前庭感覚上皮に生じたハイドロキシラジカルに対し、エダラボンを浸透させたゼルフォームを正円窓膜上に留置することで減少せしめるといったエダラボンに特徴的な作用も免疫組織学的に確認している[14)]。以上をふまえても、エダラボンが正円窓膜を通過することはほぼ間違いないと考えられる。しかしながら最終的には、エダラボンが正円窓膜を通過するのかどうかは、実際に外リンパの濃度を測定してみないと確定的なことは言えない。我々は、外リンパ中のエダラボン濃度を測定できないものか検討してみたが、今持ち合わせている技術、あるいは外注での測定いずれでも手技的に不可能であった。安全性を検討するためには、エダラボンを直接外リンパへ注入する方法も有用であると考える。我々も過去に浸透圧ポンプを用いてエダラボンを直接外リンパに投与して、音響外傷を軽減せしめたことを報告しており、直接投与法の手技は持ち合わせている[15),16)]。しかし、本研究では、将来的に実際臨床応用できる投与法での安全性を確認することを目的としており、あえてゼルフォームに浸しての経正円窓膜投与を検討した。

エダラボンは、30mg/20ml/Aの剤型で国内販売されている脳保護剤である。その作用は、ハイドロキシラジカルや、ペルオキシラジカルを消去すると言われている[17),18)]。我々は、本剤が、ストレプトマイシンによる前庭障害や、音響曝露による蝸牛障害に有効であることを基礎実験で明らかにしてきた[5)~7)]。しかし、我々が前庭障害実験で全身投与したエダラボンの濃度は、3 mg/kgであり、この濃度は、通常体重60kgの人に用いる量の6倍に相当する。この濃度の全身投与でも、ストレプトマイシン障害後の前庭眼反射の利得の低下を抑制する効果を認めたが[7)]、それに比し、3 mg/mlに調整したエダラボンをゼルフォーム片（2×2×2 mm）に浸して正円窓膜上に留置することによる局所投与は、同様の障害後の自発眼振、頭部偏奇を劇的に減少せしめた[6)]。これらのことから、エダラボンにより内耳障害を軽減させるには、全身投与であればかなりの高濃度で行う必要があり、腎機能障害の可能性が指摘されている現在、全身投与時の増量は期待できない。そういった面からは、やはり局所投与が望ましいと考える。それ故、今回安全

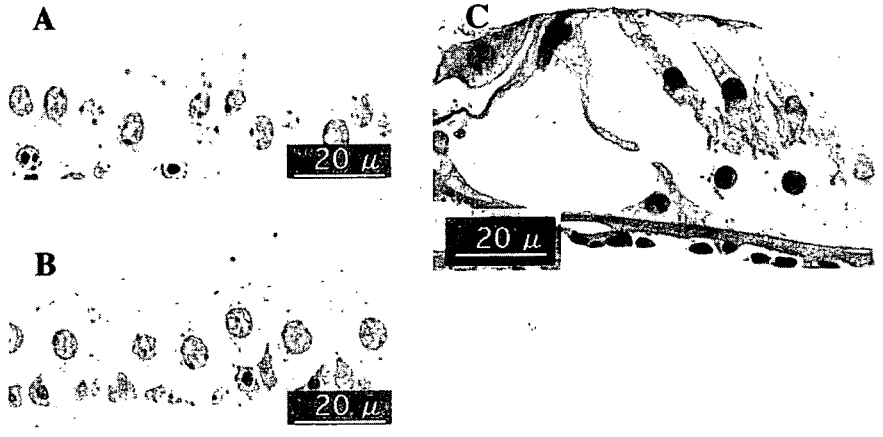

図3

手術側における術後1週間での，A 外側半規管膨大部，B 卵形嚢，C Corti器の組織を示す。末梢前庭器，Corti器ともに形態はおおむね保たれている。
Bar = 20 μm

性を検討するのに、有効であった3 mg/mlの2倍の濃度を用いた。

内耳障害に対する治療戦略としては、Kopkeらが述べているように、抗酸化剤、ヒートショックプロテイン、グルタミン酸レセプターアンタゴニスト、カルシウム拮抗剤などが挙げられる[4]。これは、とりもなおさず、虚血性神経細胞障害に対する治療戦略と同様である[19]。脳梗塞治療において、脳保護を目的として過去に様々な薬物が検討されてきた。しかし、その中でたくさんの薬物が第Ⅲ相試験で無効と判定される、あるいは副作用のため試験が中止されるといった状況で臨床応用には至らなかったようである。その中で、エダラボンは、数少ない国内で臨床試験が行われ、有効と判定された貴重な薬剤である。我々は、エダラボンが内耳局所治療法に用いることのできる有力な薬物候補であると考え、AMPAによる前庭障害に対してエダラボンを局所投与したところ、ハイドロキシラジカルを消去することで、機能的、形態的に末梢前庭保護効果を示すという実験結果を得ている。本研究で、エダラボンの局所投与が、内耳の機能、形態にはほとんど影響を与えない可能性が示唆されたので、今後はエダラボン局所投与治療の臨床応用を検討していく予定である。

結　語

本研究によりエダラボンの内耳局所投与（6 mg/ml）では、投与1週間後に内耳対する影響がほとんどなかったことが示唆された。エダラボンは内耳局所投与治療に、安全に用いることのできる薬剤である可能性が示された。

尚、本論文の要旨は第106回日本耳鼻咽喉科学会総会・学術講演会において口演した。

参考文献

1）Silverstein H：Use of a new device, the MicroWick™ to deliver medication to the inner ear. Ear Nose Throat J 78：595-600, 1999.
2）Hoffer ME, Kopke RD, Weisskopf P, et al.：Microdose gentamicin administration via the round window microcatheter: results in patients with Meniere's disease. Ann NY Acad Sci 942：46-51, 2001.
3）小川　郁：突発性難聴の治療法. 耳喉頭頸 78：205-211, 2006.
4）Kopke R, Allen KA, Henderson D, et al.：Aradical demise: toxins and trauma share common pathways in hair cell death. Ann NY Acad Sci 884：171-191, 1999.
5）Takemoto T, Sugahara K, Okuda T, et al.：The clinical free radical scavenger, edaravone, protects cochlear hair cell from acoustic trauma. Eur J Pharmacol 487：113-116, 2004.
6）Horiike O, Shimogori H, Yamashita H：Effect of edaravone on streptomycin-induced vestibulotoxicity in the guinea pig. Laryngoscope 114：1630-1632, 2004.
7）Horiike O, Shimogori H, Ikeda T, et al.：Protective effect of edaravone against streptomycin-induced vestibulotoxicity in the guinea pig. Eur J Pharmacol 464：75-78, 2003.
8）Duan ML, Canlon B：Short-ter m adaptation in the peripheral auditory system in related to the AMPA receptor. Acta Otolaryngol 121：21-27, 2001.
9）Blackley BW：Update on intratympanic gentamicin for Meniere's disease. Laryngoscope 110：236-240, 2000.
10）Slattery WH, Fisher LM, Iqubal Z, et al.：Intratympanic steroids injection for treatment of idiopathic sudden hearing loss. Otolaryngol Head Neck Surg 133：251-259, 2005.
11）澤田政道、平出文久、井上鉄三、都川紀正、椿康喜代、他：モルモット正円窓膜の透過性とその周辺組織について. Ear Res Jpn 12：84-85, 1981.
12）原田勇彦、川端五十鈴、野村恭也：蝸牛窓膜の透過性に関する実験的研究. Ear Res Jpn13：205-206, 1982.
13）Okuno T, Nomura Y：Permeability of the round window membrane. Arch Otorhinolaryngol 240：103-106, 1984.
14）Shimogori H, Takemoto T, Mikuriya T, et al.：Edaravone protects the vestibular periphery from free radical-induced toxicity in response to perilymphatic application of (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid. Eur J Pharmacol 2006, in press.
15）Takemoto T, Sugahara K, Okuda T, et al.：The clinical free radical scavenger, edaravone, protects

cochlear hair cells from acoustic trauma. Eur J Pharmacol 487 : 113, 2004.
16) Tanaka K, Takemoto T, Sugahara L, et al. : Post-exposure administration of edaravone attenuates noise-induced hearing loss. Eur J Pharmacol 522 : 116–121, 2005.
17) Watanabe T, Morita I, Nishi H, et al. : Preventive effect of MCI-186 on 15-HPETE induced vascular endothelial injury in vitro. Prostaglandins Leukot Essent Fatty Acids 33 : 81–87, 1988.
18) Yamamoto T, Yuki S, Watanabe T, et al. : Delayed neuron death prevented by inhibition of increased hydroxyl radical formation in a transient cerebral ischemia. Brain Res 762 : 240–242, 1997.
19) 山脇健盛：脳保護療法. 現代医療 33 (Suppl. 1) : 625–631, 2001.

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Edaravone protects the vestibular periphery from free radical-induced toxicity in response to perilymphatic application of (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid

Hiroaki Shimogori *, Tsuyoshi Takemoto, Takefumi Mikuriya, Hiroshi Yamashita

Department of Otolaryngology, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan

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Abstract

Intracochlear infusion of (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) was performed with a syringe pump in guinea pigs, and peripheral vestibular dysfunction was induced. Animals were administered edaravone systemically or topically. In the systemic application group, animals were administered edaravone once a day for 7 days after AMPA infusion. In the topical application group, edaravone-soaked gelfoam was placed on the round window membrane just after, 12 h after or 24 h after AMPA infusion. Spontaneous nystagmus was observed after AMPA infusion. Immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE), a marker of free radical-induced lipid peroxidation, was performed 24 h after AMPA infusion. In addition, caloric tests were performed to evaluate vestibular function 1 week after AMPA infusion. Animals in both groups showed decreased spontaneous nystagmus, but results were not significant. Animals treated topically with edaravone within 12 h of AMPA infusion showed normal morphology of the ampullar sensory epithelia of the lateral semicircular canals and showed a good response to the caloric tests. 4-HNE immunoreactivity in the sensory epithelia was very low in these animals. In contrast, untreated animals and animals treated with edaravone systemically or topically 24 h after AMPA infusion showed morphologic hair cell damage, reduced caloric response and remarkable 4-HNE immunoreactivity in the sensory epithelia. These results indicate that topical application of edaravone within 12 h after damage protects the vestibular periphery from free radical-induced toxicity in response to intracochlear infusion of AMPA.

Keywords: Edaravone; (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid; Reactive oxygen species; 4-hydroxy-2-nonenal; Caloric test; Vestibular periphery

1. Introduction

Glutamate is the major excitatory neurotransmitter in the peripheral vestibular system (Smith and Darlington, 1994), and a glutamate receptor, the (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptor, is of primary importance in neuronal transmission in the vestibular periphery (Dememes et al., 1995; Devau et al., 1993; Matsubara et al., 1999; Smith and Darlington, 1994). It is well known that glutamate induces excitotoxicity in neuronal cells in brain ischemia (Lipton and Rosenberg, 1994). AMPA induces a transient, reversible disorder of inner ear hair cells and causes ischemia-like histologic changes in the cochlea (Puel et al., 1995). Evidence for a correlation between glutamate receptor activation and reactive oxygen species in the inner ear is accumulating (Kopke et al., 1999; Tabuchi et al., 2001; Takumida and Anniko, 2000). Reactive oxygen species are common to inner ear pathologies such as those induced by aminoglycoside antibiotics and cisplatin (Fetoni et al., 2003, 2004). Therefore, reduction of reactive oxygen species generation is a candidate treatment for inner ear diseases.

Our strategy for treatment of inner ear diseases is topical application therapy. The benefits of topical application therapy are that it is possible to administer drugs at effective doses while avoiding systemic side effects and that even drugs that cannot pass the blood–inner ear barrier can be used. We have developed a model of partial vestibular dysfunction induced by topical application of AMPA (Shimogori and Yamashita, 2004). This model is suitable for studying the pharmacologic effects of drug candidates for

* Corresponding author. Tel./fax: +81 836 22 2280.
E-mail address: shimo-h@yamaguchi-u.ac.jp (H. Shimogori).

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topical application therapy, such as antioxidants. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-1) is the first free-radical scavenger to be used clinically in Japan, where it is used systemically to treat acute cerebral infarction (Yamamoto et al., 1997). Several studies have shown the effects of edaravone on the inner ear (Horiike et al., 2003, 2004; Takemoto et al., 2004; Tanaka et al., 2005). We hypothesized that edaravone would be useful as a topical, as opposed to a systemic, application therapy for inner ear diseases. However, the mechanism underlying edaravone's effects remains to be elucidated. Recently, 4-hydroxy-2-nonenal (4-HNE) was identified as a marker of free radical-induced lipid peroxidation. 4-HNE can be visualized immunohistochemically (Itakura et al., 2002; Tanaka et al., 2005; Yoritaka et al., 1996) and thus, it is a suitable marker in studies on the effects of edaravone in our model. The aims of the present study were to examine the effects of topical application of edaravone on AMPA-induced vestibular dysfunction and to assess the window of potential availability of edaravone by examining the generation of 4-HNE.

2. Materials and methods

2.1. Animals

We used 54 male Hartley guinea pigs with normal Preyer reflexes and tympanic membranes in this study. The study protocol was reviewed by the Committee for Ethics in Animal Experiments of Yamaguchi University School of Medicine and was carried out in accordance with the Guidelines for Animal Experiments of the Yamaguchi University School of Medicine and Law No. 105 and Notification No. 6 of the Japanese government.

2.2. Surgical procedure

Xylazine (16 mg/kg, i.p.)-ketamine (16 mg/kg, i.p.) anesthesia was induced, and 1.5 ml lidocaine HCl was injected into the right postauricular region for local anesthesia. Body temperature was maintained at 37 °C. The mastoid bulla was opened by postauricular incision to allow visualization of the round window with a surgical microscope. A hole was made adjacent to the round window with a perforating burr (Proxxon, 0.5-mm diameter; Kiso Power Tool, Osaka, Japan). A polyethylene catheter (0.2-mm inner diameter, 0.5-mm outer diameter; Natume Co., Ltd., Tokyo, Japan) filled with drug or vehicle and connected to a syringe filled with the same was inserted into the hole. The syringe was placed in a syringe pump (SP-70; Nipro Corp., Osaka, Japan), and infusion was performed at 0.6 ml/h for 5 min. After drug infusion, the hole was covered with a small piece of muscle and sealed with fibrin glue (Bolheal; Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan). After washing the wound with saline, a small amount of piperacillin sodium was applied to prevent infection. After closure, piperacillin sodium (40 mg/kg) was injected intramuscularly, and oxytetracycline HCl ointment was applied to the wound. Each animal was kept warm with an electric blanket for 3 h after surgery.

Forty-three animals were treated with 10 mM AMPA (Sigma–Aldrich, St. Louis, MO). In one group (group A; $n=8$), the wound was closed just after AMPA infusion. In a second group (group B; $n=9$), edaravone (3 mg/ml; Mitsubishi Pharma Corp., Osaka, Japan)-soaked gelfoam (2×2×2 mm) was placed on the round window membrane just after AMPA infusion, and the wound was closed. In a third group (group C; $n=9$), the wound was reopened 12 h after AMPA infusion, edaravone was applied in the same manner, and the wound was reclosed. In a fourth group (group D; $n=9$), edaravone was applied in the same manner 24 h after AMPA infusion. In a fifth group (group E; $n=8$), edaravone (3 mg/kg, i.p.) was administered once a day for 7 days after AMPA infusion. Edaravone was dissolved in 1 M NaOH just prior to use, and the pH was adjusted to 7.0 with 1 M HCl. Four animals (group F) were treated with topical application of edaravone alone. Seven animals (group G) were infused with artificial perilymph (113.5 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM glucose, 10.0 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid) as a control.

2.3. Immunohistochemistry for 4-HNE

Twenty-four hours after surgery, we examined 4-HNE immunostaining at the plasma membrane as a marker of free radical activity in the inner ear. Two animals each from groups A, B, C, E and G were anesthetized deeply with pentobarbital, transperilymphatic perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer was performed through the cochlear hole, and the animals were decapitated immediately. The temporal bone was dissected, and the ampulla of the lateral semicircular canal and utricular macula were excised and soaked in the same fixative for 1 h. Specimens were decalcified in 10% ethylenediaminetetraacetic acid for 1 h, rinsed in 0.01 M phosphate-buffered saline (PBS), incubated in methanol at −20 °C for 20 min and then hydrated and permeabilized in PBS plus 0.1% Triton X-100 for 10 min. Nonspecific staining was blocked by incubating the specimens in PBS/2% dried milk at 4 °C for 2 h. Specimens were then incubated in a 1:100 dilution of anti-4-HNE mouse monoclonal antibody (OXIS International, Inc., Portland, OR) at 4 °C for 12 h. Specimens were rinsed in PBS and incubated in a 1:100 dilution of Alexa Fluor® 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) at 4 °C for 8 h. Specimens were rinsed in PBS and embedded in a semi-water-soluble resin (Immuno-Bed®; Polysciences, Inc., Warrington, PA) and cut into 2-μm-thick sections. Sections were counterstained with 4′,6-diamino-2-phenylindole (DAPI) (Vectashield®; Vector Laboratories, Inc., Burlingame, CA). Immunolabeling was visualized under brightfield illumination with a fluorescence microscope equipped with a 20× objective.

2.4. Evaluation of vestibular function

We measured the frequency of spontaneous nystagmus in animals in groups A, B, E, F and G in a 80-cm square free field in the light as the number of quick-phase beats per min at 6, 9, 12, 15 and 18 h after surgery. In groups C and D, we did not analyze spontaneous nystagmus in consideration of the influence of general reinduction of anesthesia for edaravone administration. Measurements were performed three times for each observation time, and mean nystagmus beat number was

calculated for each time. Mean nystagmus beat number was calculated for each group, and differences between groups were evaluated by one-way ANOVA with significance set at $P<0.05$.

We performed caloric tests in each group 1 week after surgery. Each animal was placed in a transparent box designed to fix and hold the head and body without covering the nose or mouth. The box was positioned at a 50° angle with the animal's head facing up. Caloric tests were performed by irrigating the external auditory meatus with 5-ml ice-cold water for 10 s in the dark. Nystagmus was recorded on videotape with an infrared charge-coupled device camera, and caloric response time was measured. We calculated the time ratio as the ratio of the treated side (right) response time to the untreated side (left) response time. Differences between groups were evaluated by one-way ANOVA with significance set at $P<0.05$. Data are shown as mean±S.D.

2.5. Histopathology

After physiologic evaluation, each animal was anesthetized deeply with pentobarbital. Transperilymphatic perfusion was performed with 4% paraformaldehyde in 0.1-M phosphate buffer through the cochlear hole, and the animals were decapitated immediately. The temporal bone was dissected, and the histopathologic procedure was as described above. Specimens were embedded in Immuno-Bed®, and 2-μm-thick sections were cut, stained with hematoxylin and eosin and visualized by microscopy with a 100× oil-immersion objective.

3. Results

3.1. Vestibular function

3.1.1. Spontaneous nystagmus

In group F (edaravone alone) and group G (perilymph alone), animals showed no spontaneous nystagmus after surgery. In group A (AMPA alone), group B (edaravone just after AMPA) and group E (edaravone i.p. after AMPA), spontaneous nystagmus was observed within 18 h. Responses did not differ significantly between groups A, B and E (Fig. 1).

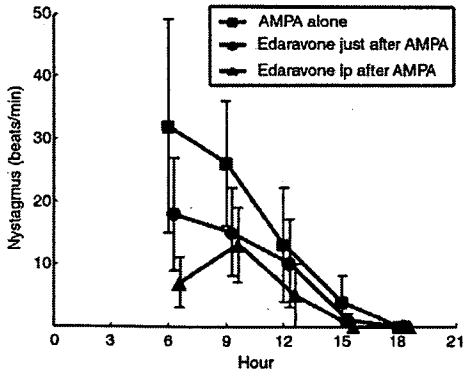

Fig. 1. Spontaneous nystagmus beats per minutes after surgery. Group mean±S.D. are plotted. Animals in the edaravone alone or perilymph alone group showed no nystagmus. No significant difference was found between the other three groups. AMPA: (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid.

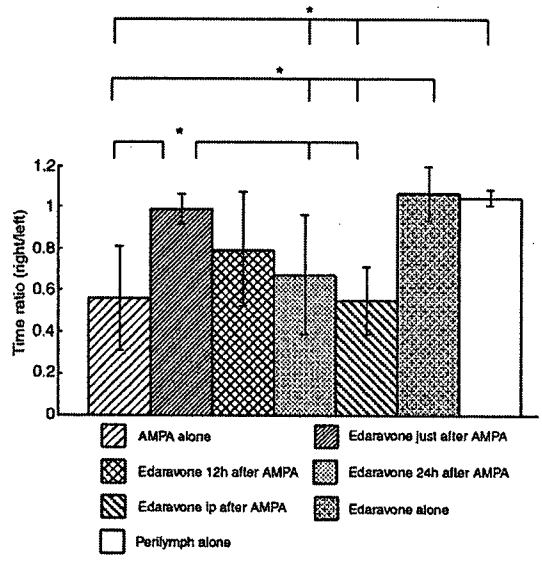

Fig. 2. Time ratio for each group 1 week after surgery. The time ratio is the ratio of the caloric response time of the treated side (right) to the caloric response time of the untreated side (left). Group means±S.D. are plotted. The time ratios of group A (AMPA alone) and group D (edaravone 24 h after AMPA) were less than the time ratio of group B (edaravone just after AMPA) (*$P=0.0001$). AMPA: (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid.

3.1.2. Caloric response

In group B (edaravone just after AMPA), group F (edaravone alone) and group G (perilymph alone), caloric response times on the treated side were similar to those on the untreated side (Fig. 2). Mean time ratios were 0.989±0.073, 1.064±0.134 and 1.043±0.037, respectively. Mean time ratios for group A (AMPA alone; 0.560±0.250), group D (edaravone 24 h after AMPA; 0.672±0.286) and group E (edaravone i.p. after AMPA; 0.548±0.162) were less than those for groups B, F and G. These differences were statistically significant ($P=0.0001$). The mean time ratio for group C (edaravone 12 h after AMPA) was 0.795±0.273. The mean time ratio did not differ significantly between groups B and C.

3.2. Immunohistochemistry for 4-HNE

Twenty-four hours after AMPA infusion, we observed remarkable 4-HNE immunoreactivity in the cytosome of hair cells and supporting cells in the ampullar and utricular sensory epithelia in group A (AMPA alone; Fig. 3a, f) and group E (edaravone i.p. after AMPA; Fig. 3d, i). In group B (edaravone just after AMPA; Fig. 3b, g) little immunoreactivity was observed in the ampullar sensory epithelia, whereas clear but less immunoreactivity than that in group A or group E was observed in the utricular sensory epithelia. In group C (edaravone 12 h after AMPA; Fig. 3c, h), clear but significantly less immunoreactivity than that in group A or group E was observed in the cytosome of hair cells in the ampullar sensory epithelia. Also in group C, in the utricular sensory epithelia, clear but less immunoreactivity than that in group A or group E was observed. Diffuse but less immunoreactivity than that in group A or group E was observed in the cytosome of hair cells and supporting cells in both ampullar and utricular sensory epithelia in group G (perilymph alone; Fig. 3e, j).

3.3. Histopathology

Histopathologic examination was performed 1 week after surgery. In group A (AMPA alone; Fig. 4a), group D (edaravone 24 h after AMPA; Fig. 4d) and group E (edaravone i.p. after AMPA; Fig 4e), we observed damaged hair cells without remarkable cilia loss. Cuticular plates were irregular but were preserved. In group B (edaravone just after AMPA; Fig. 4b) and group C (edaravone 12 h after AMPA; Fig. 4c), hair cells showed a relatively normal arrangement without cilia loss. Ballooning of probable nerve calyces was observed. In groups F and G, the sensory epithelia showed a normal appearance (data not shown).

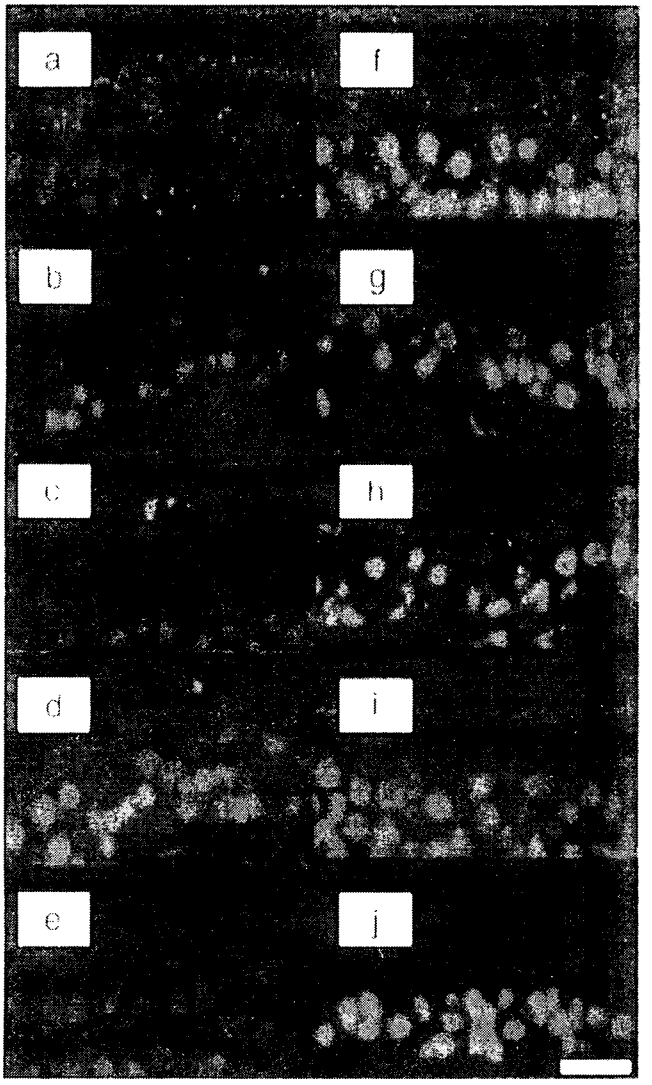

Fig. 3. Fluorescence images of 4-HNE immunoreactivity in the ampullar sensory epithelia of the lateral semicircular canals (a–e) and utricular (f–j) sensory epithelia 24 h after surgery. Figures in the left side showed ampulla and in the right side showed utricle. In group A (AMPA alone) and group E (edaravone i.p. after AMPA), strong immunoreactivity was observed in hair cells and supporting cells in both sensory epithelia (a, d, f, i). In contrast, in group B (edaravone just after AMPA), little immunoreactivity was observed in the ampullar sensory epithelium (b). In group C (edaravone 12 h after AMPA), clear but markedly less immunoreactivity than that in group A or group E was observed in the ampullar sensory epithelium (c). In the utricular sensory epithelia, group B and group C showed clear but less immunoreactivity than that in group A or group E (g, h). As a control, in group F (perilymph alone) diffuse, moderate immunoreactivity was observed in hair cells and supporting cells in both sensory epithelia (e, j). Bar, 20 μm. AMPA: (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid. 4-HNE: 4-hydroxy-2-nonenal.

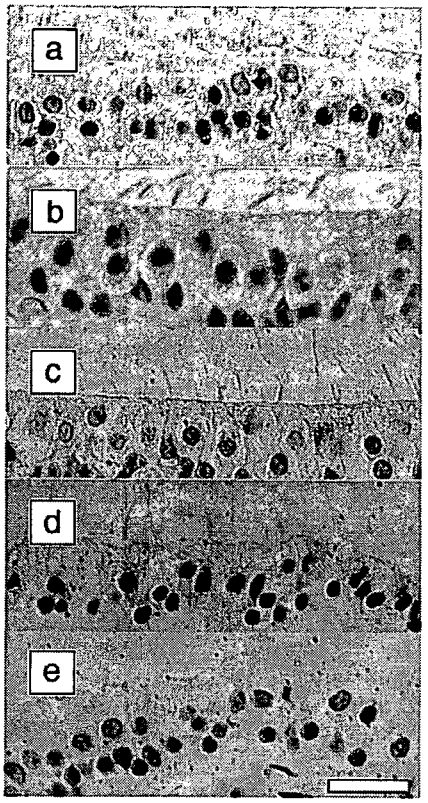

Fig. 4. Brightfield images of ampullar sensory epithelia of the lateral semicircular canals on the treated side 1 week after surgery. In groups A (AMPA alone) and E (edaravone i.p. after AMPA), we observed damaged hair cells without remarkable cilia loss (a, e). In contrast, in groups B (edaravone just after AMPA) and C (edaravone 12 h after AMPA), hair cells showed relatively normal arrangement without cilia loss. Ballooning of probable nerve calyces was observed (b, c). In group D (edaravone 24 h after AMPA), we observed damaged hair cells with cilia and preserved but irregular cuticular plates (d). Bar, 20 μm. AMPA: (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid.

4. Discussion

The main finding of the present study is the protection of vestibular periphery from AMPA-induced toxicity by edaravone topical application. Our previous study showed that intracochlear infusion of AMPA induces partial vestibular dysfunction predominantly via activation of AMPA receptors (Shimogori and Yamashita, 2004). AMPA receptors are localized to the postsynaptic region of vestibular hair cells (Dememes et al., 1995; Matsubara et al., 1999). When AMPA is applied topically in excess, activation of AMPA receptors induces excitotoxicity. When the damage is localized within the synapse, morphologic and functional recovery can occur within 7 days (Puel et al., 1997). When the damage is more severe, hair cells themselves may be involved, leading to cell death. Reactive oxygen species are generated in this process (Atlante et al., 2001; Gilgun-Sherki et al., 2002). In the present study, intracochlear infusion of AMPA led to the production of 4-HNE

in the sensory epithelia both in the semicircular canal and otolith organ, indicating the presence of free radical-induced lipid peroxidation (Itakura et al., 2002). This may represent glutamate excitotoxicity, which leads to reactive oxygen species generation and cell death (Atlante et al., 2001; Lipton and Rosenberg, 1994). The most commonly occurring cellular free radical is superoxide, which is produced when an oxygen molecule gains an electron from another molecule. Excess superoxide radicals lead to tissue damage by promoting the formation of hydroxyl radical via hydrogen peroxide. Superoxide radicals also lead to hydroxyl radical formation via interaction with endogenously formed nitric oxide. This interaction leads to the formation of peroxynitrite, which generates nitrosyl radicals that decompose to form hydroxyl radicals (Gilgun-Sherki et al., 2002). The hydroxyl radical is an extremely powerful oxidant that reacts with biologic substances indiscriminately (Tabuchi et al., 2001). Edaravone traps a variety of free radical species, and it reacts with hydroxyl and peroxyl radicals to form particular oxidized compounds (Watanabe et al., 1988; Yamamoto et al., 1997). We hypothesized that edaravone could protect the inner ear from developing free radical-induced disorders. Systemically or topically applied edaravone reduced spontaneous nystagmus after AMPA infusion, but this effect was not significant. Systemic application of edaravone resulted in no morphologic or functional recovery 1 week after AMPA infusion, whereas topical application of edaravone protected the vestibular periphery from AMPA-induced excitotoxic damage. In our previous report, systemic application of edaravone showed a protective effect against streptomycin-induced vestibulotoxicity (Horiike et al., 2003). This may be due to the different types of injury. In another study, antioxidants conferred significant protection to cultured rat hippocampal neurons against glutamate toxicity (Vergun et al., 2001). Our results are consistent with these findings.

In this study, topical application of edaravone showed morphologic and functional effects within 12 h of AMPA infusion and less effect when administered 24 h after AMPA infusion, indicating that reactive oxygen species generation in response to AMPA infusion occurs relatively early. The earlier edaravone is administered, the greater the effects may be. Many authors have reported the usefulness of 4-HNE as a marker of free radical-induced lipid peroxidation (Itakura et al., 2002; Tanaka et al., 2005; Yoritaka et al., 1996). In the absence of edaravone, 4-HNE immunoreactivity was observed 24 h after AMPA infusion. Thus, 4-HNE immunohistochemistry showed clear results and was useful in analyzing the effects of edaravone.

We also want to emphasize the characteristic of our model and our analysis methods. We have data that AMPA-induced damage is most severe in the utricular sensory epithelia and the ampullar sensory epithelia of the lateral semicircular canal shows secondary severe hair cell damage. In the ampulla, the apical and lateral parts show more severe damage than do the basal part (data not pressed). In the present study, we analyzed the lateral semicircular canal morphologically and histopathologically.

We have reported that the surgical procedures used in this study, particularly the cochleotomy, do not cause inner ear damage (Shimogori et al., 1999; Sugahara et al., 2001). In addition, our infusion method causes no pressure injury and requires no effluent hole (Shimogori and Yamashita, 2004). The concentration of AMPA used in this study was relatively high compared to that used in a previous study (Puel et al., 1997). We evaluated many AMPA concentrations with our infusion method and found that concentrations lower than 10 mM caused no obvious static symptoms such as spontaneous nystagmus. This may be due to a characteristic of our infusion method. The association of the catheter and the cochlear hole is not tight, and after 2 min of infusion, perilymph leakage occurs at the point of catheter insertion; thus, the concentration of AMPA in the perilymphatic space may be less than 10 mM.

In the present study, we used the caloric test to evaluate peripheral vestibular function and measured caloric response time. In our previous report, we used a sinusoidal rotation test at 0.1 Hz and a peak angular velocity of 60°/s and calculated vestibulo-ocular reflex (VOR) gain (Horiike et al., 2003). VOR gain is a good marker of the vestibular compensation process and we use this method to analyze the influence of systemically applied drug. In the present study, the aim was to elucidate the functional recovery of the vestibular periphery, so we selected the caloric test. In guinea pigs, caloric nystagmus occurs too quickly to analyze maximum slow-phase velocity with our eye movement analysis system (Horiike et al., 2003). We used caloric response time as a parameter instead.

With respect to therapeutic strategies for inner ear diseases, the time that the therapeutic agent is administered determines if it will be a protective, rescue or regenerative agent (Kopke et al., 1999). We also emphasize the importance of topical application therapy for inner ear diseases (Hoffer et al., 2001; Silverstein, 1999). The benefits of topical application therapy are that it is possible to administer drugs at effective doses while avoiding systemic side effects and that even drugs that cannot pass the blood–inner ear barrier can be used. Many drugs, such as calcium blockers and glutamate receptor antagonists, have been studied for their effectiveness in the treatment of ischemic brain injury, but few have been approved for clinical use because of side effects or insufficient effects (Clark et al., 2000; Lees et al., 2000). Topical application may alleviate this problem.

In conclusion, this study provides direct evidence that topical application of edaravone protects the vestibular periphery from toxicity induced by intracochlear infusion of AMPA. Thus, edaravone is a candidate for topical therapy for inner ear diseases.

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References

Atlante, A., Calissano, P., Bobba, A., Giannattasio, S., Marra, E., Passarella, S., 2001. Glutamate neurotoxicity, oxidative stress and mitochondria. FEBS Lett. 497, 1–5.

Clark, W.M., Raps, E.C., Tong, D.C., Kelly, R.E., 2000. Cervene (Nalmefene) in acute ischemic stroke: final results of a phase III efficacy study. The Cervene stroke study investigators. Stroke 31, 1234–1239.