Effects of Memantine on Aminoglycoside-Induced Apoptosis of Spiral Ganglion Cells in Guinea Pigs

Bo Young Kim, MD, PhD1, Woo Yong Bae, MD, PhD2, Dae Young Hur, MD, PhD2, Jae-Ryong Kim, MD, PhD4, Tae Kyung Koh, MD2, Tae Hoon Lee, MD, PhD5, and Ga Bin Park, PhD3

Abstract
Objective. To explore whether memantine, an N-methyl-D-aspartate receptor antagonist, exerts a neuroprotective effect against apoptosis of spiral ganglion cells (SGCs) induced by gentamicin.

Study Design. An animal experiment.

Setting. Dong-A University College of Medicine, Busan, Korea.

Subjects and Methods. Gentamicin was injected into the left cochlea of guinea pigs to induce apoptosis of SGCs; the contralateral cochleae served as controls. Memantine was intraperitoneally injected 12 hours and 1 hour prior to gentamicin injection. At 1 week after gentamicin and/or memantine injection, the cochleae were removed and stained with hematoxylin and eosin to evaluate morphologic changes and apoptosis. Western blotting was performed to measure FasL expression and the extent of caspase activation in SGCs.

Results. SGC numbers remained stable after memantine treatment. Western blotting showed that FasL expression and activation of caspases 3, 8, and 9 were reduced in SGCs after memantine treatment.

Conclusion. Memantine attenuated the gentamicin-induced apoptosis of SGCs in guinea pigs. Moreover, memantine may affect Fas-FasL signaling in the receptor-mediated apoptotic pathway and caspase activation involved in the receptor-mediated and mitochondrial apoptotic pathways.

Keywords
spiral ganglion cells, apoptosis, memantine, FasL, caspase

Received July 14, 2015; revised February 22, 2016; accepted February 25, 2016.

Spiral ganglion cells (SGCs) receive neural signals from the inner hair cells and transmit these signals to the central auditory system.1 Degeneration of SGCs, the primary sensory fibers of the auditory pathway, is an important feature of sensorineural hearing loss.2 Traumatic noise, aminoglycoside antibiotics, cochlear ischemia, or aging can cause hair cell death or excessive release of glutamate, which is the principal neurotransmitter for synapses lying between the inner hair cells and dendrites of the SGCs.3,4 Either hair cell death or oversecretion of glutamate can trigger secondary loss of SGCs.5 Although the mechanism whereby SGC numbers are reduced remains unclear, apoptosis may be a factor.6

Apoptosis may play an important role in the pathogenesis of hearing disorders. If this is the case, new therapeutic strategies should be suggested. We earlier showed that secondary apoptosis of SGCs induced by gentamicin injection may be controlled by the apoptotic Fas-FasL signaling pathway.7 Reactive oxygen species and the c-Jun N-terminal kinase signaling pathway may also be involved in apoptosis of SGCs.8,9

Using both animal and in vitro models of cultured SGCs, we showed that apoptosis of dissociated SGCs was induced by exposure to excessive glutamate and elevated activity of the Fas-FasL signaling pathway.10 Glutamate released from inner hair cells into synapses binds to N-methyl-D-aspartate (NMDA) receptors terminally located on SGCs.11 NMDA receptor overactivation, triggered by excessive glutamate, induces alterations in intercellular calcium homeostasis, in
turn activating a cascade of events culminating in apoptosis of SGCs.\textsuperscript{12,13} The NMDA receptor antagonist memantine blocks excessive calcium influx and attenuates intracellular caspase activation.\textsuperscript{14-17}

Therefore, in the present study, we explored whether memantine exerted a neuroprotective effect against SGC apoptosis induced by gentamicin in the guinea pig. In addition, we asked whether the apoptotic process was mediated via the Fas-FasL signaling pathway and whether caspase activation was attenuated by memantine.

**Materials and Methods**

**Animals**

Guinea pigs weighing 250 to 300 g (n = 10) and aged 3 to 4 weeks were purchased from Samtako Bio Korea Co Ltd (Osan, Korea). All experimental protocols were approved by the Animal Research Committee, School of Medicine, Dong-A University (Busan, Korea). Animal care was supervised by the Institute of Laboratory Animals, School of Medicine, Dong-A University. The guinea pigs were anesthetized by intramuscular injection of ketamine chlorohydrate (25 mg/kg) and xylazine (2 mg/kg). A retroauricular incision was made on the left side of each animal. We manually dissected the soft tissues to expose the bulla, and we used a microdrill to perform cochleostomy of the scala tympani. A micropipette was inserted into the cochleostomy site and 60 \( \mu \)L of a solution of the aminoglycoside antibiotic gentamicin (40 mg/mL; Shin Poong Pharm Co Ltd, Seoul, Korea) was injected into the cochlear perilymph (n = 5; gentamicin [G] group). Each hole was covered with fibrin glue. The contralateral cochleae of the M-G group were not injected with gentamicin (n = 5; memantine [M] group).

To evaluate SGC degeneration, the guinea pigs were decapitated under anesthesia and the cochleae separated from the temporal bones 1 week after gentamicin and/or memantine treatment.

**Light Microscopy**

The cochleae were fixed in 10\% (w/v) neutral-buffered formalin solution, immersed in 25\% (w/v) sucrose solution, embedded in sectioning medium, and frozen. Cryosections (8 \( \mu \)m) were prepared from the midmodiolar plane and stained with hematoxylin and eosin (H&E). We next examined the basal turn of Rosenthal’s canal.

Six histologic images from each of the left and right sides were analyzed to determine SGC survival rates. The numbers of SGCs in the basal turn of Rosenthal’s canal were counted with NDP view2 image analysis software (Hamamatsu Photonics, Hamamatsu, Japan). SGCs that had an obvious nucleolus, a cell membrane, and cytoplasm were considered viable and were counted (Figure 1):

\[
\text{survival of SGCs} = \frac{\text{number of SGCs in the basal turn}}{\text{area of Rosenthal’s canal of the basal turn}}.
\]

**TUNEL Staining**

Apoptotic cells of cryosectioned tissues were stained with a TUNEL kit (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; Merck, Darmstadt, Germany). Briefly, specimens were air-dried, fixed in 1\% (v/v) paraformaldehyde for 10 minutes, and then postfixed in ethanol:acetic acid (2:1, v/v) for 5 minutes. After quenching of endogenous peroxidase with 3\% (v/v) hydrogen peroxide (\( \text{H}_2\text{O}_2 \); Fluka Chemie AG, Buchs, Switzerland) for 5 minutes, the sections were stained with the ApopTag kit as described above. The sections were washed, and color was developed.
with diaminobenzidine tetrahydrochloride (Sigma) with 0.03% H$_2$O$_2$ in phosphate-buffered saline (0.05 M, pH 7.4). The images were digitized with a Hamamatsu Nanozoomer 2.0 HT whole-slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) and visualized at high resolution with the aid of NDP View software (Hamamatsu Photonics). Control specimens were prepared and examined with the same techniques.

**Western Blotting**

For Western blotting, we collected midmodiolar tissue with the method of Szabo et al.\textsuperscript{18} First, we decapitated each animal. We separated the temporal bones from the rest of the skull and isolated 3 cochleae from each group. We next delicately dislodged the spiral lamina from the rest of the bony cochlea. After removal of the spiral lamina, we carefully dissected the modiolus, until only the modiolus protruded from the temporal bone. It was then easy to remove the modiolus from the residual temporal bone. Each cochlea was then broken into several small pieces with fine forceps. These samples were lysed in NP-40 buffer (Elpis Biotech, Daejeon, Korea) containing a protease inhibitor cocktail (Sigma). Protein concentration was determined with a BCA assay kit (Pierce, Rockford, Illinois). Each protein sample (10 μg) was immediately heated for 5 minutes at 100°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% (w/v) reducing acrylamide gels. The separated proteins were transferred to nitrocellulose membranes (Millipore Corp, Billerica, Massachusetts). The membranes were blocked with 5% (w/v) skim milk and Western blotting performed. Chemiluminescent signals were detected with the aid of an ECL kit (Advansa Corp, Menlo Park, California) and the multiple Gel DOC system (Fujifilm, Tokyo, Japan). The following primary antibodies were used: anti-caspase 8, anti-caspase 3, anti-caspase 9, and anti-β-actin from Cell Signaling Technology (Beverly, Massachusetts) and anti-FasL from Santa Cruz Biotechnology (Santa Cruz, California). β-actin served as the internal control. Data were analyzed with Image J 1.38 software (National Institutes of Health, Bethesda, Maryland).

**Control Study**

We used a control group to explore the effects of the operation and the use of fibrin glue to seal the cochleostomy site. After cochleostomy, 60 μL of 0.9% (w/v) normal saline were injected into the cochleostomy site (n = 5; saline [S] group). Each hole was covered with fibrin glue. The contralateral cochleae served as controls (n = 5; control [C] group).

**Statistical Analysis**

The numbers of SGCs stained by H&E were statistically compared. Analysis of variance was used to identify significant differences among groups. Scheffé’s test was employed to identify groups that differed significantly from other groups. The statistical significance of differences in FasL and caspases 8, 9, and 3 staining densities upon Western blotting was evaluated with the Kruskal-Wallis test. Differences were deemed significant at a P value <.05.

In the control study, the numbers of SGCs stained by H&E were also statistically compared. The t test was used to identify significant differences among groups. The statistical significance of differences in FasL and caspase 3 upon Western blotting was evaluated with the Mann-Whitney U test. Differences were deemed significant at a P value <.05.

**Results**

**Effects of Gentamicin and Memantine-and-Gentamicin Treatment on SGC Morphology**

Light microscopy of H&E-stained SGCs in midmodiolar sections showed that gentamicin reduced SGC numbers (Figure 2A) and that the remaining cells appeared abnormal in comparison with those of the C and M groups (Figure 2B, 2D). SGC loss was reduced in the M-G group versus the G group (Figure 2C).

SGC survival rates were calculated by histologic imaging (Figure 3). The number of SGCs in the G group (649.01 ± 112.32) was significantly lower than that of the other groups: C (1156.61 ± 117.92), M (1294.14 ± 169.16), and M-G (1009.96 ± 115.70; P = .05).

**Effects of Gentamicin and Memantine-Gentamicin Treatments on Apoptosis**

TUNEL staining showed that SGCs underwent apoptotic death after gentamicin treatment. TUNEL-positive SGC cells were evident in the G group (Figure 4A) but not in the C or M group (Figure 4B, 4D). Fewer TUNEL-positive cells were evident in the M-G group (Figure 4C). These results were supported by Western blotting.

**Effects of Gentamicin and Memantine-and-Gentamicin on FasL Expression**

FasL expression increased in the G group (2.18 ± 0.76–fold vs control) and decreased in the M-G group (0.93 ± 0.43–fold; Figure 5). Gentamicin increased FasL expression when compared with the control (0.57 ± 0.37–fold; P = .05; Figures 5 and 6). Memantine significantly inhibited gentamicin-induced FasL overexpression (P = .05; Figure 6A).

**Effects of Gentamicin and Memantine-and-Gentamicin on Caspase Activation**

We next examined whether caspase activation upon Fas-FasL-mediated apoptotic signaling was attenuated by memantine, because caspases are important mediators of apoptosis. The proapoptotic caspases include initiator caspases (2, 8, 9, and 10), which cleave and activate effector or executioner caspases (3, 6, and 7).

Activation of the initiator caspases 8 and 9 and the executioner caspase 3 was explored by Western blotting. The G group exhibited initial activation of caspase 8, as revealed by the appearance of a large cleavage fragment (41-43 kDal) and an 18-kDal active cleavage product.
Caspase 9 was also activated, with production of the p35 and p37 subunits, and caspase 3 was cleaved into 17/19-kDal active products (Figure 7). Cleavage of caspases 8, 9, and 3 was reduced in the M-G group as compared with the G group (Figure 7).

Gentamicin (G group) increased activation of caspases 8 (P = .02), 9 (P = .02), and 3 (P = .03) as compared with the control. Memantine significantly reduced caspase activation during gentamicin-induced apoptosis of SGCs (P = .05; Figure 6B-6D).

Results of Control Study

The number of SGCs in the C’ group (1159.72 ± 66.82) did not differ significantly from that in the S group (1108.48 ± 61.33; P = .1095; Figure 8). The FasL expression level in the C’ group (0.05 ± 0.01–fold that of the control) did not differ significantly from that of the S group (0.05 ± 0.01–fold; P = 1.00). The extent of caspase 3 activation in the C’ group (1.12 ± 0.05–fold) did not significantly differ from that of the S group (0.92 ± 0.09–fold; P = .14).

Figure 2. Hematoxylin and eosin staining of midmodiolar sections showed that the number of spiral ganglion cells was significantly less in the (A) gentamicin group compared with the other groups: (B) control, (C) memantine and gentamicin, and (D) memantine.

Figure 3. The number of spiral ganglion cells in the basal turn of Rosenthal’s canal in the G group was significantly less than that in the M-G, C, and M groups. *P < .05. C, control; G, gentamicin; M-G, memantine and gentamicin treatment.

Figure 4. TUNEL-positive cells (arrows) were evident in both the (A) gentamicin and (C) memantine-and-gentamicin groups but fewer in number in the other groups: (B) control and (D) memantine. Inset: the midregion of panel A.
Discussion

We explored whether the NMDA receptor antagonist memantine attenuated gentamicin-induced apoptosis of SGCs. In the control study, we observed no changes after saline injection. Therefore, neither the operation nor fibrin glue affected SGC apoptosis (Figure 8).

Light microscopy of H&E-stained SGCs showed that SGC numbers fell after gentamicin treatment (Figure 2A) and that the cells were abnormal in appearance when compared with the C and M groups (Figure 2B, 2D). The number of SGCs in the G group fell significantly as compared with the C, M, and M-G groups (Figure 3). This excitotoxic effect of gentamicin is presumably associated with excessive glutamate release into the synaptic cleft; glutamate then binds to NMDA receptors of the afferent dendrites. Excessive glutamate triggers NMDA receptor overactivation, which in turn causes associated neuronal ion channels to open, facilitating entry of extracellular calcium and sodium.12

Basile et al proposed that aminoglycosides cause polyamine-like enhancement of glutamate NMDA receptor activity, triggering excitotoxicity and eventual hair cell death. They showed that NMDA antagonists prevented loss of hair cells.19 However, the possible effects of NMDA antagonists on SGCs were not studied. SGCs are a critical relay point between the peripheral auditory organ and the central auditory system. This is particularly important because NMDA receptors are expressed primarily by SGCs, not hair cells.20

We found that memantine significantly increased SGC survival (Figures 2 and 3) and reduced apoptosis (Figure 4C). Our data suggest (albeit indirectly) that NMDA receptors on SGCs play a major functional role in the SGC apoptotic pathway.

We sacrificed the guinea pigs 1 week after drug administration, based on preliminary experiments (not shown) indicating that a glutamate antagonist did not seem to inhibit

Figure 5. Western blotting to detect FasL. FasL expression was significantly higher in the G group than the C group and was reduced in the M-G group. C, control; G, gentamicin; M-G, memantine and gentamicin.

Figure 6. Levels of (A) Fas L, (B) caspase 3, (C) caspase 8, and (D) caspase 9 were higher in the G group when compared with the C and M-G groups. *P < 0.05. C, control; G, gentamicin; M-G, memantine and gentamicin.

C; control, G; Gentamicin treatment, M-G; memantine and gentamicin treatment
SGC apoptosis 2 weeks after gentamicin treatment. The maximum effect of memantine pretreatment was evident after 1 week.

Memantine (3,5-dimethyl-1-adamantanamine), an NMDA receptor antagonist with moderate affinity for the receptor, has recently received Food and Drug Administration approval as a treatment for Alzheimer’s disease. Memantine exhibited neuroprotective features in several in vitro and in vivo models of excitotoxicity and neuronal apoptosis. The drug effectively blocked NMDA receptor activity in the (prolonged) presence of excess glutamate; memantine preferentially blocked extrasynaptic NMDA receptor channels and spared normal synaptic activity. This may explain why the drug has few side effects.

We found that memantine did not induce morphologic changes in or apoptosis of SGCs (Figure 2D, 4D). Although we did not measure electrophysiologic parameters, our results suggest that memantine could be used to treat inner ear disease, with few side effects. Memantine reduced clinical deterioration in patients with moderate to severe Alzheimer’s disease and induced meaningful cognitive improvement in patients with mild to moderate vascular dementia. Drug tolerability was excellent.

Although the neuroprotective effects of NMDA receptor antagonists, including memantine, have been extensively investigated in patients with brain disease, there are few studies on their effects on inner ear disease. Duan et al suggested that the NMDA receptor antagonist MK801 protected against noise-induced excitotoxicity in the cochlea, whereas combination treatment with neurotrophin 3 and MK801 preserved both auditory physiology and morphology from aminoglycoside-induced toxicity.

Basile et al showed that the noncompetitive NMDA receptor antagonist dizocilpine given to streptomycin-treated rats not only rescued a substantial proportion of the sensory hair cells of the cristae but also prevented attendant hyperkinesis and vestibular impairment. These results indicate that excitotoxic mechanisms contribute to aminoglycoside-induced vestibulotoxicity and that NMDA antagonists may be useful to attenuate such otoxicity.

Topdag et al evaluated the effect of intratympanic memantine on cisplatin ototoxicity in rats and found that the drug significantly protected inner ear cells from apoptosis.

In the present study, we found that memantine reduced the expression of FasL and activation of caspases 3, 8, and 9 in SGCs (Figures 5-7). In terms of SGC survival, the number of SGCs in the C group did not differ significantly from that in the M group (Figure 3). Therefore, we did not perform Western blotting of FasL and caspases on M group material.

Two major pathways of apoptosis have been identified in neuronal cells: the receptor-mediated pathway initiated by ligand binding to death receptors, such as Fas and tumor necrosis factor receptor 1, and the mitochondrial pathway initiated by translocation of cytochrome c. We earlier showed that the FasL level increased over time after gentamicin treatment, whereas Fas was expressed constitutively even in control cochlea; the level did not change significantly after gentamicin treatment.

Apoptosis of SGCs induced by gentamicin may thus reflect the activity of FasL and not Fas. Therefore, we evaluated FasL expression and not that of Fas. The FasL level increased upon gentamicin treatment; this effect was attenuated by memantine (Figure 5). However, the mechanism underlying attenuation of FasL expression by memantine remains to be identified. It is possible that FasL stimulates the entry of extracellular calcium into SGCs. In the receptor-mediated pathway, signals from the Fas-FasL pathway activate the initiator caspase 8. This caspase is activated by cleavage into a large fragment of 41 to 43 kDal and a small fragment of 18 kDal (the active product). This initiates a cascade that processes the effector caspases 3, 6, and 7, which in turn cleave several protein substrates.
mitochondrial pathway, mitochondria release apoptogenic factors, including cytochrome c, which induce activation of procaspase 9 and activate the downstream execution phase of apoptosis.  

Caspase 9 is activated by cleavage to p35 and p37 subunits. Caspase 3 is cleaved into active 17/19-kDa fragments. Caspase 3 activation is common to the receptor-mediated and mitochondrial apoptotic pathways.

In the present study, memantine attenuated activation of the initiator caspases 8 and 9 and reduced activation of the executioner caspase 3. Therefore, we suggest that memantine attenuated the receptor-mediated and mitochondrial apoptotic pathways, which may be associated with an increase in the intracellular calcium level.

Although the mechanism underlying the neuroprotective effect of memantine has not yet been identified, we provide evidence that memantine attenuates the gentamicin-induced apoptosis of SGCs. Moreover, memantine may affect Fas-FasL signaling in the receptor-mediated apoptotic pathway and caspase activation in the receptor-mediated and mitochondrial apoptotic pathways.

**Conclusion**

Memantine attenuated gentamicin-induced apoptosis of SGCs. Memantine may affect Fas-FasL signaling in the receptor-mediated apoptotic pathway and caspase activation in the receptor-mediated and mitochondrial apoptotic pathways.

**Author Contributions**

Bo Young Kim, drafted the article, acquisition of data, interpretation of data, revised the article; Woo Yong Bae, design of study, drafted the article, interpretation of data, revised the article; Dae Young Hur, acquisition of data, revised the article; Jae-Ryong Kim, analyzed data, drafted the article; Tae Kyung Koh, acquisition of data, revised the article; Tae Hoon Lee, acquisition of data, drafted the article; Ga Bin Park, acquisition of data, drafted the article.

**Disclosures**

Competing interests: None.

Sponsorships: None.

Funding source: This research was supported by the Basic Science Research Program of the National Research Foundation of Korea, funded by the Ministry of Education (grant NRF-2013R1A1A2007173).

**References**


