Abstract: Objective: This study is intended to contribute to the knowledge regarding the prevention and treatment of ototoxicity due to various drugs and chemicals. 
Material and Methods: In this study, the effects of dexamethasone, memantin, and piracetam applied via the intratympanic route on preventing cellular apoptosis due to ototoxicity were histologically evaluated and compared in 36 rats. 
Results: The effects of dexamethasone and memantin on stria vascularis, organ of Corti, and spiral ganglion were found to be significant (p<0.05). Although the apoptosis rate was decreased, the effect of piracetam was not found to be significant (p>0.05). 
Conclusion: Dexamethasone and memantin were found superior to piracetam in preventing apoptosis due to ototoxicity. Further studies implementing electron microscope and ABR are needed in this subject.
TITLE: THE COMPARISON OF THE EFFICACY OF INTRATYMPANIC DEXAMETHASONE, MEMANTIN, AND PIRACETAM COMPOUNDS AGAINST OTOTOXIC COCHLEAR DAMAGE

RUNNING TITLE: INTRATYMPANIC TREATMENT AGAINST OTOTOXIC COCHLEAR DAMAGE

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Keywords: Ototoxicity, Dexamethasone, Memantin, Piracetam
INTRODUCTION

The prevention and treatment of ototoxic inner ear damage is currently a popular research field.

Ototoxicity develops mostly after oncologic treatments. The ototoxic effects of cisplatin and carboplatin are well known. There are many studies in the literature on various chemoprotective agents used to prevent cisplatin-based ototoxicity.

Intratympanic steroid applications in the treatment of cochlear disorders began to gain popularity in the recent years. The main advantage of intratympanic injection is in achieving high local active substance concentration in the ear without systemic side effects.

In this study, our aim was to reduce the negative effects of ototoxicity on cochlea using various compounds. Ototoxic inner ear damage was created in rats, which was followed by intratympanic injection of dexamethasone, memantine and piracetam. The animals were sacrificed and levels of apoptosis were determined histopathologically at the stria vascularis, the spiral ganglion, and organ of corti. The obtained data were compared with the protective effects of used agents.

MATERIAL and METHODS

The study was approved by the Kocaeli University Ethical Committee for Experimental Animals. This study was performed on 36 healthy adult female Wistar albino rats.

Rats weighing 240–330 g, were housed under standard conditions (12 h light-12 h dark cycle and 21-22°C temperature, with free access to water and food ad libitum. In the course of the study, animals which died or developed otitis media were excluded. Rats with normal otoscopic examination were included. Animals were divided into three groups each consisting of 12 rats. The left ear was used for intratympanic treatment and right ears served as the control ears in which saline was injected. In all groups, under ether anesthesia, rats were injected intraperitoneally with 16 mg/kg Cisplatin. 48 h after these injections, intraperitoneally (i.p.) 45 mg/kg ketamin +5 mg/kg xylazine was applied for general anestesia and dexamethasone (4mg/ml), piracetam (200mg/ml), memantine hydrochloride (10mg/ml) were administered by the intratympanic route. 0.2 ml solutions were applied at each
injection. The injections were repeated 4 times with 48 hour intervals. Animals were sacrificed by decapitation under anesthesia using i.p. 100 mg/kg ketamine on the 10th day after cisplatin administration. Inner ears were removed and fixed in 4% neutral formaline. After the fixation, tissues were decalcified with Decal for 2 h and, were embedded in paraffin and cut in serial sagittal sections (3 μm). Paraffin sections were stained with Haematoxylin Eosin (H&E) and “Mouse anti-single-stranded DNA monoclonal antibody” (Millipore, MAB 3299; LV1505484, 2002-2007) assay was performed. Immunoreactivity was examined in different cochlear regions by light microscopy (BX50F-3; Olympus, Tokyo, Japan). Serial cochlea sections stained with H&E and mouse anti-single-stranded DNA monoclonal antibody immunostaining were examined by light microscopy. Images were collected using a microscope and camera, and apoptotic cells were counted using semi-quantitative methods.

Mouse anti-single-stranded DNA monoclonal antibody positive cells were counted in the spiral ganglion, organ of corti, and stria vascularis. Cell counts were performed on digital images captured at 100× magnification. Counts were performed at 10 locations for each slide.

Apoptotic cells were counted in selected sections independently for each ear regions including the spiral ganglion, organ of corti, and stria vascularis by three investigators, who were blinded to the group. Apoptotic cells were counted in ten regions for each animal in all groups.

RESULTS

Different regions of the cochlea were examined for anti-ss DNA in the right ear serving as the control group and in the left ear as the treatment group using light microscopy. Anti-ss DNA monoclonal antibody positive cells were discriminated with brown nuclei in the spiral ganglion, organ of corti, and stria vascularis in all groups (figures 1-5). Non-apoptotic cells were discriminated with dark blue nuclei stained with Mayer’s Haematoxylin in the above mentioned cochlear regions in all groups (figure 6).

Most of anti-ss DNA positive cells were determined in organ of corti from different regions of cochlea (Table 1-4).

Apoptotic cell numbers (ACN) were significantly lower in the dexamethasone and memantin groups than the piracetam group and the control group (P < 0.05). The
number of apoptotic cells from the spiral ganglion, organ of corti, and stria vascularis of all the groups were shown in Table 1-4.

A significant decrease in the number of apoptotic cells was observed in the spiral ganglion, organ of corti, and stria vascularis in the dexamethasone group compared with the control group (figure 3), (Table 1 ; $P < 0.05$). Similarly, a significant decrease in the number of apoptotic cells was observed in the spiral ganglion, organ of corti, and stria vascularis in the memantine group compared with the control group (figure 4), (Table 2 ; $P < 0.05$). In addition to these findings, a slight decrease in apoptosis rate was seen in the organ of corti and stria vascularis in the piracetam group compared with the control group, but this difference was not significant (figure 5), (Table 3).

It was determined that dexamethasone and memantin were more effective than piracetam in preventing apoptosis resulting from ototoxicity. When comparing the dexamethasone and memantin treatment groups, dexamethasone was found more effective than memantin on the spiral ganglion, but there was no difference in the organ of corti and stria vascularis (Table 4).

**DISCUSSION**

Ototoxicity is the general name of the damage in the cochlear and vestibular organs as a result of exposure to therapeutic and chemical substances.

The ototoxicity of cisplatin causes blockage of the ion channels in the membranes of external hair cells and increases the hyperpolarization and auditory threshold. In his studies, Peter supported this mechanism (1). It has been shown that cisplatin ototoxicity is related to the malfunction of the anti-oxidant system leading to an increment of the peroxidation of the lipids in the cochlear tissues (2).

The other mechanism of cisplatin toxicity is the formation of reactive oxygen radicals in the cochlea. Especially, free radicals like superoxide anion causes a
decrease in the intracellular anions (3). Free radical formation takes place as a result of the decrease in intracellular glutathion levels and changes in the activities of antioxidant enzyme activities. In several studies, among the rats that have undergone 16mg/kg cisplatin treatment, a 53% decrease compared with the control group was found in the cochlear glutathion levels. A 165% increment in the malonaldehyde amount was observed as evidence of the cellular free radical oxidation of lipids (4). The derangement in the antioxidant defense system causes an increment in the lipid peroxidation and therefore leads to apoptosis in the hairy cells, support cells, stria vascularis, and auditory nerves (5).

Several agents showing protective effects against cisplatin ototoxicity have been reported. Among those are; sodium thiosulfate (6), diethyldithiocarbamate, and D-Methionine (7). Previous studies have shown the efficacy of tocopherol, vitamin C, melatonin, sodium salicylate, N-acetylcysteine (8) and lactate (9) against cisplatin cytotoxicity. Nevertheless, an effective and ideal treatment applied by the intratympanic route is still sought.

In this study, the protective effects of memantin which is a blocker of the NMDA type glutamate receptors and calcium sensitive nicotinic acetyl choline receptors, dexamethasone, which has a proven anti-inflammatory efficacy, and piracetam, which is an antioxidant, trombocyte antiagregant, and neuroprotective compound, against the inner ear damage by cisplatin ototoxicity is examined in an animal model.

Steroids (prednisolone, dexamethazone, methylprednisolone) are traditionally used in the treatment of various ear diseases (10). Dexamethasone’s intratympanic usage has also started to increase. In the immunohistochemical studies; dexamethasone has been marked in high concentrations in the spiral ligament, basillary membrane, corti organ and spiral ganglion. Glucocorticoids have various physiologic effects on the cochlear tissues (11).

Common use of glucocorticoids in otology is related to their effects on cochlear immune suppression and their anti-inflammatory functions (12, 13, 14). For example, transcription factor-nuclear factor-κβ by the general immune response regulates the synthesis of several cytokines (15, 16), and this transcription factor is suppressed by glucocorticoids (17, 18). Specific compartments within the inner ear of
this nuclear factor κβ inhibition by glucocorticoids is thought to be responsible for the repercussions of hearing loss.

Besides their restorative effects on the dysfunctional autoimmunity, the effect of corticosteroids on the ion transport in the stria vascularis of mice have also been shown (19). The presence of glucocorticoid receptors in a large number of spiral ligament cells support the potential role of K+ ion haemostasis. The same way the damage of the cochlea due to ototoxicity is shown to be reduced by glucocorticoids, (20), ischemia (21), mechanical damage (22), and the noise (23, 24) induced cochlear damage is also shown to be decreased by these agents.

Adamantane derivative 3,5-Dimethyl-1-Adamantane (Memantine) is a blocker of calcium-permeable ion channels such as nicotinic acetylcholine receptors or NMDA-type glutamate receptors. In mice studies, it was given intraperitoneally at a dose of 5mg/kg or 20 mg/kg subcutaneously. Intratympanic application has not been tested previously. A very recent study of memantine in the cortical neuron cell cultures have shown the compound to be protective against glutamate toxicity (25).

The loss of function of the outer hairy cells is known to be the major cause of sensorineural hearing loss associated with various detrimental stimulations created by noise or ototoxic agents. The influx of Ca through nicotinic receptors inflicts structural changes in external hair cells. Memantine prevents massive influx of Ca into the cell through NMDA receptors, and thus, improves cochlear physiology (26).

Piracetam is a drug with a large spectrum of effects. It has been used in sudden hearing loss with favorable results (27). Piracetam (2-oxo-1-pyrolidine-acetemine) is a gamma amino butyric acid derivative with low molecular weight. It has anti-inflammatory, antiapoptotic, cytoprotective, and immune modulatory effects. Literature presents no information on piracetam that is used in the treatment of acute hearing loss. The efficiency of intraperitoneal piracetam was evaluated in cochlear alterations after radiotherapy in guinea pigs with cranial and cervical cancer. In this study, the damage to the stria vascularis, spiral ganglion, inner hairy cells and outer hairy cells of the cochlea in the animals that were subjected to 60 Gy cranial radiotherapy was histopathologically shown and piracetam was shown to reduce the cochlear damage associated radiation (28). Another study determined that piracetam prevented the ototoxic effects of cisplatin-gentamycin combination in the auditory tract extending from the cochlea to the midbrain (29). In these studies, it has been
suggested that piracetam, with its reological effects, increases oxygenation, and with its antiapoptotic effects, prevents apoptosis in healthy cells.

Using “Mouse anti single-stranded DNA Monoclonal Antibody”, apoptotic cells with heavily stained nuclei were differentiated in the stria vascularis, corti organ, and spiral ganglion. This antibody does not define the double stranded DNA conformations; it was the choice of preference because it is free of internucleosomal DNA fragmentations and is useful in determining different stages in different cell types and it is a cellular marker specific to apoptotic death. This antibody reacts with deoxycytidine and requires an ss DNA flexibility of minimum 25-30 base for binding. In our study, apoptotic cells reacting with this antibody have been counted in specific areas calculated from the tissue samples.

On the microscopy, the highest rate of ototoxicity was noted with ssDNA (+) stained neurons in the right cochlea of the control group. The highest rate of apoptotic (+) staining was determined in the corti organ among different areas.

The number of apoptotic cells was reduced in the spiral ganglion, corti organ, and stria vascularis of the rats in the treatment group. While this reduction was statistically significant for the dexamethasone and memantine groups, it was not significant in the piracetam group. However, we believe that interpreting this result obtained with piracetam as inefficient would be wrong. Higher doses and more frequent use of piracetam, may show increased efficiency.

When the reduction in apoptosis rates of the memantine and dexamethasone applied groups were compared, the difference in the corti organ or stria vascularis was not statistically significant, but dexamethasone was more effective in the spiral ganglion. However, this finding alone should not lead to the assumption that dexamethasone is more effective in preventing apoptosis resulting from cisplatin ototoxicity.

Memantine, like NMDA type of glutamate receptors, is a Ca channel blocker. Abnormally high levels of Ca induce degeneration of external hairy cells. We believe memantine, binding with glutamate NMDA receptors, may be much more effective in preventing the damage to the external hairy cells, particularly in the corti organ by blocking the degenerative mechanism due to cellular Ca increase through prevention of massive Ca infiltration into the cells before the mechanism is triggered and may restore the cochlear physiology.
REFERENCES


**FIGURES LEGENDS**

**Figure 1.** Anti-ss DNA (+) cells (arrows) on the spiral ganglion of the control group, Anti-ss DNA stain, 100X.

**Figure 2.** Anti-ss DNA (+) cells (arrows), spiral ganglion (sg), and tectorial membrane (tm) within the cochlea of the control group, Anti-ss DNA stain, 40X.

**Figure 3.** Anti-ss DNA (+) cells (arrows), and spiral ganglion (sg) on the dexamethasone treated ear, Anti-ss DNA stain, 40X.

**Figure 4.** Scala media (sm), scala tympani (st), and spiral ganglion (sg) on the memantine treated ear, Anti-ss DNA stain, 40X.

**Figure 5.** Spiral ligament (sl) and stria vascularis (sv) on the piracetam treated ear, Anti-ss DNA stain, 100X.

**Figure 6.** Scala tympani (st), tectorial membrane (TM), basilar membrane (bm) on the piracetam treated ear, H&E stain, 40X.
Table 1. The number of apoptotic cells of different regions from the cochlea in the control and dexamethasone groups and their respective p values

<table>
<thead>
<tr>
<th>Tissues/Groups (mean±S.E.M)</th>
<th>Control</th>
<th>Dexamethasone</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral ganglion</td>
<td>0.18±0.02</td>
<td>0.08±0.01</td>
<td>0.00*</td>
</tr>
<tr>
<td>Organ of corti</td>
<td>1.44±0.53</td>
<td>0.67±0.71</td>
<td>0.02*</td>
</tr>
<tr>
<td>Stria vascularis</td>
<td>0.78±0.44</td>
<td>0.22±0.44</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

*p<0.05  significantly different from the control value
Table 2. The number of apoptotic cells of different regions from the cochlea in the control and memantine groups and their respective p values

<table>
<thead>
<tr>
<th>Tissues/Groups (mean±S.E.M)</th>
<th>Control</th>
<th>Memantine</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral ganglion</td>
<td>0.15 ± 0.018</td>
<td>0.11 ± 0.01</td>
<td>0.001*</td>
</tr>
<tr>
<td>Organ of corti</td>
<td>1.33 ± 0.50</td>
<td>0.67 ± 0.70</td>
<td>0.039*</td>
</tr>
<tr>
<td>Stria vascularis</td>
<td>1.11 ± 0.60</td>
<td>0.33 ± 0.50</td>
<td>0.013*</td>
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</tbody>
</table>

*p<0.05 significantly different from the control value*
Table 3. The number of apoptotic cells of different regions from the cochlea in the control and piracetam groups and their respective p values.

<table>
<thead>
<tr>
<th>Tissues/Groups (mean±S.E.M)</th>
<th>Control</th>
<th>Piracetam</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiral ganglion</td>
<td>0.17 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.164</td>
</tr>
<tr>
<td>Organ of corti</td>
<td>1.22 ± 0.66</td>
<td>0.89 ± 0.78</td>
<td>0.337</td>
</tr>
<tr>
<td>Stria vascularis</td>
<td>0.78 ± 0.83</td>
<td>0.44 ± 0.52</td>
<td>0.406</td>
</tr>
</tbody>
</table>

*p<0.05 significantly different from control the value*
Table 4. The number of apoptotic cells of different regions in the cochlea in treatment groups and p values

<table>
<thead>
<tr>
<th>Tissues/Groups (mean±S.E.M)</th>
<th>Stria vascularis</th>
<th>spiral ganglion</th>
<th>Organ of corti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>0,22±0,44</td>
<td>0,07±0,01</td>
<td>0,66±0,70</td>
</tr>
<tr>
<td>Memantine</td>
<td>0,33±0,50</td>
<td>0,11±0,01</td>
<td>0,66±0,70</td>
</tr>
<tr>
<td>P</td>
<td>0,730</td>
<td>0,000*</td>
<td>1,00</td>
</tr>
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*p<0,05 significantly different from the control value