Effect of Intratympanic Dexamethasone Administration on Cispaltin-Induced Ototoxicity in Adult Guinea Pigs, Is It Time-Dependent? Audiological and Histological Study

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Abstract: Introduction: Cisplatin is a cornerstone chemotherapeutic drug often dose-limited by ototoxicity. Many trials have been introduced for a complete cure or prevention of cisplatin-induced ototoxicity but unfortunately, with un-satisfactory results. Intratympanic steroids have been recently tried and shown competitive results in terms of reduction of ototoxicity. However, perfect timing of drug administration remains controversial.
Aim of the work: To evaluate the effect and safety of intratympanic dexamethasone administration on cisplatin-induced ototoxicity in adult male guinea pigs and to assess the differences between early and late protection from this ototoxicity.
Materials and methods: Forty eight adult male guinea pigs were divided as follows: Group I served as control group. Group II was subjected to intratympanic saline (subgroup IIa) or dexamethasone (subgroup IIb) injection. Group III was intraperitoneally injected with cisplatin. Groups IV and V were subjected first to intratympanic dexamethasone administration in both ears for 5 days starting 1 day and 1 hour -respectively- before cisplatin intraperitoneal injection.
Results: Dexamethasone intratympanic injection revealed similar functional and structural results compared with control. Cisplatin intraperitoneal injection resulted in a profound cochlear functional and structural damage in group III. Limited otoprotection resulted from intratympanic dexamethasone administration one day before cisplatin. Intratympanic dexamethasone injection one hour before cisplatin treatment resulted in a significant preservation of the functional and structural properties of the cochlea.
Conclusion: Intratympanic dexamethasone administration is a safe, easy and efficient way to protect from cisplatin ototoxicity especially when administered one hour before cisplatin treatment.
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Key words: dexamethasone, cisplatin, ototoxicity, guinea pigs.

1. Introduction:

Cisplatin is a common chemotherapeutic agent essentially used to treat many different types of cancer including neuroblastoma, osteosarcoma, testicular, ovarian, cervical, bladder, lung, and head & neck cancers. It has several side effects stemming from its non-specific cytotoxic action. The most striking dose-limiting side effect of cisplatin therapy is ototoxicity **(1)**. Cisplatin-induced ototoxicity generally manifests as tinnitus and sensorineural hearing loss which starts in the high frequencies. Nevertheless, it extends into lower frequencies that are important for speech perception. This hearing impairment is dose related, cumulative, bilateral and usually permanent **(2)**. Moreover, ototoxicity caused by cisplatin may occur within hours to days after drug administration **(3)**. Dozens of experimental studies have attempted to find an ideal otoprotectant by administration of antioxidants at an early stage in the ototoxic pathways. Unfortunately, many of these agents have been found to inhibit the tumoricidal effects of cisplatin **(4)**.

Systemic glucocorticoids are currently in use for treatment of hearing loss in a variety of cochlear disorders such as autoimmune inner ear, Meniere's disease, tinnitus and cases of sudden or idiopathic hearing loss when etiology is unclear **(5)**. Unfortunately, corticosteroids also down-regulate apoptosis genes in tumor cells. Therefore, their systemic application to protect against cisplatin-induced ototoxicity may result in decreased efficacy of cisplatin's tumoricidal properties **(6)**.

Intratympanic administration of drugs is a contemporary method of locally treating inner ear disorders, allowing diffusion across the round window into the inner ear where it can exert its effect. Specifically, steroids placed into the middle ear have been shown to diffuse across the round window into the inner ear and bathe the inner ear structures **(7)**. This method allowed concentration of steroid to much higher levels within the inner ear

compared to oral or parenteral routes **(5)**. Also, local administration prevented systemic absorption avoiding the common systemic side effects of steroids including hyperglycemia, peptic ulcers, hypertension and osteoporosis. Most importantly it prevented the more problematic reduced efficacy of chemotherapeutic agents **(6)**. Intratympanic administration of steroids has been used to safely treat other inner ear disorders such as sudden sensorineural hearing loss **(8)** and Méniere's disease for many years **(9)**.

So, our desired goal is to evaluate the safety of the intratympanic dexamethasone therapy, from both audiological and histological points of view as a steroid-protectant from cisplatin ototoxicity and to assess the ultimate timing of drug application for satisfactory protective results.

2. Materials and Methods:

Animals

Forty eight adult male guinea pigs were used in this study. They were housed under standard conditions of boarding in wire mesh cages with food and water *ad libitum.* The experiment was done in the Medical Research Center and in the Audiology Department, Faculty of Medicine, Ain Shams University.

Experimental groups

Guinea pigs were allocated into five groups, 8 animals each; except for group II it consisted of 16 animals:

Group I (Control):

Guinea pigs received single intraperitoneal injection of 1 ml of saline and served as a control group.

Group II:

After the first Auditory Brainstem Response (ABR) measuring, this group was divided into 2 subgroups each consisted of 8 animals:

Subgroup IIa: (Saline)

Guinea pigs were subjected to intratympanic saline injection in both ears once daily for 5 days.

Subgroup IIb: (Dexamethasone):

Guinea pigs were subjected to intratympanic dexamethasone injection in both ears once daily for 5 days **(10)**.

Group III (Cisplatin):

A single intraperitoneal injection of cisplatin at a dose of 8 mg/kg (**11)** was administered to guinea pigs of this group. It was purchased as Cytoplatin-50 Aqueous, CIPLA LTD, Verna Industrial Estate, India.

Group IV (Dexamethasone 1 day before cisplatin):

Guinea pigs were subjected to intratympanic dexamethasone injection in both ears once daily for 5 days, starting 1 day before a single cisplatin injection at a same dose and route as group III.

Group V (Dexamethasone 1 hour before cisplatin):

Guinea pigs were subjected to same doses of dexamethasone as group IV, in both ears for the same period, starting 1 hour before a single cisplatin injection at the same dose and route as group III.

Procedure of Intra-tympanic injection:

Prior to injection, the animals were examined for any evidence of tympanic membrane perforation, middle ear infection, effusion, and/or debris in the external auditory canals. Intratympanic injections of dexamethasone (with a concentration of 4 mg/ml) or 0.9% saline in guinea pig ears were carried out under light ether anesthesia using a 30 degree pediatric nasal endoscope. After the tympanic membrane was visualized, a sterile 22-gauge canula connected to a 1ml syringe was passed through the inferior portion of the tympanic membrane. About 0.5 - 0.7 ml (enough to fill the middle ear) of either solution was injected into the middle ear space **(10)**. Dexamethasone was purchased as dexamethasone sodium phosphate ampoules (Amriya pharmaceutical industries, Alexandria- Egypt).

I-Audiological study:

Auditory Brainstem Response (ABR) was measured in all guinea pigs before injection of cisplatin or intra-tympanic saline or dexamethasone. Moreover, ABR was repeated after saline or dexamethasone intratympanic injection in guinea pigs of subgroup IIa and subgroup IIb, respectively. All recordings were conducted under anesthesia by ketamine hydrochloride (Sigma), 40 mg/kg **(12)** in a soundproof chamber.

Stimulus parameters:

The ABRs were generated in response to 100 μs alternated clicks at a range of 2-4 KHz. The stimulus was presented at a rate of 21 pulses / second. Monaural thresholds were obtained via headphone at 10 dB steps between 100 dBSPL down to threshold.

Recording parameters:

The ABRs were recorded by means of three platinum-iridium needle electrodes, placed sub-dermally over the vertex (positive), the mastoid (negative) and the contra-lateral mastoid (ground). The recording window included a 10-millisecond post-stimulus times. ABRs were amplified 20000-fold and filtered from 30 Hz to 3000 Hz. At least two

repeatable traces with approximately 1000 response sweeps for each trace were collected for each subject. The test session including electrode application and evoked response recording for each subject lasted for about 30 minutes.

Response analysis:

The ABRs was defined by three positive peaks (I, III, V) at supra-threshold intensity (100 dB SPL). Three recording parameters were analyzed. Absolute and inter-peak latencies for wave I, III and V measured and threshold. Threshold was defined as the lowest intensity capable of producing a visually detectable, reproducible wave V.

Eight days post cisplatin injection; ABR in all guinea pigs in groups III, IV and V were re-measured by the same procedure. Moreover, ABR was repeated simultaneously in subgroups IIa and IIb. Hearing loss induced by single-dose cisplatin in guinea pig has been found to be stabilized by 5-7 days after cisplatin injection **(13, 14)**.

II- Histological study:

At the end of the final ABR recording session, all animals were anesthetized with ketamine hydrochloride 40 mg/kg **(12)**. The animals were decapitated, the temporal bone was taken, and the cochleae were dissected carefully. The right cochleae of all groups were processed for light microscopic examination (LM). Moreover, the left cochleae of all groups were processed for scanning electron microscopic examination (SEM). A tiny opening was made in the apical turn of the cochleae by a curved stapes pick. The proper fixative was gently forced through the preformed apical opening by a fine needle fitted onto a tuberculin syringe allowing for good fixation.

A-Light microscopic study (LM):

The cochleae were fixed in 10% formalin for 2 days. Decalcification was done using the chelating agent, ethylene-diamine-tetra-acetic acid (EDTA) in the form of its disodium salt (5.5 g EDTA in 90 ml distilled water and 10 ml formaldehyde, 37:40%). Decalcification was done for 4 weeks with daily change of the solution until softening of specimens was obtained. Specimens were processed to form paraffin blocks. Serial longitudinal sections passing parallel to the modiolus were cut at sections of 5µm thickness and subjected to Haematoxylin and Eosin (H&E) stain **(15)**.

B- Scanning electron microscopic study (SEM):

The cochleae were fixed in 1.5% gluteraldehyde in Phosphate buffered saline (pH=7.4) for 2 hours at room temperature. The cochleae were then washed in Phosphate buffered saline, transferred to 1% osmium solution. Longitudinal micro-dissection of the cochleae was done using extra sharp forceps and microsurgical scissors. They were then dried and gold-coated using sputter coated SCD/005. Tissues were mounted in copper stup and viewed using scanning electron microscope (XL30) in the Anatomy Department, Faculty of Medicine, Ain Shams University **(16)**.

III-Morphometric and Statistical study:

A- Auditory brainstem response (ABR) thresholds and threshold shifts were expressed as mean ± SEM.

B- The cochleae were examined in serial H&E-stained sections from all guinea pigs in each group (five high power fields /section) to measure the thickness of the central part of the fibrous connective tissue meshwork underneath the stria vascularis. The measurements were performed using Image Analyser (Olympus Image J, NIH, 1.41b, USA) in the Oral Pathology Department, Faculty of Dentistry, Ain Shams University. The mean values of different fields from serial cochlear sections were estimated.

The standard error of means (SEM) of all data was calculated and statistical analysis was carried out using SPSS statistical program version 17; IBM Corporation, Route 100 Somers, NY 10589. Data were evaluated by using the one-way analysis of variance test (ANOVA). Comparison of measurments between all groups was done by post hoc least significant difference. As regard the probability, the least significant level used was at P value less than 0.05.

3. Results:

I-Audiological results:

Forty eight adult guinea pigs were enrolled in the present study (groups I, II, III, IV and V). Prior testing, all animals showed normal mobile tympanic membranes together with normal ABR morphology and thresholds (Diagram 1).

As shown in table (1), all Animals of all groups showed normal mean hearing thresholds with normal mean absolute latencies of waves I, III and V & normal inter-peak latencies (I-III, III-V and I-V). There was non-significant statistical difference ($p>0.05$) in all groups of the study prior to cisplatin intraperitoneal injection and prior to saline or dexamethasone intratympanic injection. This emphasized that all animals were normal hearers before any intervention.

Cisplatin injection resulted in a death rate of 2 animals (25%) in each of groups III, IV and V,

leaving 6 animals in each of these groups continuing the experiment.

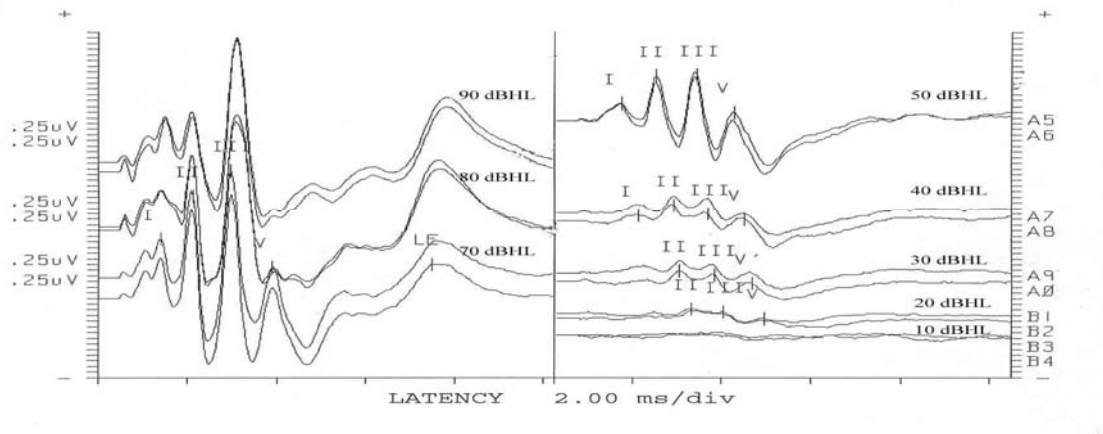


Diagram 1: showing different ABR parameters of a control guinea pig.

Table (1): Showing the Mean ± SEM of different ABR parameters and comparison between all groups: (Before administration of drugs)

	Group I	Group II	Group III	Group IV	Group V
Threshold	11.25 ± 2.39 (8)	10.00 ± 2.89 (16)	10.00 ± 2.04 (8)	13.75 ± 1.25 (8)	10.00 ± 2.04 (8)
I Lat.	1.48 ± 0.14 (8)	1.46 ± 0.13 (16)	1.57 ± 0.15 (8)	1.43 ± 0.14 (8)	1.58 ± 0.13 (8)
III Lat.	2.73 ± 0.13 (8)	2.74 ± 0.15 (16)	2.80 ± 0.23 (8)	2.65 ± 0.13 (8)	2.73 ± 0.17 (8)
V Lat.	3.90 ± 0.11 (8)	4.10 ± 0.18 (16)	3.80 ± 0.31 (8)	3.95 ± 0.12 (8)	4.00 ± 0.11 (8)
V Lat Ths	4.90 ± 0.17 (8)	4.48 ± 0.17 (16)	4.77 ± 0.29 (8)	4.78 ± 0.19 (8)	4.73 ± 0.18 (8)
I-III	1.25 ± 0.06 (8)	1.35 ± 0.08 (16)	1.47 ± 0.27 (8)	1.20 ± 0.07 (8)	1.23 ± 0.09 (8)
III-V	1.18 ± 0.08 (8)	1.25 ± 0.09 (16)	1.10 ± 0.06 (8)	1.28 ± 0.09 (8)	1.18 ± 0.08 (8)
I-V	2.38 ± 0.13 (8)	2.58 ± 0.19 (16)	2.60 ± 0.31 (8)	2.53 ± 0.18 (8)	2.40 ± 0.16 (8)

-Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs.

Table (2): Showing the Mean ± SEM of different ABR parameters and comparison between all groups: (After administration of drugs)

	Group I (Control)	Group IIa (Saline)	Group IIb (Dexa)	Group III (Cisplatin)	Group IV (Dexa 1 day before Cisplatin)	Group V (Dexa 1 hour before Cisplatin)
Threshold	11.25 ± 2.39 (8)	11.25 ± 1.25 (8)	12.50 ± 1.44 (8)	58.33 ± 4.41 [a] (6)	26.67 ± 3.33 [ab] (6)	11.67 ± 1.67 [bc] (6)
I Lat.	1.48 ± 0.14 (8)	1.45 ± 0.12 (8)	1.58 ± 0.14 (8)	1.43 ± 0.03 (6)	1.23 ± 0.03 (6)	1.47 ± 0.09 (6)
III Lat.	2.73 ± 0.13 (8)	2.78 ± 0.05 (8)	2.75 ± 0.17 (8)	2.70 ± 0.06 (6)	2.73 ± 0.09 (6)	2.90 ± 0.26 (6)
V Lat.	3.90 ± 0.11 (8)	4.23 ± 0.20 (8)	3.98 ± 0.13 (8)	3.90 ± 0.00 (6)	3.90 ± 0.21 (6)	4.03 ± 0.28 (6)
V Lat Ths	4.90 ± 0.17 (8)	4.43 ± 0.15 (8)	4.83 ± 0.17 (8)	4.83 ± 0.23 (6)	4.93 ± 0.24 (6)	5.03 ± 0.47 (6)
I-III	1.25 ± 0.06 (8)	1.33 ± 0.08 (8)	1.18 ± 0.09 (8)	1.27 ± 0.03 (6)	1.37 ± 0.09 (6)	1.43 ± 0.19 (6)
III-V	1.18 ± 0.08 (8)	1.45 ± 0.18 (8)	1.30 ± 0.11 (8)	1.20 ± 0.06 (6)	1.17 ± 0.22 (6)	1.13 ± 0.12 (6)
I-V	2.38 ± 0.13 (8)	2.78 ± 0.19 (8)	2.65 ± 0.17 (8)	2.47 ± 0.03 (6)	2.33 ± 0.12 (6)	2.57 ± 0.23 (6)

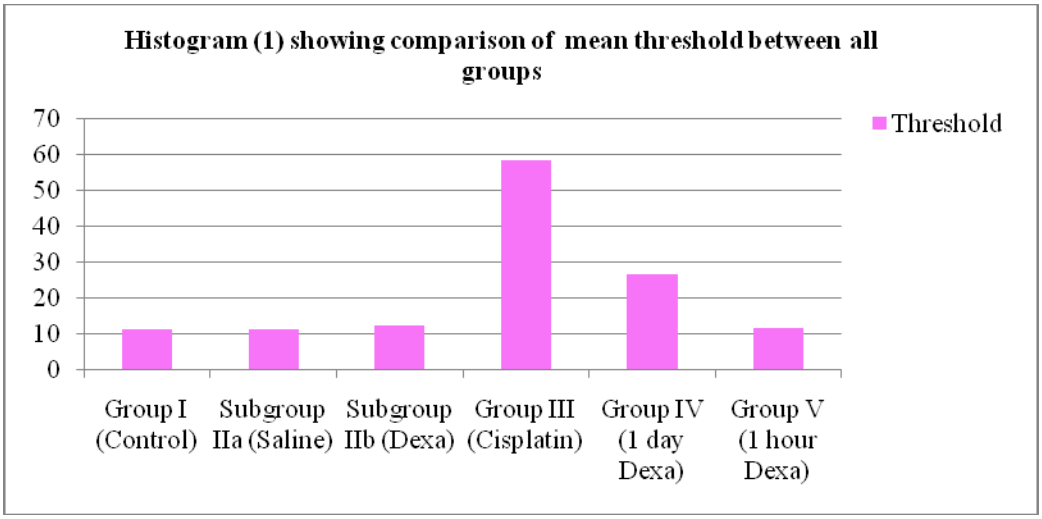
-Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs. -Dexa=Dexamethasone
- **a:** significance of difference by LSD from Group I (Control) at least $p<0.05$.
- **b**: significance of differences by LSD from Group III (Cisplatin) at least $p<0.05$.
- **c**: significance of differences by LSD from Group IV (dexamethasone 1 day before cisplatin) at least $p<0.05$

As shown in table (2) and histogram (1), guinea pigs of subgroups IIa and IIb given either saline or dexamethasone intratympanically respectively, showed non-significant statistical difference ($p>0.05$) in mean threshold and all other parameter means of ABR testing compared with group I (control). This documented that intratympanic dexamethasone is safe.

On the other hand, both groups III and IV showed statistically significant elevated mean threshold parameter only ($p<0.05$) with non-significant statistical difference ($p>0.05$) as regards the other parameters means of ABR testing compared with group I (control). Moreover, group IV (administered dexamethasone 1 day before cisplatin) showed significant statistical difference in mean threshold parameter only ($p<0.05$) with non-significant statistical difference as regards the other parameters means of ABR testing ($p>0.05$) compared with group III (not protected from cisplatin ototoxicity). This documented the limited protective effect of dexamethasone (injected 1 day prior to cisplatin administration) on cisplatin ototoxicity in both ears in group IV.

On the other hand, group V (administered dexamethasone 1 hour before cisplatin) showed non-significant statistical difference ($p>0.05$) in mean threshold and all other parameters means of ABR testing compared with group I (control). Moreover, group V (administered dexamethasone 1 hour before cisplatin) showed significant statistical difference in mean threshold parameter only ($p<0.05$) with non-significant statistical difference as regards the other parameters means of ABR testing ($p>0.05$) compared with group III (not protected from cisplatin ototoxicity). This documented the marked protective effect of dexamethasone (injected 1 hour prior to cisplatin administration) on cisplatin ototoxicity in group V.

Additionally, group V (administered dexamethasone 1 hour before cisplatin) showed significant statistical difference in mean threshold parameter only ($p<0.05$) with non-significant statistical difference as regards the other parameters means of ABR testing ($p>0.05$) compared with group IV (administered dexamethasone 1 day before cisplatin). This result pointed to better protection achieved by administration of dexamethasone one hour (total protection) prior cisplatin administration than one day (partial protection).



Dexa= Dexamethasone

II-Histological results:

No histological differences were found in the structure of the cochleae of the control group (given single intraperitoneal saline injection) and in both subgroups of group II (given either intratympanic saline or dexamethasone injections).

A) Light microscopic results:

Group I (Control):

Examination of H&E-stained sections of control group showed the wedge-shaped cochlear duct housed in the boney cochlea. Reissner's membrane was noticed with its two layers of simple squamous epithelium roofing the cochlear duct and separating it from the scala vestibuli, while the basilar membrane made its floor separating it from

the scala tympani. The basilar membrane extended from the spiral lamina medially to the spiral ligament on the lateral wall, supporting the organ of Corti (Figure 1). Neuroepithelial cells of the organ of Corti were seen as three outer hair cells (OHCs) with acidophilic cytoplasm and basal rounded vesicular nuclei and one inner hair cell (IHC) with central rounded vesicular nucleus. Outer phalyngeal cells supported the OHCs and the inner phalyngeal cell was seen supporting the IHC. Outer and inner pillars surrounded the tunnel of Corti. Other supporting cells as Hensen, Claudius, Böttcher cells laterally and border cells medially could be recognized. The tectorial membrane was seen hanging over the hair cells as homogenous acidophilic structure (Figure 2). Stria vascularis covered the lateral wall of the cochlear duct and was made of three layers of epithelium; marginal, intermediate and basal with obvious capillaries, melanin pigments and fibrous connective tissue meshwork underneath (Figure 3).

Group III (Cisplatin):

Cisplatin intraperitoneal injection resulted in cytoplasmic vacuolization and degeneration of OHCs in most of the sections in this group (Figures 4 & 5). Widely spaced OHCs were also seen in most of those sections (Figure 6). Absent OHCs was noticed in some sections, especially in the third row, with degeneration of the remaining OHCs (Figure 7). The IHCs were seen apparently less affected compared with OHCs showing slight cytoplasmic vacuolization in some sections (Figure 6). Most of the supporting cells in all sections examined were highly vacuolated, swollen and showed pyknotic nuclei (Figures 4, 5, 6 & 7). Regarding the stria vascularis, vacuolated epithelial cells could be seen involving the three layers. Dilated congested intraepithelial capillaries could be easily noticed. The fibrous connective tissue meshwork underneath the stria vascularis showed apparent increase in thickness as compared with the control sections (Figure 8). It is noteworthy that most of these findings were seen in the basal and middle turns of the cochlea with little affection of the apical turns.

Group IV (Dexamethasone 1 day before cisplatin):

Intratympanic administration of dexamethasone, one day before intraperitoneal cisplatin injection, H&E-stained sections of the cochleae showed moderate protection against cisplatin ototoxicity. Widely spaced OHCs with slightly vacuolated cytoplasm were detected (Figure 9). Other sections showed degenerated OHCs with pyknotic nuclei together with slight vacuolization of the IHC (Figure 10). Vacuolization of the cytoplasm of the supporting cells was also observed (Figures 9 & 10). The fibrous connective tissue meshwork underneath the stria vascularis showed apparently decreased thickness as compared with Group III (Cisplatin group). Cytoplasmic vacuolization was also noticed in the epithelial cells of the stria, especially in the intermediate and basal layers (Figure 11).

Group V (Dexamethasone 1 hour before cisplatin):

Intratympanic administration of dexamethasone, one hour prior to cisplatin intraperitoneal injection markedly protected the hair cells from cisplatin ototoxicity. The OHCs and IHCs structure was seen comparable to the control. The supporting cells were slightly vacuolated (Figure 12), while stria vascularis and the thickness of its underlying fibrous connective tissue meshwork were seen comparable to the control group (Figure 13).

Scanning electron microscopic results:

Group I (Control):

Examination of the organ of Corti of the control group showed the stereocilia of OHCs arranged in three rows as W-shaped organized structures. The IHCs were arranged in one row with their U-shaped organized stereocilia (Figure 14).

Group III (Cisplatin):

After cisplatin intraperitoneal injection, the arrangement of the three rows of OHCs showed disarray in some specimens (Figure 15). The stereocilia of OHCs were seen lost in many places. Most of the remaining stereocilia showed disarrangement, fusion and loss of their regular W-shaped pattern (Figure 16). Many of stereocilia of OHCs showed formation of membrane blebs and others were lost (Figure 17). On the other hand, no loss of IHCs stereocilia was noticed in this group. However, disarrangement, backward deflection and membrane blebs of some IHCs' stereocilia were obvious (Figure 17). Moreover, membrane blebs were seen on the top surface of the supporting cells (Figures 16 &17). Again these findings were mostly seen in the basal and middle turns of the cochlea.

Group IV (Dexamethasone 1 day before cisplatin):

Dexamethasone intratympanic administration, one day before cisplatin injection in this group showed loss of some stereocilia of OHCs. Stereocilia of some IHCs were seen disarrayed. The top surface of some supporting cells showed few blebbings (Figure 18).

Group V (Dexamethasone 1 hour before cisplatin):

Intratympanic administration of dexamethasone, one hour before cisplatin intraperitoneal injection in this group, resulted in protection of the hair cells and their stereocilia as they appeared similar to the control group (Figure 19

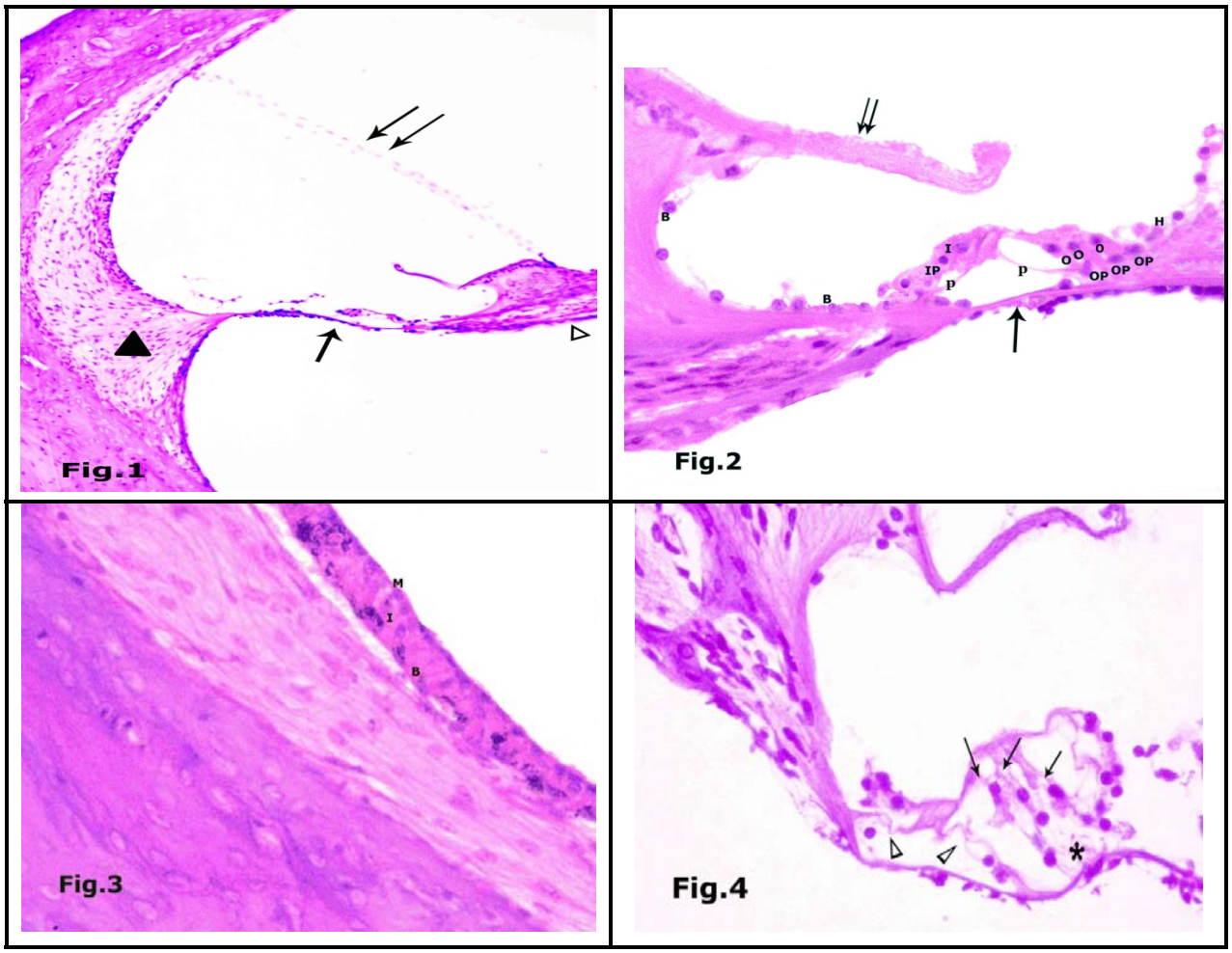


Fig. 1: Showing the wedge-shaped cochlear duct with scala vestibuli above and scala tympani below it. Basilar membrane (↑) is seen extending between spiral ligament (▲) and the spiral lamina (Δ). Reissner's membrane is clearly seen as 2 layers of simple squamous cells (↑↑).

(*Group I: H&E × 250)*

Fig. 2: Showing the organ of Corti resting on the basilar membrane (↑). OHCs (O), IHC (I), pillar cells (P) can be seen. Other supporting cells as outer phalyngeal (OP), inner phalyngeal (IP), border cells (B), Hensen cells (H) can be noticed. Notice the homogenous acidophilic tectorial membrane hanging over the hair cells (↑↑).

(*Group I: H&E × 640)*

Fig. 3: Showing the marginal cells (M), intermediate cells (I) and basal cells (B) of stria vascularis epithelium. Notice the fibrous connective tissue meshwork underneath.

(*Group I: H&E × 640)*

Fig. 4: Showing cytoplasmic vacuolization of OHCs (↑). Notice the vacuolated pillar cells (Δ) and outer phalyngeal cells (*). (*Group III: H&E × 640)*

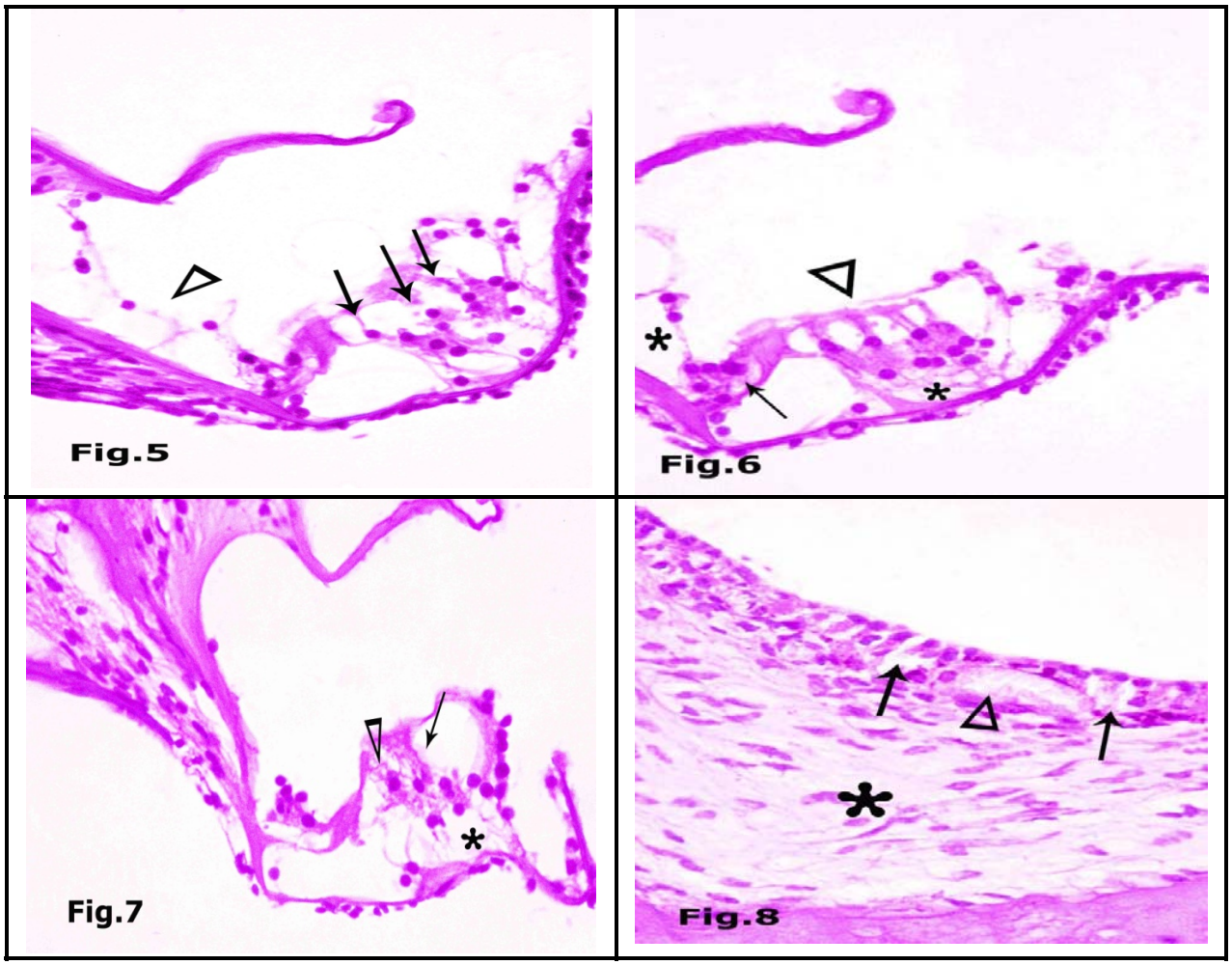


Fig. 5: Showing degenerated OHCs (↑) and swollen vacuolated supporting cells with pyknotic nuclei (Δ).
(*Group III: H&E × 640)*

Fig. 6: Showing widely-spaced OHCs (Δ) and slightly vacuolated IHC (↑). Vacuolated supporting cells can also be seen (*).
(*Group III: H&E × 640)*

Fig. 7: Showing absent OHC in the third row (↑) and degeneration of the other OHCs (Δ). Notice the vacuolated supporting cells (*).
(*Group III: H&E × 640)*

Fig. 8: Showing vacuolated cells in the stria vascularis (↑). Apparently thickened fibrous connective tissue meshwork (*) compared with control and dilated congested intraepithelial capillaries (Δ) can be seen.
(*Group III: H&E × 640)*

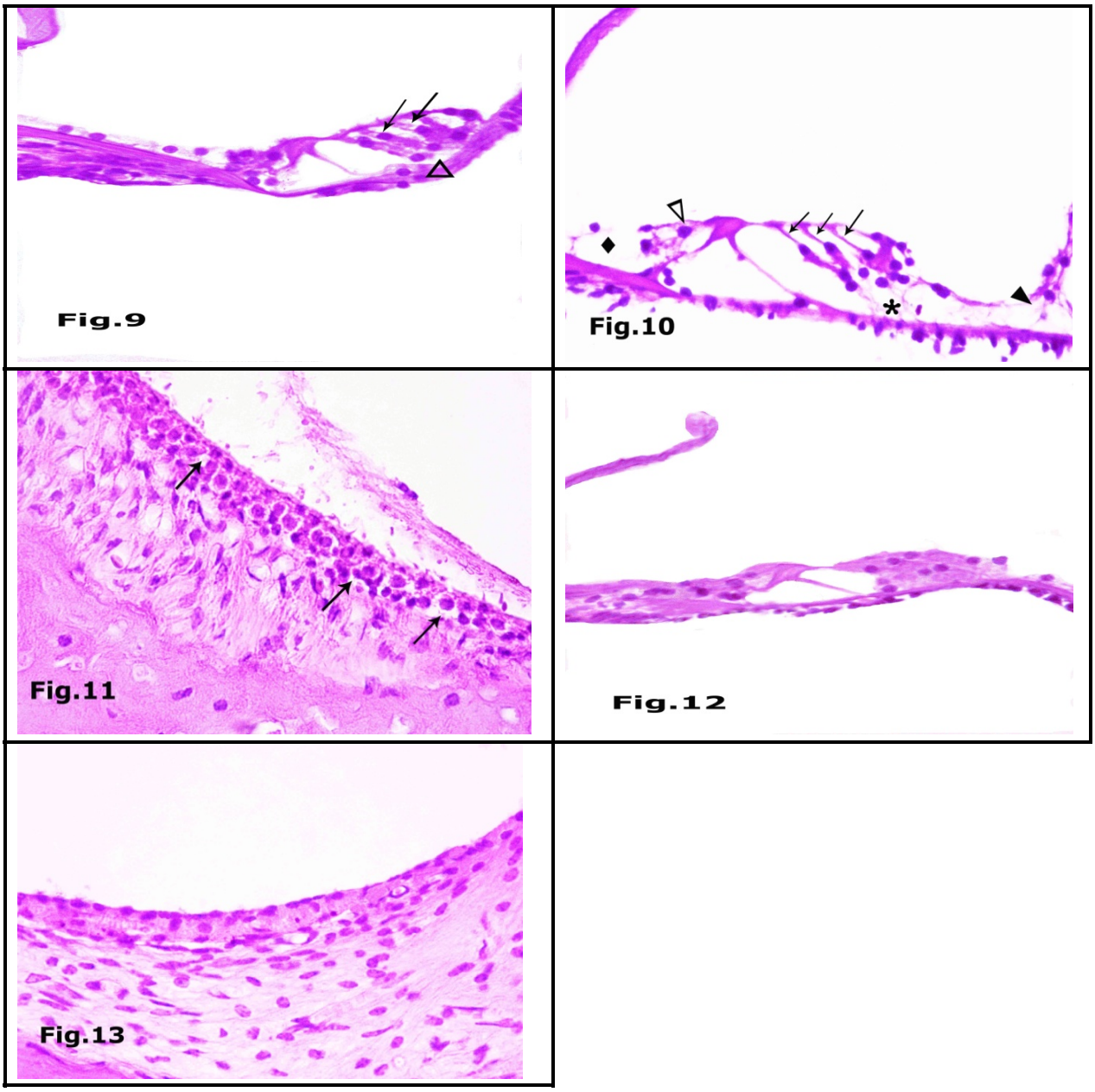


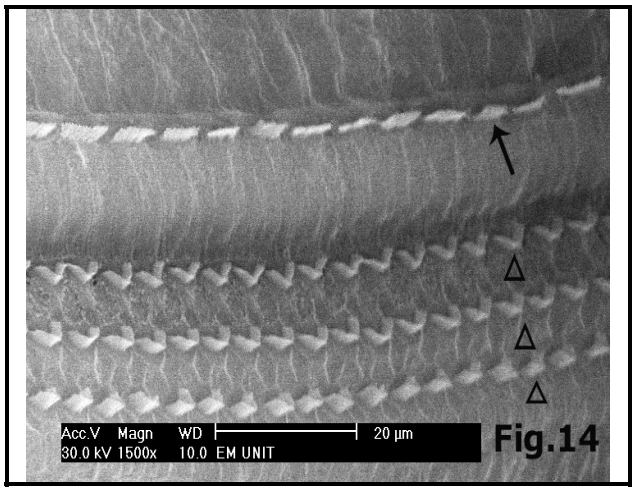
Fig. 9: Showing widely spaced and slightly vacuolated OHCs (↑). Slightly vacuolated supporting cells can be seen (Δ). *(Group IV: H&E × 640)*

Fig. 10: Showing degenerated OHCs with pyknotic nuclei (↑). Notice the vacuolated IHC (Δ), outer phalyngeal cells (*), Hensen cells (▲) and border cells (♦). *(Group IV: H&E × 640)*

Fig. 11: Showing apparently decreased thickness of the fibrous connective tissue meshwork underneath the stria vascularis compared with group III. Cytoplasmic vacuolization can be seen in the cells of the intermediate and basal layers (↑). *(Group IV: H&E × 640)*

Fig. 12: Showing the OHCs and IHC comparable to the control group. *(Group V: H&E × 640)*

Fig. 13: Showing apparently normal stria vascularis as compared with the control group. *(Group V: H&E × 640)*



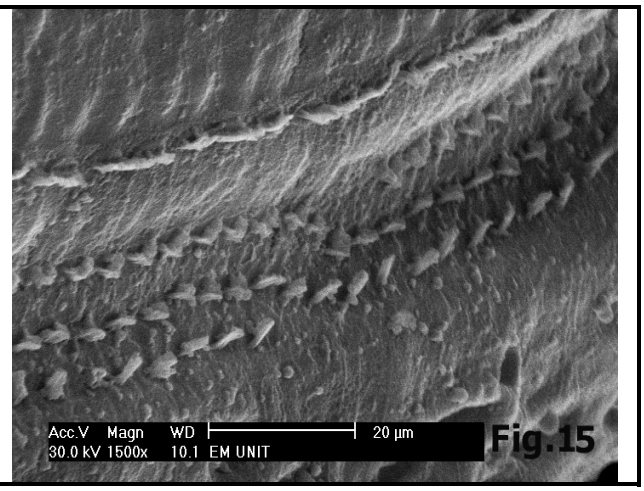
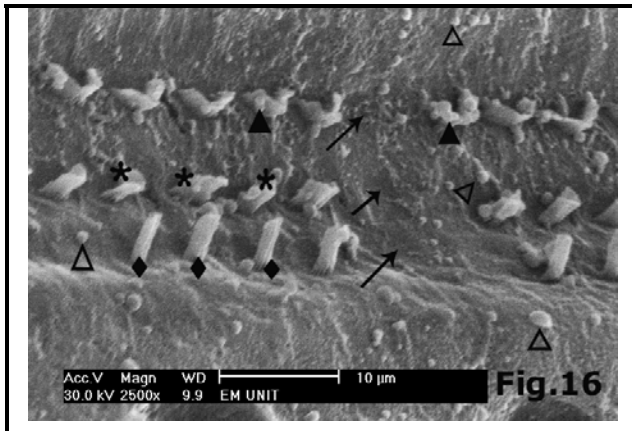
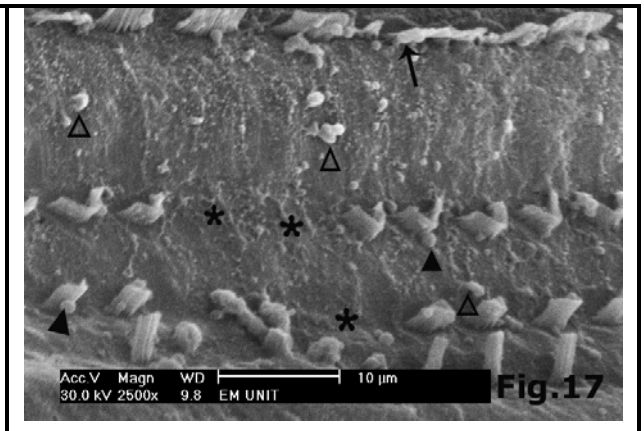
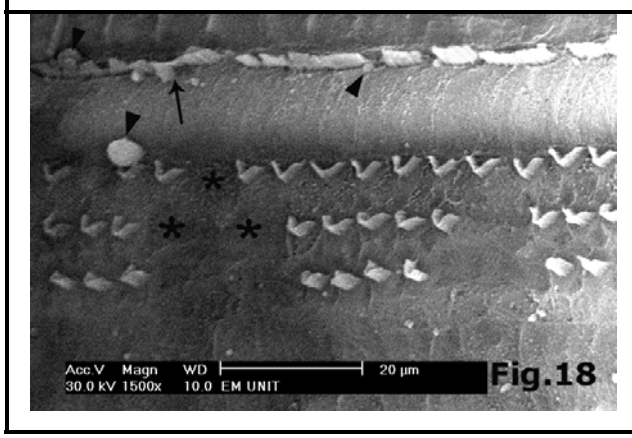


Fig. 14: Showing the W-shaped arrangement of the stereocilia of the three rows of OHCs (Δ). The stereocilia of the IHC row is seen as U-shape (↑). *(Group I: SEM × 1500)*

Fig. 15: Showing disarray of the three rows' arrangement of OHCs. *(Group III: SEM × 1500)*







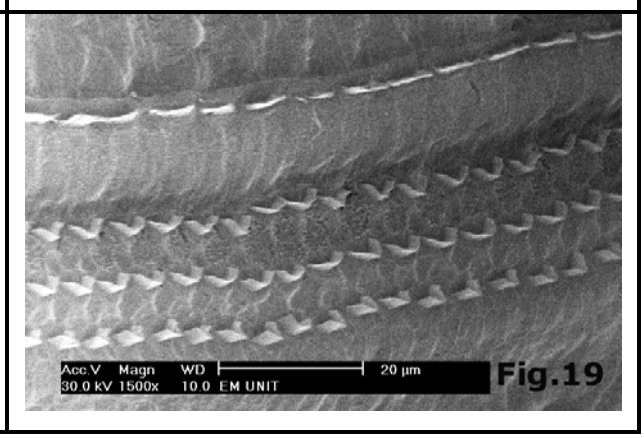


Fig. 16: Showing lost (↑), disarranged (*) and fused (♦) stereocilia of some OHCs. Multiple blebs can be easily seen in the stereocilia of hair cells (▲) and in the top of supporting cells (Δ). *(Group III: SEM × 2500)*

Fig. 17: Showing backward deflection and blebbing (↑) of IHC stereocilia. Some stereocilia of OHCs show blebs (▲), whereas others are lost (*). Notice the blebbing in the top of the supporting cells (Δ). *(Group III: SEM × 2500)*

Fig. 18: Showing loss of stereocilia of some OHCs (*****). Disarray (↑) of stereocilia of some IHCs can be seen. Notice the few blebs in the top of some supporting cells (▲). *(Group IV: SEM ×1500)*

Fig. 19: Showing apparently normal organization of stereocilia of OHCs and IHCs. *(Group V: SEM × 1500)*

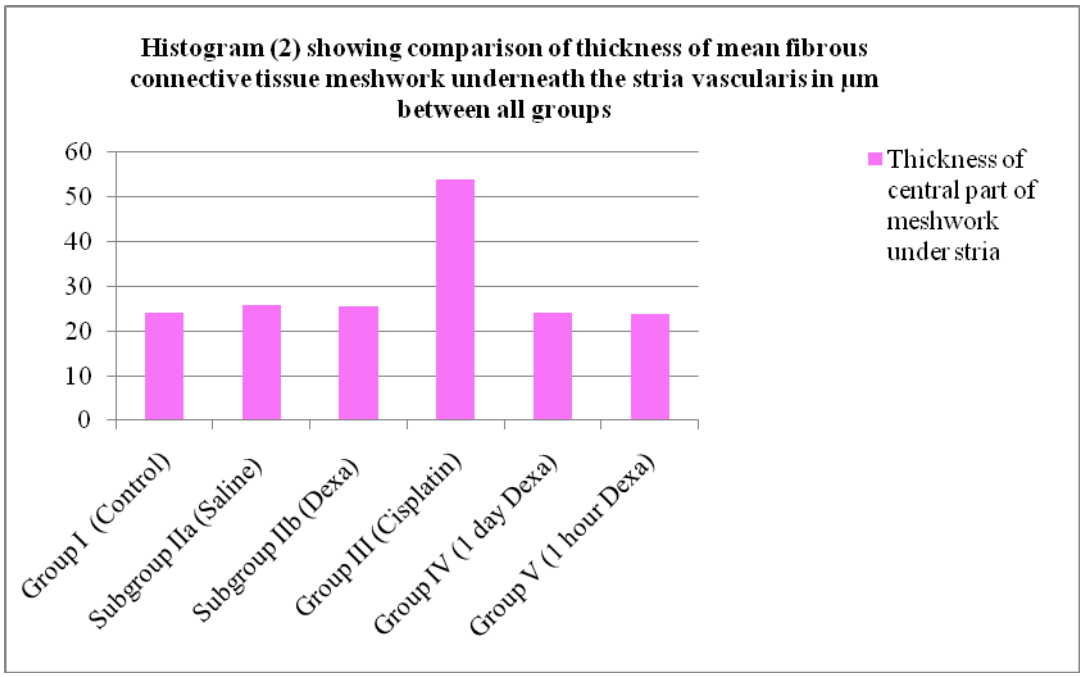
***B)* Morphometric and Statistical histological results:**

Table (3): Showing the Mean ± SEM of thickness of the central part of the fibrous connective tissue meshwork underneath the stria vascularis in µm and comparison between all groups: (After administration of drugs)

	Group I (control)	Subgroup IIa (Saline)	Subgroup IIb (Dexa)	Group III (Cisplatin)	Group IV (Dexa 1 day before Cisplatin)	Group V (Dexa 1 hour before Cisplatin)
Thickness Of meshwork	24.05 ± 0.85 (8)	25.81 ± 1.47 (8)	25.40 ± 1.56 (8)	53.71 ± 2.02 [a] (6)	24.06 ± 1.55 [b] (6)	23.78 ± 1.44 [b] (6)

-Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs. Dexa= Dexamethasone
- a: significance of difference by LSD from Group I (Control) at least $p<0.05$.
- b: significance of differences by LSD from Group III (Cisplatin) at least $p<0.05$.

As shown in table (3) and histogram (2), both subgroups of group II showed statistically non-significant difference in the mean thickness of the fibrous connective tissue meshwork underneath the stria vascularis ($p>0.05$) compared with the control. On the other hand, group III (Cisplatin group) showed significant increase in the mean thickness of the fibrous connective tissue meshwork underneath the stria vascularis ($p<0.05$) compared with the control. However, each of groups IV (administered dexamethasone 1 day before cisplatin) and group V (administered dexamethasone 1 hour before cisplatin) showed non-significant statistical difference in the mean thickness of the fibrous connective tissue meshwork underneath the stria vascularis ($p>0.05$) compared with the control. On the other hand, each of groups IV (administered dexamethasone 1 day before cisplatin) and group V (administered dexamethasone 1 hour before cisplatin) showed significant decrease in the mean thickness of the fibrous connective tissue meshwork underneath the stria vascularis ($p<0.05$) compared with group III (cisplatin group).



Dexa= Dexamethasone

4. Discussion:

Cisplatin ototoxicity continues to be a challenging side effect affecting a majority of cancer patients receiving it as chemotherapy **(17)**. The present study tried to investigate the cisplatin ototoxicity using a dose equivalent to that used in humans undergoing cisplatin chemotherapy worldwide **(18, 19)**. On the other hand, the concentration of dexamethasone used in the current study is the only one available in the Egyptian market and used by clinicians in a variety of diseases.

In the present study, before administration of cisplatin and any intratympanic injection, all guinea pigs were evaluated using ABR to ensure normal hearing. On the other hand, cisplatin presented its ototoxic effect detected in the significant increase in the mean ABR threshold compared with the control. This coincides with the presence of histological degenerative changes in group III in this study particularly in the basal turns of the cochlea.

The OHCs were the most affected by cisplatin ototoxicity in group III in this study especially in the lower turns. This agreed with recent studies reporting similar findings in OHCs, spiral ganglion cells and neurons in the auditory brainstem nuclei. The damage was noticed primarily in the high frequency region of the cochlea **(10, 20, 21)**. This may be due to a progression of drug ototoxicity in a base to apex gradient **(10)**. This could be attributed to lower levels of antioxidants found in the base, hence, increased this zone's susceptibility to free radicals **(22)**. Coinciding with the results of the present study, some authors reported that the most important finding after cisplatin administration was the loss of OHCs' stereocilia starting in the third row at the basal turn **(23)**. They added that the stereocilia were disarrayed and fused which agrees with the results of group III in the present study. This might be caused by loss of lateral cross links connecting the tips of stereocilia of the same or adjacent rows to make them function as a unit **(24)**.

The IHCs were far less intensely affected by cisplatin ototoxicity in group III in the present work. This was in agreement with a number of studies reporting that IHCs where noticeably less affected by cisplatin ototoxicity **(23, 25)**.

Vacuolization of supporting cells of the organ of Corti was noticed in cisplatin group (group III) in the present study. This extends the results of some investigators who attributed this finding to swelling of mitochondria **(26)**. Others reported that the supporting cells were more sensitive than hair cells that the ultrastructural changes preceded any detectable affection of OHCs **(18)**.

The thickness of the fibrous connective tissue meshwork underneath the stria vascularis was also found apparently increased after cisplatin injection in group III of the present work. This was confirmed by the significant increase in its mean thickness in group III compared with group I. This could be attributed to edema formation in this tissue. In agreement, a previous study reported edema formation in the connective tissue underneath the stria vascularis followed by severe atrophy after cisplatin therapy **(27)**. This might be secondary to immune-mediated cochlear destruction induced by ototoxic drugs, leading to cochlear inflammatory process as reported in a recent study **(28)**. Other investigators reported that proinflammatory cytokines as tumour necrosis factor-alpha (TNF-α), interleukin-1beta, and interleukin-6 and nuclear factor kappa B (NFκB) are upregulated by cisplatin treatment in vitro and in the rat cochleae **(29)**. Other authors reported injury of stria vascularis in a time course paralleling OHCs loss, suggesting that cisplatin targets it directly **(30)**. Coinciding, group III in this study showed that cisplatin-induced vacuolar degeneration in the epithelial cells of the stria vascularis and showed dilated congested intraepithelial capillaries.

Several mechanisms might be involved in cisplatin-induced ototoxicity. Recent studies showed that a trans-membrane trimer localized in the hair cells (copper transporter; ctr1) enhanced the cellular uptake and influx of cisplatin, hence increasing its cytotoxicity **(31, 32)**. Upon entering the cells, cisplatin integrates into the DNA causing its cross linking and damage that result in accumulation of dysfunctional protein and inefficient enzyme synthesis **(33)**. Progressively, the cochlea becomes unable to flush out the accumulated toxins at a rapid rate, thus reactive oxygen species (ROS) overloads. Combined with the expected depletion of antioxidants, cell injury and apoptosis is the result **(34)**. Cisplatin-mediated ROS generation depends on induction and activation of a permeant channel used by cisplatin called "the transient receptor potential vanilloid 1 (TRPV1) channel. This activation might enhance Ca^{++} influx and overload **(35)**. The resulting increase in intracellular Ca++ might inhibit a group of ATPases known as aminophospholipid translocases (APTLs) that maintain the normal asymmetrical distribution of phosphatidylserine and phosphatidylethanolamine across the plasma membrane **(36, 37)**. At the same time, this activates phospholipid scramblases that randomize all membrane phospholipids between leaflets resulting in a complete loss in cell membrane lipid asymmetry **(38)**. This might cause disturbance of cell membrane and consequently formation of membrane blebs that

was noticed by SEM examination in the stereocilia of OHCs, IHCs and in the top surfaces of several supporting cells in the present study.

Furthermore, cisplatin was found to activate big conductance K^+ channels (BK) in the fibrocytes of the spiral ligament of cochlear lateral wall. This led to disruption of the electrochemical gradient by K^+ efflux, decreasing intracellular K^+ levels causing disturbance of the ionic concentration essentially needed for hair cell function. The final expected result is triggering apoptosis and cell death **(39)**.

To our knowledge, few investigators tried the intratympanic way of steroid administration for protection from cisplatin-induced ototoxicity. They suggested that this way of local drug application was safe and feasible, avoiding systemic side effects without compromising the tumoricidal efficacy of chemotherapy **(10, 28)**.

Recently, a study reported that intratympanic injection ensured a high concentration of the protective drug to enter the inner ear directly aiming the target organ **(40)**. These authors added that steroids injected into the middle ear could reach scala tympani within minutes, mainly through the round window membrane and minimally through oval window membrane. It then quickly reaches scala vestibuli through the spiral ligament laterally or Rosenthal canal medially. The communication routes between scala tympani and the organ of Corti and spiral ganglion assure that hair cells and nerve cells will rapidly be exposed to drugs delivered through the round window. The presence of drug within the scala media demonstrated transport into the endolymphatic spaces as well **(41)**. Dexamethasone, after its intratympanic injection, had a higher rate of endocytosis, hence greater intracellular efficacy in contrast to other steroids as methyl prednisolone (**42**). This was the reason behind our choice of dexamethasone in particular in the present work.

In the present study, intratympanic dexamethasone alone (subgroup IIb) had non-significant statistical difference in comparison with the saline administration (subgroup IIa) and with control animals (group I) in relation to ear functional assessment by ABR. This also matched the histological finding as no apparent structural abnormality was observed in the structure of the cochleae by either H&E stain or by SEM examination of dexamethasone treated cochleae of subgroup IIb compared with control. Moreover, subgroup IIb showed non-significant statistical difference in the mean thickness of the fibrous connective tissue meshwork underneath the stria vascularis compared with group I. All these results revealed the safety of dexamethasone on cochlear function and structure. This coincides with other study that reported no change in the cochlear construction and function after intratympanic dexamethasone application in guinea pigs **(43)**.

The results of the present study showed that intratympanic dexamethasone played a protective role against cisplatin-induced ototoxicity by reducing ABR threshold shifts and improving the histological structure. Prior to this study, there were no combined histological and audiological investigations concerning the time dependent effect in better cochlear protection. As shown in the audiological and histological results of the present study, administration of dexamethasone one hour prior to cisplatin intake (Group V) showed much better protection than one day (Group IV). This might be explained by the findings of another study that observed significant levels of dexamethasone in the perilymph within one hour, and decreased by 50-100 folds within 12 hours in guinea pigs **(44)**.

Dexamethasone had multiple effects as an otoprotectant, acting on ion homeostasis and immune suppression **(45)**. Regarding ion homeostasis, dexamethasone increased the expression of Na^+/ K^+ channels **(46)** and of active water channels (aquaporins) in the endolymphatic sac and the tissues surrounding endolymphatic spaces **(47)**. It was documented that the stria vascularis pumps K^+ into the endolymph to maintain the endolymphatic potential critical for normal hearing. This process requires K^+ recycling from the base of hair cells through the spiral ligament via intercellular gap junctions **(28)**. Any pathologic process that interferes with this movement of K^+ or stria vascularis function will cause hearing loss. Through both Na^+/ K^+ and aquaporins channels, dexamethasone could effectively maintain the tightly regulated ion transport mechanisms critically needed for auditory and vestibular hair cell functions **(46)**. As for immune suppression, intratympanic dexamethasone down regulated greater number of proinflammatory cytokine genes in the cochlear tissues than if delivered systemically **(48).** Immune-mediated cochlear tissue destruction resulting from ototoxic drugs usually initiated cochlear inflammatory processes that are responsive to glucocorticoid treatments. Therefore, immune suppression is a key factor in protecting the ear or reversing hearing loss **(28)**. This may explain the absence of edema noticed in the cochleae of groups IV and V, administered intratympanic dexamethasone one day and one hour respectively before cisplatin in the present study. This was reflected by the significant decrease in the mean thickness of the fibrous connective tissue meshwork underneath the stria vascularis compared with group III (cisplatin group). However, each of groups IV and V showed non-significant statistical

difference in the mean thickness of the fibrous connective tissue meshwork underneath the stria vascularis compared with the control. In addition, dexamethasone reported to act as a slowly-acting free radical scavenger greatly accounting for cisplatin ototoxicity **(49)**. Thus, all these functions (ion homeostasis, immune suppression and free radical scavenging) seem to be quite interlinked as regard to maintenance of the endolymphatic potential of fluids around auditory and vestibular hair cells.

Conclusion:

It is concluded that cisplatin ototoxic insult is planned and predictable that possible protection by intratympanic dexamethasone administration should be given precisely in good timing before the insult. Giving the drug as early as one hour yielded marvelous protective effect and turned to be a perfect timing of interference before cisplatin chemotherapy treatment session begins. We recommend following this way of protection as it proved to be safe, easily performed by an otolaryngologist in the same clinic in which cisplatin injection is to be performed.

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We dedicate this work to the soul of our beloved, most respectable colleague Mirahan Thabet, lecturer of audiology, Faculty of medicine, Ain Shams University. Without her great participation as a main author in this study, this work was never to be done.

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