Synergistic protective effect of dexamethasone and erythropoietin on experimentally induced cochlear toxicity in albino rats [Histological and immunohistochemical study].

Background: Cisplatin (CIS) is averse -neoplastic substitute pro the therapy of solid tumors. Conversely, an extraordinary incidence of harsh ototoxicity is prevalant in patients remedied by CIS. Dexamethasone (DX) is a synthetic steroid equivalent expended for the treatment of distinctive inner ear diseases. Erythropoietin (ER) had well-established erythropoietic function plus strong anti-inflammatory, anti-apoptotic validity.

Purpose: The principle of this study was to assess the otoprotective effects of dexamethasone (DX) and erythropoietin (ER) administration in the CIS- aggravated ototoxicity in rats.

Methods: Thirty-five rats apportioned into: **Control group, CIS group, CIS + DX group** received an intraperitoneal injection of dexamethasone before CIS for 3 consecutive days, **CIS + ER group,** received an intraperitoneal injection of ER before CIS for 3 days and **CIS + DX + ER group,** cisplatin obtained and pretreated with dexamethasone and erythropoietin. The cochleae were subjected to the histological and immunohistochemical methods.

Results: The DX group exhibited a moderate protective effect against CIS-provoked cellular ototoxicity and remarkably reduced the histopathological finding. Pretreatment with ER effectually preserved inner and outer hair cells and supporting cells in the organ of Corti as compared to the effects distinguished with CIS. Quantification of spiral ganglion neurons by immunohistological anti-caspase 3 significantly shown that ER-treated rats had a significantly less spiral ganglion neuron harm than CIS rats. The synergistic dose of DX and ER have better picture nearly near normal histological structure.

Conclusion: These results demonstrated that each DX and ER revolutionize the histological represent of cochlea simply together were effective and better in protection and treatment of cisplatin- provoked ototoxicity.

Keywords: Erythropoietin, Cisplatin-induced cochlear injury, Dexamethasone, Ototoxicity.

Introduction:

Ototoxicity denotes to depredation of inner ear organizations (i.e., cochlea and stria vestibulé) then malfunction (balance and audible range functions) with subsequent coverage to in-hospital prescriptions (i.e., solvents, antibiotics and chemotherapy) [1]. Ototoxicity is a predictable side outcome of these platinum- established agents, chiefly cisplatin [2,3]. Cisplatin (*cis*-diamine-dichloride-platinum (II), *cis*-$[PtCl_2(NH_3)_2]$) is a pioneer of anticancer drugs, highly effective chemotherapeutic agent [3]. Damage in the organ of Corti, stria vascularis, spiral ganglion, and spiral ligament by CIS has been proved in many researches [4,5]. The dose and duration of treatment by CIS and its associate radiotherapy therapy is related to the level of hearing toxicity with increased pathology with collective dosing, long duration therapy and history of noise contact [6].

Autoimmune diseases, gastrointestinal tract ulceration, rheumatism, tumors and organ transplantation were treated mainly by steroids specially dexamethasone. Dexamethasone (DX) is a synthetic steroid equivalent expended for the treatment of distinctive inner ear diseases including abrupt idiopathic sensory neural hearing loss, Meniere's syndrome in addition Bell's palsy [7].

The hormone erythropoietin (ER), which is produced by the kidneys, is supposed to be the key regulator in red blood cells production. It was discovered that a wide range of non-hematopoietic tissues had its receptor. This implies that ER serves purposes beyond its well-established erythropoietic function. ER has strong anti-inflammatory, anti-apoptotic, neuro defensive, and neurotrophic properties [8].

The key controller of red blood cells synthesis is thought to be the hormone erythropoietin (ER), which is secreted by the kidneys. Many different types of non-hematopoietic tissues were revealed to have its receptor. This implies that ER serves purposes beyond its well-established erythropoietic function. ER has strong anti-inflammatory, anti-apoptotic, neuroprotective, and neurotrophic properties [8].

Materials and Methods

Animals

Thirty-five adult male albino rats weighing 160–200 g used in this study. They were acquired from the Centre of Laboratory Animal, Faculty of Veterinary Medicine, Benha University, Egypt. All animals will be allowed to acclimate for two weeks at a warmth of about 25 °C and a 12/12 sun/moon cycle before the experiment begins. Throughout the experiment, rats will be fed a typical commercial laboratory meal and given unlimited access to water. The study protocol and use of rats had ethical approval from the Benha University. Faculty of Medicine's Ethics Committee (Approval no. RC 11-10-2024).

Chemicals

Cisplatin was obtained from Fujiuan Gutien Pharmaceutical Co., Ltd (Fuzhu, China). Dexamethasone- sodium -phosphate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Erythropoietin was acquired from (NeoRecormon, Roche,Mannheim, Germany).

Experimental design

-Group I: (Control group): (7 rats)

Three of them were received intraperitoneal injection of 1mm of normal saline 1% for 3- consecutive days and four of them left without any treatment.

- Group II: (CIS group): (7 rats)

A single intraperitoneal injection of 15 mg/kg of CIS was administered to the rats [9].

-Group III (CIS + DX group): (**7 rats)**

Rats were given an intraperitoneal injection of dexamethasone (15 mg/kg/d) 90 minutes prior to receiving 8 mg/kg/day of CIS (IP) for three consecutive days [10].

-Group IV (CIS + ER group): (7 rats)

Rats received an intraperitoneal injection of Erythropoietin (5000IU/kg) daily 90 min prior to CIS (IP) at 8 mg/kg/day for 3 consecutive days [11].

-Group IV (CIS + DX + ER group): (7 rats)

Rats received an intraperitoneal injection of DX +ER before Cis for 3 consecutive days, the dose as in groups II, III and IV.

On the 5th day, the animals were sacrificed.

Sample collection:

At the end of the experiment, all animals were put to sleep with phenobarbital sodium and sacrificed via cervical dislocation. A one milliliter syringe needle was used to make a tiny hole at the apex of the cochlear capsule after careful removal of cochleae from the temporal bone. To make sure the right fixative had fully entered the cochlea, the fixative was then carefully pushed in with a tiny needle. The animals' remains were burned in an incinerator [12].

Light microscopic study

Formalin EDTA was used to fix and decalcify the specimens for four weeks. Every day, the solution was modified. A sufficient amount of decalcifying suspension — as a minimum 30–50 times the bulk of tissue—was utilized [12]. After being decalcified, the specimens were cleaned and prepared for paraffin block creation. They were then encased with paraplast. Mid-modiolus longitudinal sections that were approximately 5 μm thick were cut into serial sections and processed to H&E. Other slides were prepared for immunohistochemical staining by the rabbit polyclonal caspase-3 antibodies [E83-77] (marker for apoptosis) (Labvision Corporation, Catalogue Number ab208003, Fremnt, CAO, USA) which was afforded on a dilution of (1:100) [13]. The deparaffinized sections (thru xylene), were dunked in 3 percent hydrogen- peroxide to extinguish endogenous peroxidase activity then microwaved in sodium citrate liquid (pH= 6.8) pro 16 min pro antigen retrieva1. The tissue portions were simmered with avidin– biotin peroxidase system. The primary antibodies were subsisted and expended. At that point, the sections stained with pro Meyer’s Haematoxylin as a counter stain. Phosphated saline buffer

was used in place of the primary antibody in the negative assessment. The positive control was of mouse tonsil [13].

Morphometric study

- Degeneration indicators were graded independently, including cytoplasmic vacuolization, apoptotic cells, cell degeneration and nerve degeneration. For indications of degeneration, each rat was scored on a five-point scale: 0 for normal, 1 for very minimal, 2 for mild, 3 for moderate, and 4 for severe.

-Evaluation of the positively defiled cells of anti-caspase-3 was computed. Seven slides (n=7) from each group were measured in 10 non-overlapping fields at ×400 magnification with Leicaa Qwinn 500C (image analysis, Leicca Micrὸsystems computer organization) (LItd., Cambridge, UK) on the Pathology Unit, Faculty of Medicine, Benha University [14].

Statistical analysis

For Windows, SPSS edition 23 (SPSS Inc., Chicago, IL, USA) was utilized to analyze the data. The means ± standard error of measurement were used to present the results. The post hoc LSD test, a one-way analysis of variance, was utilized to assess all the data; a p-value to a lesser extent than 0.01 was deemed significant.

Results

The normal histological architecture of control cochlea detected within the organ of Corti, with hair cells and supporting cells (Fig.1a), stria vascularis with melanin pigments and small blood capillaries (Fig.1b) plus spiral ganglion with its vesicular nuclie (Fig.1c) with nearly negative cells for caspase 3 (Fig.1d). The light microscopic H&E appraisal of the organ of corti in CIS group showed that the tissue's overall structure had deteriorated. Degenerative cell separation and increased cytoplasmic vacuolization were noted. The inner and outer hair cells' extensions plus cell bodies displayed progressive detachment and local swelling (Fig.2a). Vascularization with congested blood capillaries and increased melanin were observed in stria vascularis (Fig.2b). The spiral ganglion cells' cytoplasm displayed vacuolization, and their nuclei were found to be highly acidophilic and somewhat pyknotic with hemorrhage (Fig.2c). Pyknotic cells confirmed by high expression in caspase-3 immunostaining (Fig.2d).

The DX group's light microscopic analysis showed that there was moderate degenerative cell separation and cytoplasmic vacuolization. Moderate local swelling and degenerative separation were observed in the extension in addition cell body of the inner plus outer hair cells, despite the organ of Corti remaining intact. The cytoplasm of the cells displayed vacuolization, and the nuclei were found to be mildly acidophilic (Fig. 3a, b). There was some vacuolization in the supporting cells' cytoplasm despite the nucleus being somewhat basophilic. The spiral limbus locality 's vacuolization revealed split-ups as dilatation (Fig. 3a, b). Comparing the array and nucleus view of the cells that compose the stria vascularis and spiral ligament of the control group, the former displayed moderate pathologic picture. Cell degeneration was found to cause vacuolations and nucleus loss. At the stria vascularis plus spiral ligament, there was increased vacuolization and some degree of cell degeneration. spiral ganglion and vesicular nuclei had moderate vacuolations (Fig. 3c). There was moderate degeneration of the cochlear nerve fibres and spiral ganglion. Axonal degeneration and vacuolization were found moderately in these cells with increased cells expressed by caspase-3 (Fig.3d).

The light microscopic evaluation of the The ER group showed that the tissue's overall structure had somewhat better appearance than the DX group. Cytoplasmic vacuolization and some degenerative cell separations were also noted in some areas. The extension and cell body of the inner and outer hair cells had little enlargement and degenerative separation, despite the fact that the organ of Corti had retained the semi normal structure. (Fig. 4a, b). A little degree of vacuolization was seen in the cytoplasm of the supporting cells, despite the nuclei being somewhat basophilic. The spiral limbus region's vacuolization revealed separations as dilatation (Fig. 4 a, b). The cells that make up the spiral ligament and stria vascularis were seen from the array and nucleus. There was moderate degeneration of the cochlear nerve fibres and spiral ganglion. Minimal axonal degeneration and vacuolization were found in pseudo unipolar neurons with minimal caspase-3 expression (Fig. 4.c, d).

The light microscopic valuation of the ER+DX group revealed nearly normal structure of organ of corti (Fig. 5a), stria vascularis (Fig. 5b) and spiral ganglia (Fig. 5c, d).

Morphometric and statistical results:

- Table 1 summarizes the histological blind grading of the groups based on the findings of cytoplasmic vacuolization, nerve degeneration, cellular degeneration and

apoptotic cell. The histopathological blind grading results showed that there was no significant difference in the degeneration findings between the IV and V groups and the control group (P<0.01). All evaluation criteria bared significant differences between the CIS group and the control group (P<0.01).

-The mean ± SD of area % intended for anti-caspase-3 in all groups were represented in Table 2 and Histȯgram 2. There was a significant (P<0.01) increase in caspase 3 expression in group II compared with control group. There was a significant (P<0.01) decrease in caspase 3 expression in group III, IV, V compared with group II with better results in V group.

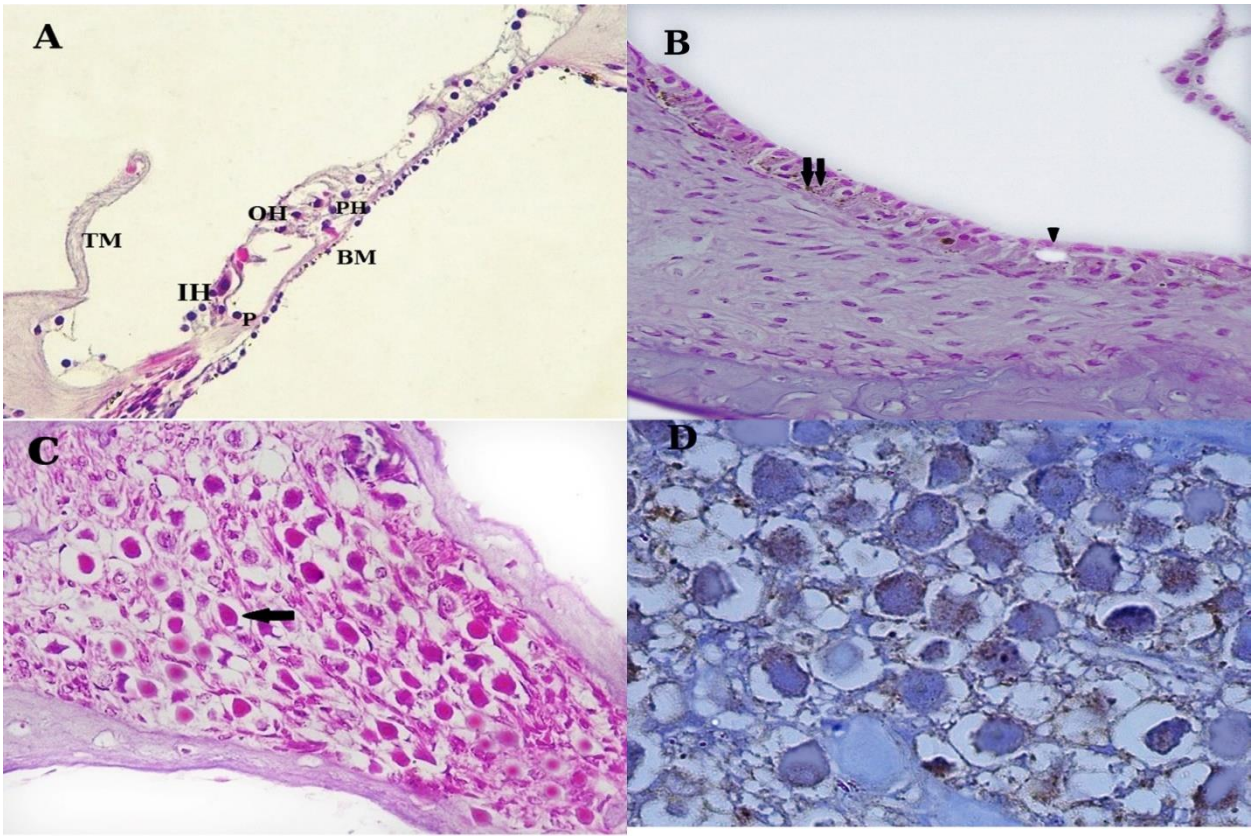


Figure 1: Control group: (A) organ of corti, intact basilar membrane (BM) with inner (IH) &outer hair cells (OH) with their supporting cells, as phalangeal cells (PH) and pillar cells (p). (B) stria vascularis containing melanin granules (double arrow) with intraepithelial capillaries (head arrow). (C) spiral ganglion and their

vesicular nuclei (arrow). (hematoxylin & eosin stain, ×400). (D) spiral ganglion (caspase-3, x400**)**

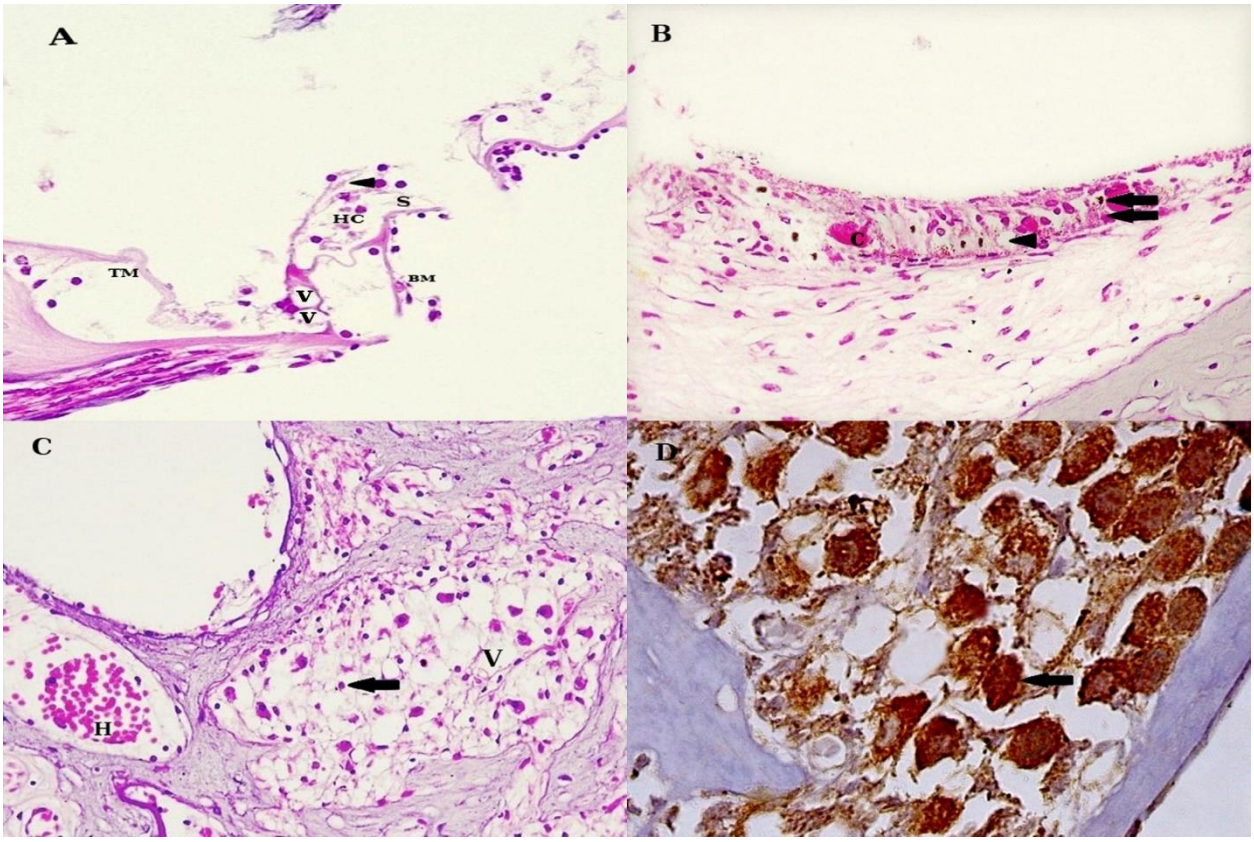


Figure 2: CIS group: (A) organ of corti with dispersed hair cell (HC) and supporting cells (s), vacuolization (v) and distorted basilar membrane (BM). (B) stria vascularis thickened with vacuolations (head arrow) and congested blood capillaries (C) with increased melanin granules (double arrow). (C) spiral ganglion with small dark pyknotic nuclei (arrow), vacuolations (V) and haemorrhage (H) (hematoxylin eosin stain, ×400). (D) spiral ganglion with many cells strong positive for caspase -3 (arrow) (caspase-3, x400**).**

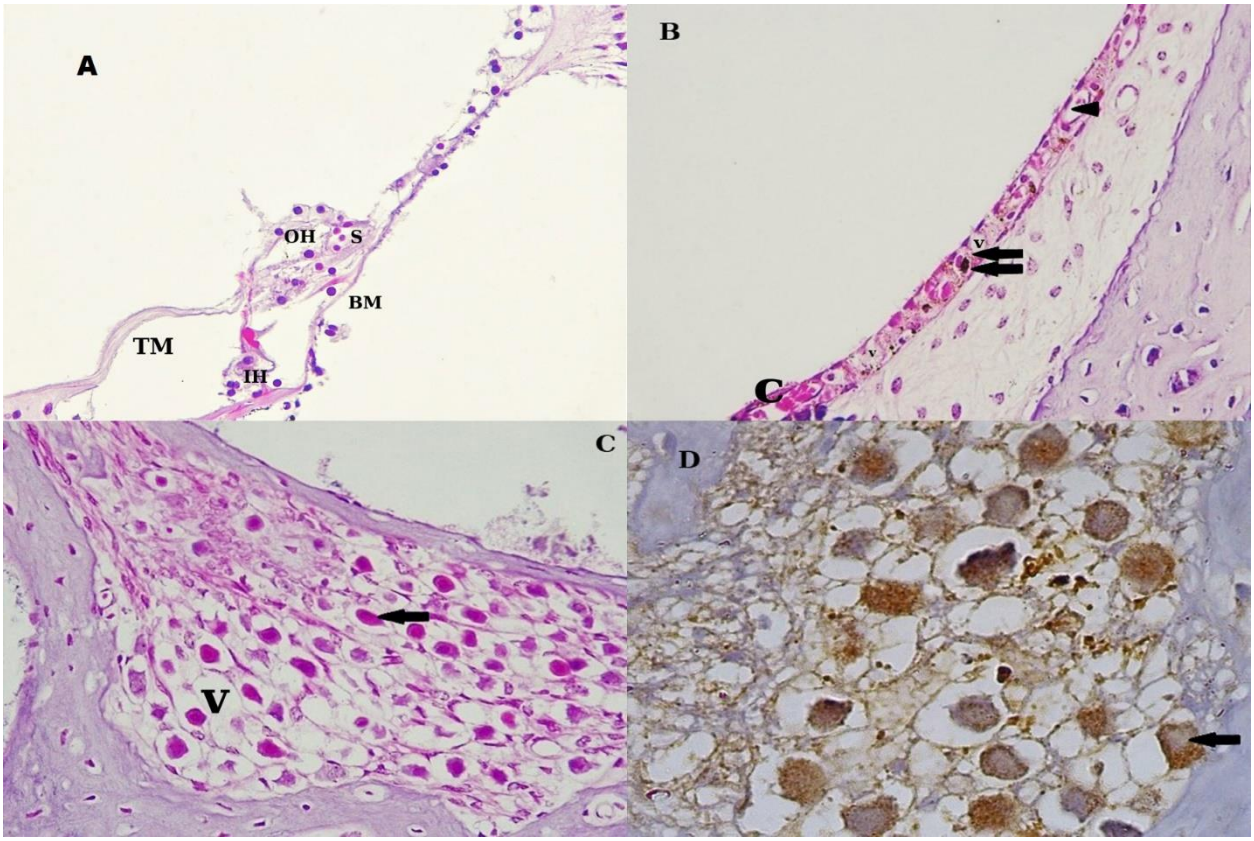


Figure 3 CIS + DX group: (A) organ of corti, intact basilar membrane (BM) with inner (IH) & outer hair cells (OH) with supporting cells (s). (B) stria vascularis with moderate vacuolations (V) and congested blood capillaries (C) with melanin granules (double arrow). (C) spiral ganglion with nuclei (arrow) and moderate vacuolations (V). (hematoxylin & eosin stain, ×400). (D) spiral ganglion with some moderately positively stained caspase -3 cells (arrow) (caspase-3, x400**).**

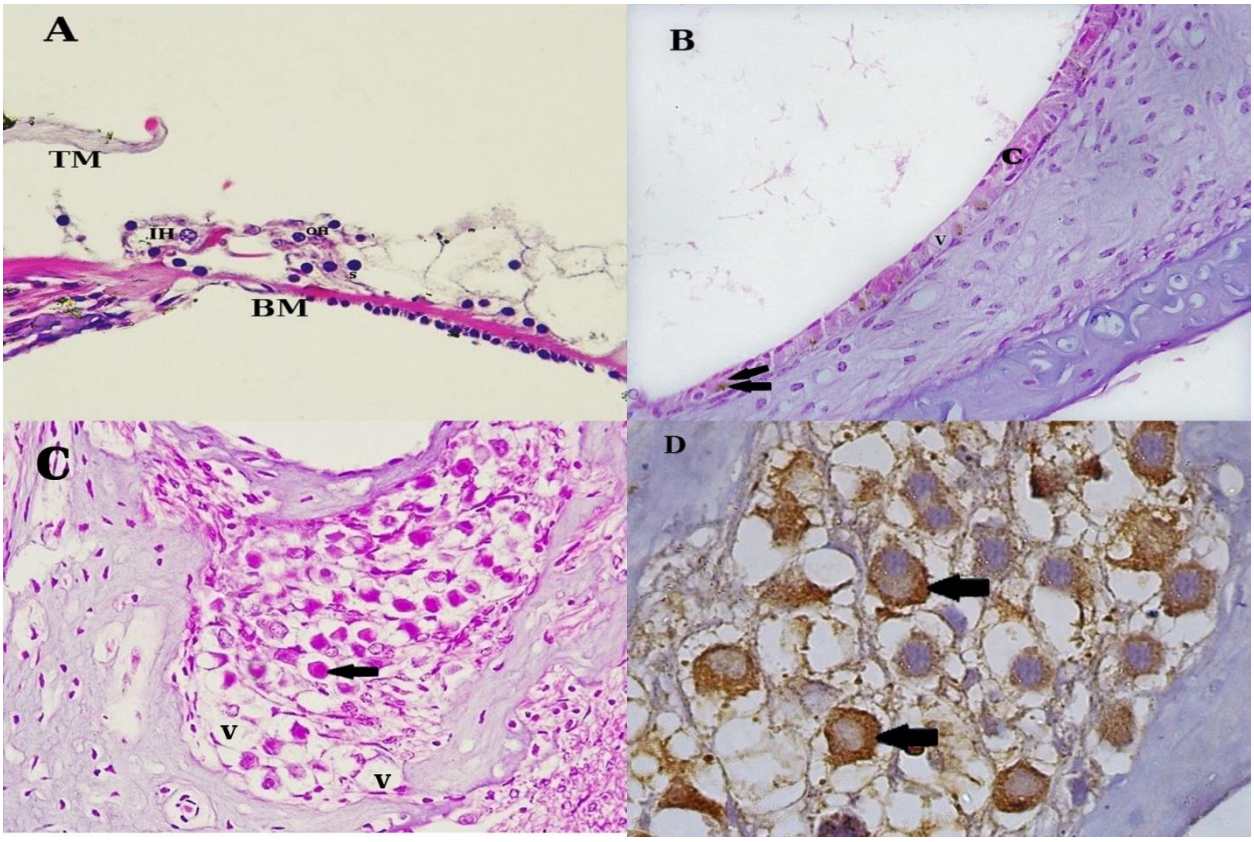


Figure 4 CIS + ER group: (A) organ of corti, intact basilar membrane (BM) with inner (IH) &outer hair cells (OH) and supporting cells (s). (B) stria vascularis with minimal vacuolations (V) with little congested capillaries (C) and melanin granules (double arrow). (C) spiral ganglion with minimal vacuolations (V). (hematoxylin & eosin stain, ×400). (D) spiral ganglion with few caspase-3 positively cells moderately stained (arrow) (caspase-3, x400**).**

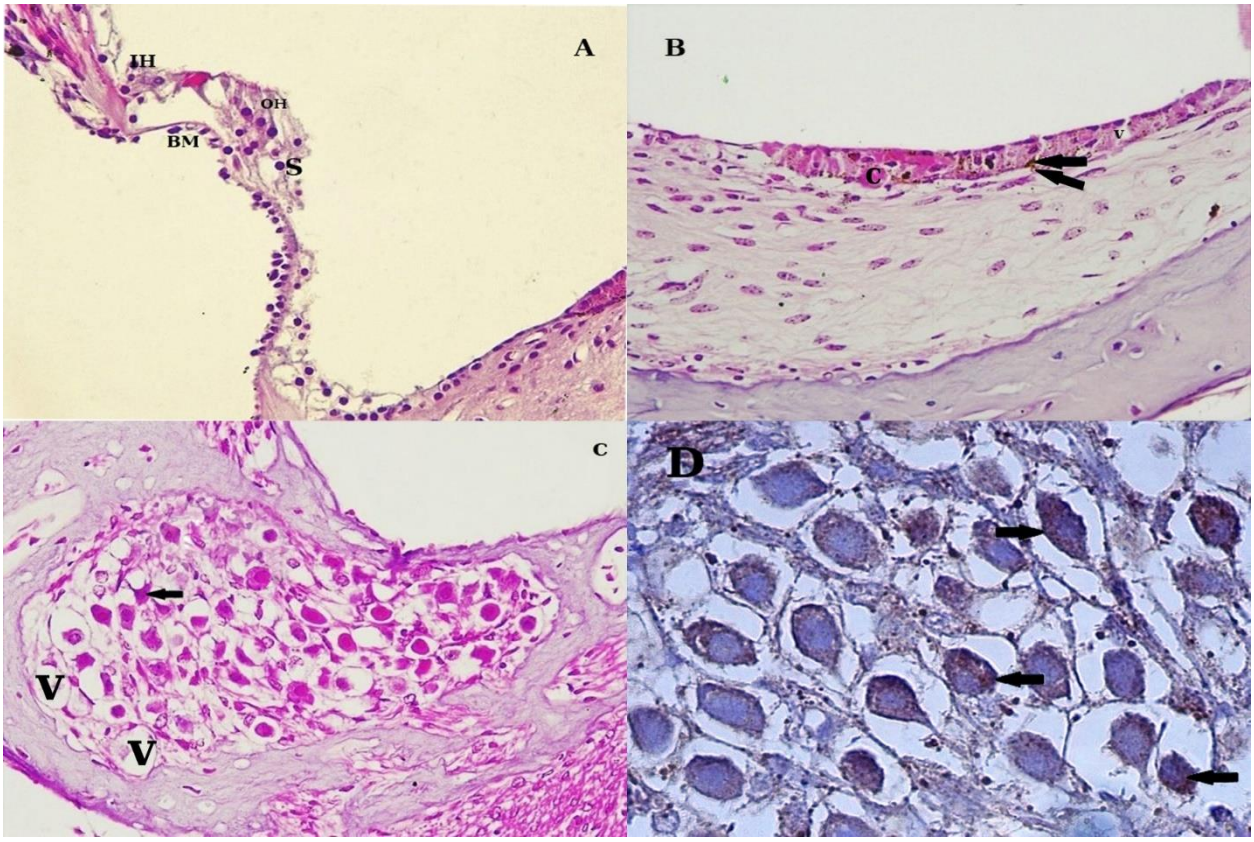


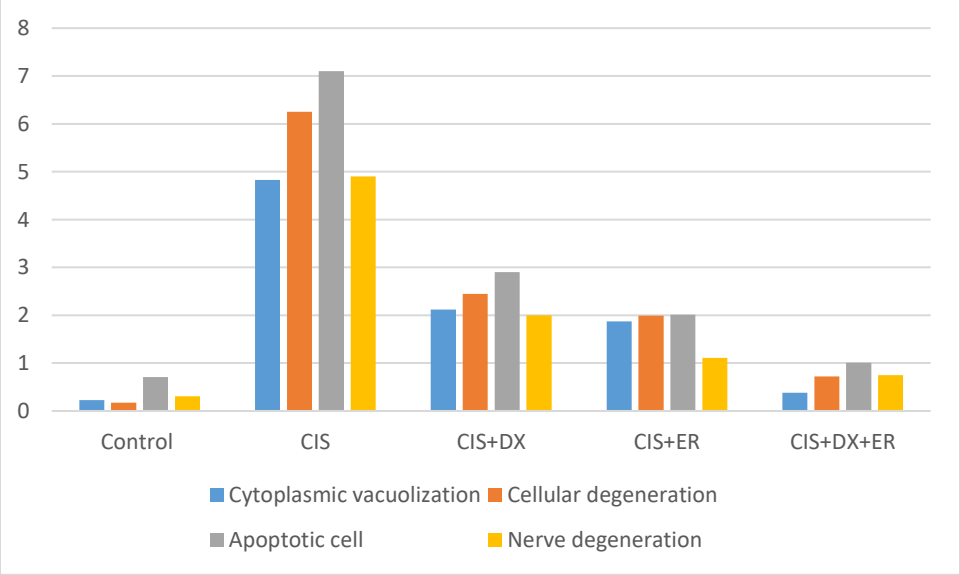
Figure 5 CIS + DX + ER group: (A) nearly normal organ of corti resting on intact basilar membrane (BM) with inner (IH), outer hair cells (OH) and supporting cells. (B) stria vascularis containing melanin granules (double arrow) with intraepithelial capillaries (head arrow). (C) spiral ganglion and vesicular nuclei (arrow) with minimal vacuolations (V). (hematoxylin & eosin stain, ×400). (D) spiral ganglion with few mildly stained caspase-3 positive cells (arrow) (caspase-3, x400**).**

Groups	Cytoplasmic vacuolation	Cellular degeneration	Apoptotic cell	Nerve degeneration
Control	0.23 ± 0.36	0.17 ± 0.37	0.71 ± 0.45	0.31 ± 0.42
CIS	4.83 ± 0.59	6.25 ± 0.45	7.10 ± 0.00	4.90 ± 0.43
CIS+DX	2.12 ± 0.44	2.45 ± 0.21	2.90 ± 0.11	2.00 ± 0.33
CIS+ER	1.87 ± 0.55	1.99 ± 0.47	2.01 ± 0.35	1.11 ± 0.25
CIS+DX+ER	0.38 ± 0.52	0.72 ± 0.46	1.01 ± 0.46	0.75 ± 0.71

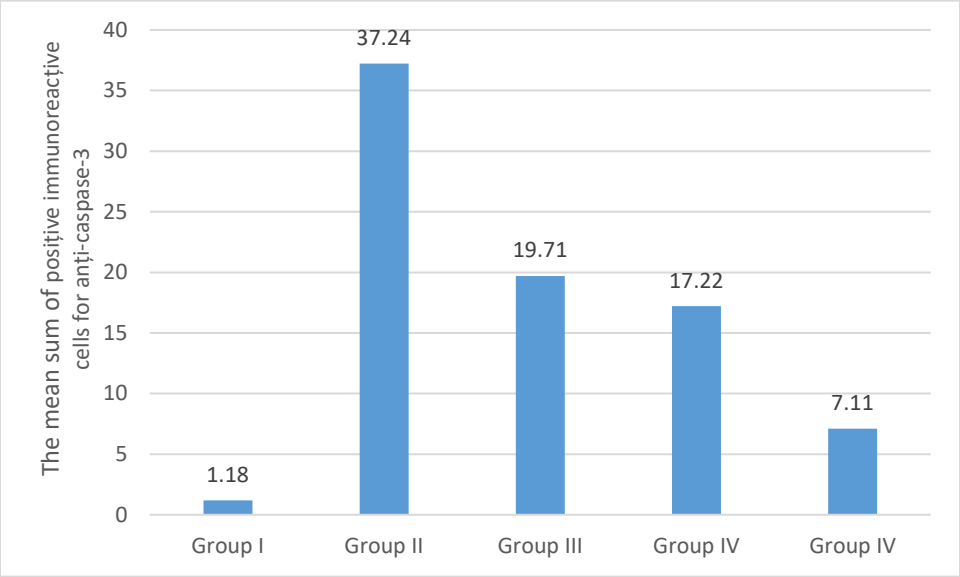
TabIe (1): Histopathological blind grading findings of each group.

	Group I	Group II	Group III	Group IV	Group IV
mean	1.18	37.24	19.71	17.22	7.11
SD	0.0578	0.6377	0.8344	0.7833	0.6562
Sig.	II,,III	I, ,III,IV,V	I, ,II	I,II	I,II
P-value < 0.01					

TabIe (2): The mean sum and SD of positive immunoreactive cells for anti-caspase-3 in all groups.



Histogram (1): Histopathological blind grading findings of each group.



Histogram (2): The mean sum of positive immunoreactive cells for anti-caspase 3 in entirely groups.

Discussion

Treatment by cisplatin (CIS) in various pediatric and adult cancers is very effective as a chemotherapeutic agent. Inappropriately, it has dose- qualifying restrictive side effects, comprising renal toxicity and ototoxicity [15]. In clinical and experimental trials, ototoxicity appeared as a side effect of CIS [16]. It exhibits sensorineural hearing loss [17]. The Early diagnosis of CIS ototoxicity is achievable by high-frequency audiologic checking; still in spite of this, inner ear damage and hearing loss is the end results [18]. For this intention, our research is focused on prevention and early treatment of CIS ototoxicity. The primary mechanism of CIS ototoxicity was the induction of cochlear cell death and apoptosis [19], which appeared in this study to be severely increased in histopathological grading of organ of corti, stria vascularis and spiral ganglia with increased caspase-3 expression in spiral ganglia contrasted to control group. These finding were explained according to the hypothesis which described CIS attributed apoptosis and cell death in cochlea specially hair cells (HCs), which are related to the over generation of reactive oxygen species (ROS) commanding proliferated oxidative stress, lipid peroxidation and

caspase initiation [20]. The stimulated influx of calcium ions by CIS leading to prompt upsurge in HC Ca2+ intracellular concentration [21]. The inner ear has expression of Ca2+-sensitive neuropeptide [22]. Hayashi,et.al [23], observed that cisplatin significantly increased proinflammatory cytokine levels for example TNF-α and IIL-1β in cochlea with evidence of increasing TNF-α and IL-1β expression in the cochlea [24].

Glucocorticoids comprising prednisones, dexamethasone (DX), and methylprednisolones is repeatedly used for innumerable inner ear diseases cure (autoimmune inner ear, Meneiere's disease, tinniitus, acoustic trauma, ototoxicity and sudden sensorineural hearing loss) [25]. The function of DX is known to be via the signal transduction pathway and glucocorticoid receptor promote inflammation [26]. for instance, Dinh et al. [43], has publicized the preventive consequence of DX against the organ of corti toxicity induced by TNF-α leading to apoptosis via the inflection of nuclear factor kappa B (NFK-B) through increased in the inner ear ROS concentration [27]. In our study, organ of Corti treated with DX flaunted moderate improvement in histological grading and moderate decrease of caspase 3 expression compared with CIS group. These results of DX were coinciding with that of previous study which explained, DX had protective effect on cochlear apoptosis generated by gentamycin (exceeding 5 μg/mL) by inhibition of antioxidant and proinflammatory cytokines (TNF-α, IL-1β) overproduction [28]. In addition, DX had estimated to diminish the expression of apoptotic activator especially Bax in cochlear hair cells programmed cell death by gentamycin. In a study by Ju et al, 2017 [29] who verified that DX had suppressive influence on hair cells apoptosis thru Bax expression adjustment in ototoxicity exemplary and improve hearing loss. Another theory of that study suggest that the protective effect of DX in ototoxicity is by triggering the PI3K–Akt pathway activity and diminishing caspase activity, in which DX was more efficacious in a comprehensive kind of cell types protection over different mechanisms especially, the anti-apoptotic action by inhibiting standardized cell death effectors, including BAD, Bax, FKHR, caspase-3 and caspase7.

A glycoprotein called erythropoietin promotes the ability of erythroid progenitor cells to survive, develop, and differentiate while controlling the erythropoiesis process [30]. In addition to its erythropoietic properties, ER has been shown to have antiapoptotic properties that provide cytoprotection in a variety of cellular injury scenarios [31]. The neuroprotective benefits of ER are mediated by a number of mechanisms, such as direct antioxidant effects, apoptosis suppression, and a decrease in inflammatory responses [32]. According to Olgun et al. [33], ER avoided

hypoxic-ischemic encephalopathy and reduced the apoptotic cell death that the condition caused in neonatal rats. Rjiba-Touati et al. [34] showed that ER therapy reduced kidney damage and CIS apoptotic tubular cell death in rats. Additionally, they demonstrated that when ER was administered 24 hours prior to CIS treatment, its antiapoptotic effect was more noticeable. Additionally, ischemic brain, spinal cord, and retinal injury experimental models have shown that ER has neuroprotective effects through antiapoptosis [35]. The existence of ER receptors in many cell types inside the guinea pig cochlea (including spiral ganglia, the stria vascularis, and the organ of Corti) was shown by Caye-Thomasen et al. [36] and Monge et al. [37]. Our findings demonstrate that ER has a protective effect on inner ear neurosensory cell, as evidenced by a noticeably better blinded grading of histopathological findings and lower expression of caspase-3 when compared to CIS group (P-value < 0.01). According to research by [38], when ER binds to cochlear cells, it triggers a number of intercellular pathways, such as the PI3K/AKT/mTOR and Ras/MAPK pathways, the Wnt signaling, in addition to the Jak2/STAT5 signaling cascade. These pathways have been demonstrated to contribute to ER-mediated (neuro-)protection by preventing apoptosis, among other effects. The primary mechanism of hair cell loss in CIS ototoxicity is apoptosis, which exposes hair cells to oxidative stress and produces reactive oxygen species [22]. It's interesting to note that ER has been demonstrated to possess antioxidant qualities due to its direct ability to stop oxidative damage [39]. ER has been shown to enhance the survival of isolated neurons and to stimulate neurite outgrowth in the study of (40). Furthermore, it has been demonstrated that ER and angiogenic genes are expressed greater following hypoxia damage. In conclusion, the inner ear's ER may prevent harm to spiral ganglion neurons and hair cells by both directly preventing oxidative stress and preventing apoptosis.

Concomitant administration of ER and DX in Group V significantly reduced apoptosis and cell damage in blinding histopathological grading of cochlear cells and decreased caspase-3 expression in spiral ganglia compared to CIS group (P-value<0.01). Our findings demonstrate that ER+DX has a protective effect on inner ear neurosensory cell, as evidenced by a noticeably better blinded grading of histopathological findings and lower expression of caspase-3 when compared to CIS groups (P-value < 0.01). According to these findings, ER's antiapoptotic properties help to lessen the ototoxic side effects of CIS and have antioxidant consequences plus anti-inflammatory -antiapoptotic effects of DX increased efficacy of ER role.

In study of [41] improved effects of ER by anti-inflammatory drugs in regeneration of sciatic nerve.

In conclusion, data from the current investigation indicate that DX and ER promote improvements in cochlear membrane histological picture and spinal ganglia regeneration so that facilitate functional recovery of the cochlea in rats with better results when administered both together. So, it is recommended to administrate both of DX and ER in patient treated by CIS to protect them from cochlear ototoxicity and hearing loss. Further human experimental studies are needed in the future.

Conflicts of interest

No conflict of interest.

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