Araştırma Makalesi
Research Article

Protective Role Of Darbepoetin In Cisplatin-Induced Ototoxicity

SİSPLATİNE BAĞLI OTOTOKSİSİTEDE DARBEPOETİNİN KORUYUCU ROLÜ

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ABSTRACT

Aim: It was aimed to evaluate in-vivo whether darbepoetin-alpha (DPO) application has a preventive role in the cisplatin-induced ototoxic effect.

Materials and Methods: In the study, four groups were formed using 28 Wistar albino rats. Group 1 (intraperitoneal) IP saline given control group, Group 2 cisplatin (16mg/kg IP single dose), Group 3 DPO (25 µg/kg IP), and Group 4 is the group in which a single dose of 25 µg/kg IP DPO was administered 24 hours before and half an hour after cisplatin administration. Distortion product otoacoustic emission (DPOAE) and brainstem auditory evoked potentials (BAEP) were performed on rats before agent administration and on the 7th day of the experiment. After hearing measurements, the rats were sacrificed. Apoptotic cell death in ear tissue, caspase-3, -8, -9 expression, Inducible Nitric Oxide Synthase (iNOS), Neuronal Nitric Oxide Synthase (nNOS) expression levels, antioxidant Nrf2 (Nuclear factor (erythroid-derived 2)) - like 2) and related Heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQQ1) were studied by immunohistochemical method. Serum glutathione (GSH) and anti-inflammatory TNF-α and IL-1β protein levels were determined using ELISA kits.

Results: The hearing loss was detected at all frequencies showing ototoxic effects compared to control in hearing measurements with cisplatin application. In the examinations performed at the immunohistochemical tissue level, structural changes in the inner ear, necrosis, and necroptosis in the brain and nerve tissues were detected in the cisplatin-administered group. The preventive effects of darbepoetin on the inner ear and brain damage induced by cisplatin are detected by apoptotic protein expressions and oxidative stress-related markers, iNOS and nNOS, Nrf-2 and related HO-1, NQO1, serum GSH, and TNF-α and IL-1β proteins.

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Conclusion: In this study, the protective effects of DPO in ototoxicity caused by cisplatin were demonstrated by reducing apoptosis, increasing antioxidant Nrf-2 and target proteins and glutathione levels, and through anti-inflammatory proteins.

Keywords: Darbepoetin, Cisplatin, Ototoxicity, Nrf2, antioxidant system

ÖZ

Amaç: Darbepoetin-alfa (DPO) uygulamasının sisplatine bağlı ototoksik etkiyi önleyici rolü olup olmadığının in-vivo olarak değerlendirilmesi amaçlandı.

Gereç ve Yöntem: Çalışmada 28 adet wistar albino rat kullanılarak 4 grup oluşturuldu. Grup 1 (intraperitoneal) IP salin verilen kontrol grubu, Grup 2 sisplatin (16mg/kg IP tek doz), Grup 3 DPO (25 µg/kg IP), Grup 4 sisplatin verilmeden 24 saat önce ve verildikten yarım saat sonra 25 µg/kg tek doz IP DPO uygulanan grup. Ratlara ajan uygulamalarından önce ve deneyin 7. gününde distorsiyon ürünü otoakustik emisyon (DPOAE) ve İşitsel uyarılmış beyin sapı potansiyelleri (ABR) yapıldı. İşitme ölçümleri sonrasında ratlar sakrifiye edildi. Apoptotik hücre ölümü kulak dokusunda kaspaz-3, -8, -9 ve Nrf2 ve Hem oksijenaz-1 (HO-1), NQQ1, Glutatyon (GST) gibi Nrf2 ilişkili antioksidan ve iNOS, nNOS ekspresyon düzeyleri immünohistokimyasal yöntemle çalışıldı. Serum glutatyon (GSH) ve anti-inflamatuar TNF-α ve IL-1□ protein düzeyleri ELISA kitleri kullanılarak belirlendi.

Bulgular: Sisplatin uygulaması ile işitme ölçümlerinde kontrole göre ototoksik etkileri gösteren tüm frekanslarda işitme kaybı saptandı. İmmunohistokimyasal doku düzeyinde gerçekleştirilen incelemelerde sisplatin uygulanan grupta iç kulakta strüktürel değişiklikler, beyin ve sinir dokularında nekroz ve nekroptoz saptandı. Darbepoetinin sisplatinin ortaya çıkardığı iç kulak ve beyin hasarlarını önleyici etkileri hem apoptotik protein ekspresyonları hem de oksidatif stres ile ilişkili belirteçler olan Nrf-2 ve ilişkili HO-1, NQO1, iNOS ve nNOS, GST ve anti-inflamatuar proteinleri üzerinden saptandı.

Sonuç: Bu çalışmada sisplatinin oluşturduğu ototoksisitede DPO'in koruyucu etkileri apoptozun azaltılması, antioksidan Nrf-2 ve hedef proteinler ve glutatyon düzeylerinin artışı yanında anti-inflamatuar proteinler üzerinden geliştiği gösterilmiştir.

Anahtar Kelimeler: Darbepoetin, Cisplatin, Ototoxicity, Nrf2, antioxidant system.

Although cisplatin (CDDP) is used to treat many adult and childhood cancers, its ototoxic, neurotoxic, and nephrotoxic side effects require dose adjustment in clinical applications. CDDP causes Corti-organ damage, which starts in the first line cells in the basal fold of the cochlea and progresses to the outer hair cells higher up, and affects the inner hair cells. CDDP ototoxicity occurs due to reactive oxygen species (ROS) and oxidative mitochondrial damage (1-2). As a result of mediators inducing infiltration of inflammatory cells, fibrocyte damage occurs, and the function of the cochlea is impaired. Although the immune function of the inner ear is crucial in protecting against infectious diseases such as labyrinthitis, immune-related inflammatory responses often lead to cochlear degeneration in the inner ear and permanent hearing loss (3-4-5-6).

Many protective agents are being studied for protection from ototoxicity (7-8-9). There are also studies showing the autoprotective effects of erythropoietin against inner ear damage (10-11-12). Darbepoetin (DPO) is an active synthetic form of erythropoietin with a longer half-life and more effective in-vivo and is an agent used in the treatment of anemia due to cancer treatment and chronic renal failure. It has also been used to treat anemia due to CT in patients with solid tumors (13-14). In addition, it has been reported to be protective against neurotoxicity and nephrotoxicity (15-16-17). However, there is no study in the literature examining the effects of DPO on CDDP-induced inner ear damage and its mechanism. In this study, the effect of DPO on CDDP-induced ototoxicity was investigated based on the neuroprotective properties of DPO detected in previous studies. It has been studied whether DPO is protective in CDDP-induced ototoxicity and whether the possible protective mechanism is related to Nrf2 and its target proteins, apoptosis, and anti-inflammatory proteins.

MATERIALS AND METHODS

Ethics committee approval (protocol no:16/2016) was obtained from DEU Animal Experiments Local Ethics Committee for the study. 28 Wistar-type Albino female rats, each 6–8 weeks old, average weight 200–250g, reared by inbreeding, were obtained from Dokuz Eylül University Medical Faculty Experimental Animals Research Laboratory (DEUTFDHAL). During the study, the rats were kept at room temperature (20 ± 2 ºC) and in a 12-hour light/dark environment and fed with standard pellet rat chow, allowing them to access water freely. The ears of each rat were inspected by otomicroscopy (Opmi 1, Zeiss, Germany) to remove residues and debris, confirming that there were no visible external ear canal, tympanic, or middle ear anomalies.

Experimental Groups

Four groups of seven Wistar rats were formed, each randomly selected.

Group I (n: 7) Control: Control group treated with physiological saline

Group II (n: 7) Cisplatin: Rat group treated with cisplatin (16 mg/kg 1-hour IP infusion) (Single dose) (18)

Group III (n: 7) Darbepoetin-alpha: DPO administered rat group (25 μg/kg intraperitoneally (IP) (19).

Group IV (n: 7) Darbepoetin-alpha-Cisplatin: Rat group in which a single dose of 25 μg/kg DPO was administered 24 hours before and half an hour after CDDP administration. CDDP (Koçak) was freshly prepared by diluting with physiological saline in each study, and Darbepoetin-alpha (Aranesp, Amgen) was used. Before the auditory functions were evaluated, rats were anesthetized by administering Ketamine hydrochloride (40mg/kg) and Xylazine hydrochloride (5mg/kg) IP.

The hearing functions of all animals in each group were evaluated before agent administration and on the seventh day. Distortion product otoacoustic emission (DPOAE) and brainstem auditory evoked potentials (BAEP) were tested in each rat as hearing tests.

Animals were sacrificed on the 7th day after agent applications. Apoptotic cell death in ear tissue, cochlea, brain stem regions; caspase 3, 8, 9 levels were studied by immunohistochemical method, Nrf2-related antioxidant such as Nrf2 and Hemoxygenase-1, NQQ1, and iNOS, nNOS protein expressions in tissue were studied by immunohistochemical method. Antioxidant glutathione levels and inflammatory protein levels were determined in their serum with rat compatible ELISA kits (Thermo). Each group was evaluated by comparison within itself.

Evaluation of Auditory Functions

Hearing functions of rats in all groups were evaluated with DPOAE and BAEP tests at the beginning of the study (day 0) and seven days after agent administration. The tests were conducted in quiet rooms. The body temperatures of the rats were maintained at 37°C-38°C. The rats were placed in a lying position on the polyurethane platform, their heads were fixed with tape, and it was ensured that they did not move and that the probe was positioned appropriately. The right and left ears of the rats were measured separately.

The distortion product otoacoustic emission test was tested using the Otodynamics ILO 88 Echoport V6

software programmed version. For the DPOAE test, the ratio between f2 and f1 frequencies (f2/f1) was adjusted to be 1.22. The difference between L1-L2 levels was kept at 10 dB SPL (L1 = 65 dB SPL, L2 = 55dB SPL). Signal-to-noise ratios at 1000, 1500, 2000, 3000, 4000, 6000, and 8000 Hz were recorded in the geometric mean of DPOAE, f1, and f2. DPOAEs were measured at the 2f1-f2 frequency. The values obtained with the signal-to-noise ratio of the response greater than 3 dB peSPL at each frequency were considered to have OAE.

Intelligent Hearing Systems Smart EP-10 was used for brainstem auditory evoked potential testing. The BAEP test was performed in alternating polarity at 8,12,16,20, and 32 kHz. Sub-dermal needle electrodes were used during recording. The active electrode was placed on the vertex, the reference electrode was placed on the test ear, and the ground electrode was placed under the opposite ear. Electrode resistances were kept below 1 Kohm. The bioelectrical responses collected by the electrodes were converted from analog to digital at a sampling rate of 31.3 microseconds. In order to narrow the frequency spectrum of the stimulus, a tone burst stimulus with 1000 ms up-and-down time was used with the Blackman envelope. The lowest intensity level, at which the third wave was obtained, was accepted as the hearing threshold of the rat at that frequency. The results of DPOAE and BAEP tests of rat groups were evaluated comparatively within and between groups.

Biochemical Analysis

Total GSH in blood and anti-inflammatory TNF-α and IL-1β protein levels in serum were studied following the working conditions of ELISA kits (Invitrogen). GSH, TNF-α, and IL-1 β levels were expressed as micromole/mL and (pg/mL), respectively.

Detection of Apoptotic Cell Death in Tissue Samples and Immunohistochemical Analysis

Both ears' cochlea, brain, and brain stem were removed, fixed in formol and decalcified, and embedded in paraffin after tissue follow-up. Sections of 5-micrometer thickness were taken on positively charged slides and evaluated morphologically and histopathologically. Dyeing processes were performed with an automated Ventana Discover device. 5 micrometer thick sections prepared on + loaded slides before the procedure were kept in a 37-degree oven for 12 hours. After deparaffinization, fixation, decreasing alcohol series, distilled water, and PBS washing, blocking antibody was applied. At this stage, primary antibodies were kept in the appropriate dilution for 1 hour without washing. After washing with PBS, streptavidin-biotin secondary antibody stages, staining with DAB, and background staining with hematoxylin, the slides were applied with increasing alcohol series and cleared with xylol, covered with entelan and coverslip, and evaluated under the light microscope. Caspase-3 (Bioss), caspase-8 (Bioss), caspase-9 (Bioss), iNOS (Abcam), nNOS (Bioss), Nrf2 (Bioss), HO-1 (Bioss) and NQO1 (Abcam) are the primary antibodies administered .

If inflammation is observed in the tissues examined, it is classified as acute and chronic and scored as mild, moderate, and severe according to its severity. Again, when fibrosis was observed in the examined tissues, Masson's trichrome connective tissue histochemical staining was performed, and fibrosis was graded as mild to moderate.

Statistical Analysis

Statistical analysis of the research results was analyzed using the 26.0 version of the Windows statistical program for SPSS at a significance level of $p<0.05$. Since there were more than two groups in the numerical data, the Non-parametric Kruskal Wallis test and the Mann-Whitney-U test with Bonferroni correction were used to compare the binary groups. The Chi-square test was applied to the data we evaluated as yes or no or more or less.

RESULTS

Distortion Product Otoacoustic Emission and Brainstem Auditory Evoked Potential Test Results

In all groups, a strong signal-to-noise ratio was detected at all frequencies in the baseline DPOAE test. Signal-to-noise ratio was obtained in the DPOAE test on days zero and seven in the control and DPO groups. No difference was found between the control and DPO groups regarding signal-to-noise ratio in the DPOAE test ($p>0.50$). In the DPOAE test performed on the seventh day in the CDDP applied group, it was observed that the signal-to-noise ratios disappeared at all frequencies. The signal-to-noise ratio was statistically significantly reduced at all frequencies compared to the control group ($p=0.000$). All BAEP measurements were normal on days zero and seven in the control and DPO groups. No difference was found between the control and the DPO groups regarding hearing thresholds ($p>0.50$). Hearing loss was detected in all frequencies in the BAEP measurements performed on the seventh day in the CDDP group, and it was observed that the hearing thresholds of all frequencies increased statistically significantly compared to the control group ($p=0.000$). BAEP results support the ototoxicity induced by CDDP. In the group in which DPO and CDDP were applied together, hearing loss was detected in all frequencies on the seventh day. Although there is a difference between the hearing thresholds in the baseline and seventh-day measurements, the difference is not statistically significant ($p=0.24$). It suggests that DPO attenuates the ototoxic effect of CDDP and has protective effects (Figure 1).

Figure-1

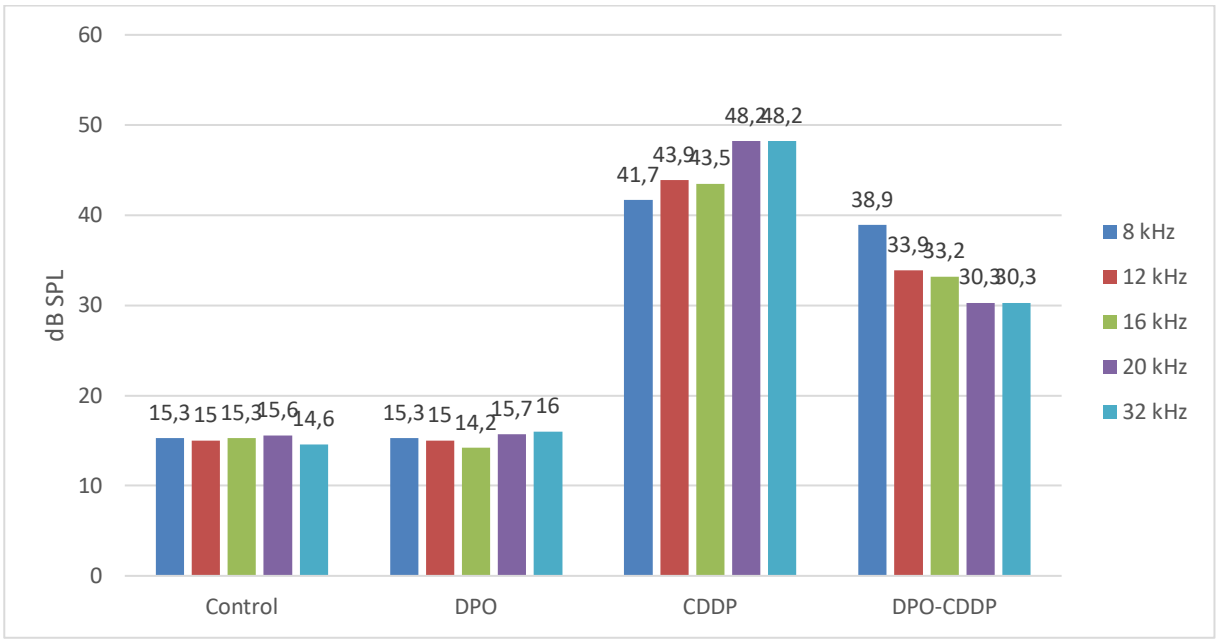


Figure 1. Seventh-day BAEP results of all groups. All BAEP measurements were normal in the control and DPO groups. Hearing loss was detected in all frequencies in the CDDP group ($p=0.000$). Although there was a difference between hearing thresholds at all frequencies in the group in which DPO and CDDP were applied together, the difference was not statistically significant ($p=0.24$).

Biochemical Results

While serum GSH levels decreased in the CDDP group, there was a slightly significant increase in the DPO added group ($p<0.05$) (Figure 2). While serum TNF-α and IL-1β levels increased in the CDDP group, there was a significant decrease in the CDDP group with added DPO ($p<0.05$) (Figure 3).

Figure-2

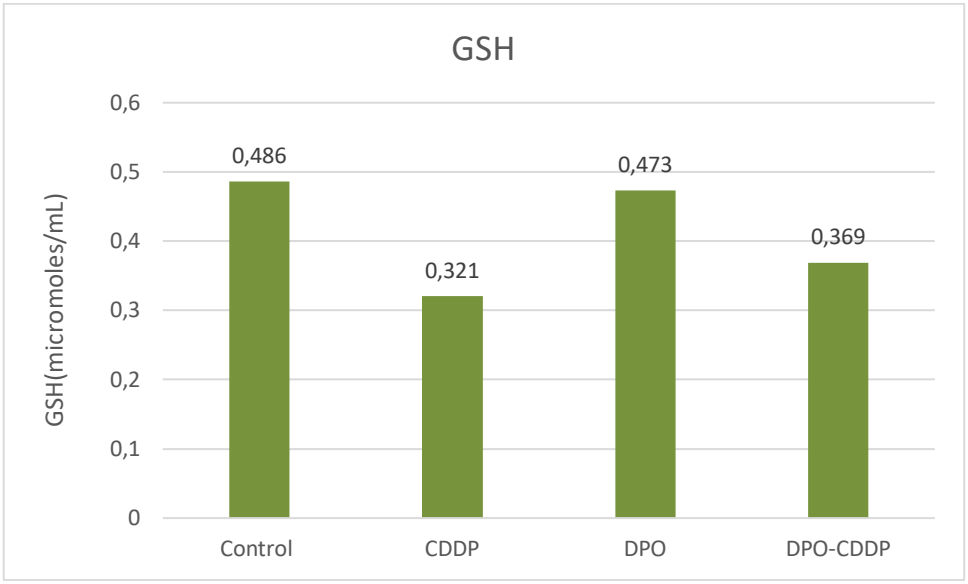


Figure 2. Changing serum GSH values depending on the agents in the groups; While it decreased in the CDDP group, there was a significant increase in the DPO added group ($p<0.05$).

Figure- 3

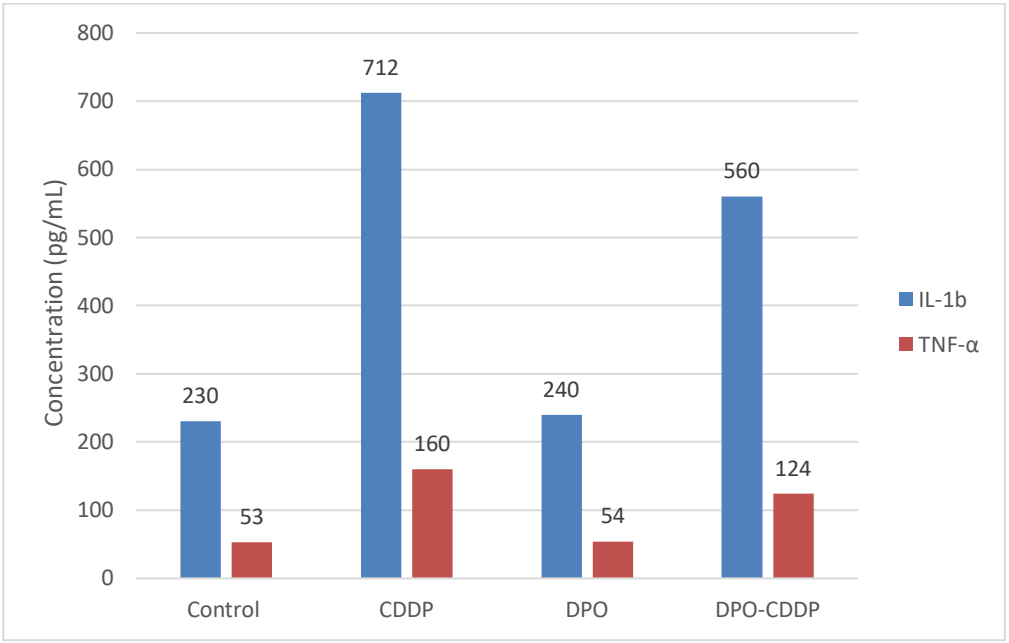
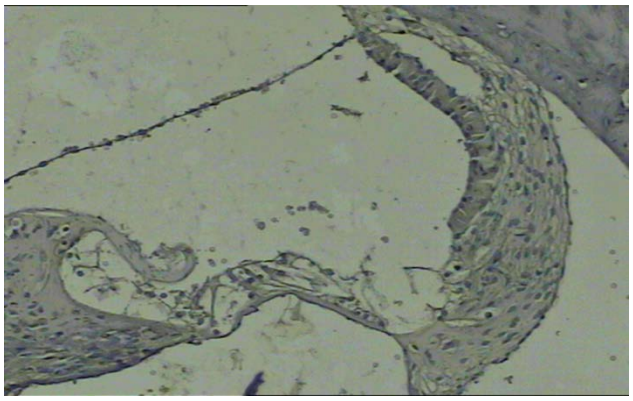


Figure 3. It was determined that serum IL-1β and TNF-α levels were significantly increased in the CDDP group compared to the control group ($p<0.05$). It was found to be decreased in the DPO-CDDP group compared to the CDDP group ($p<0.05$).

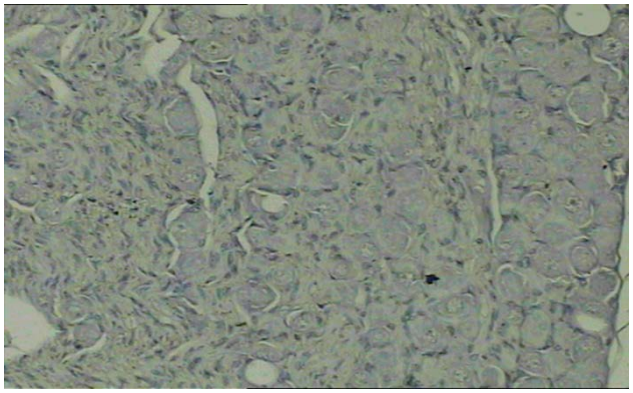
Immunohistochemical Results

Immunohistochemically, structural changes in the inner ear, necrosis, and necroptosis in the brain and nerve tissues were lower in the DPO-CDDP group than in the CDDP group. While it was shown that DPO prevents inner ear and brain damage caused by CDDP by apoptotic protein expressions and Nrf-2 and related HO-1, NQO1, which are oxidative stress-related markers, its effects via the nitric oxide system were also detected (Figure 4,5).

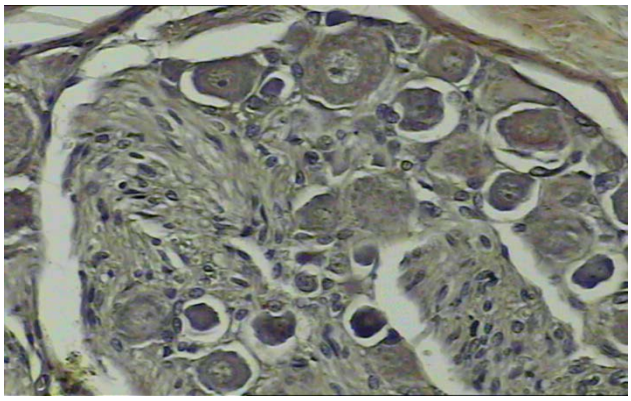
Figure-4 a,b,c,d



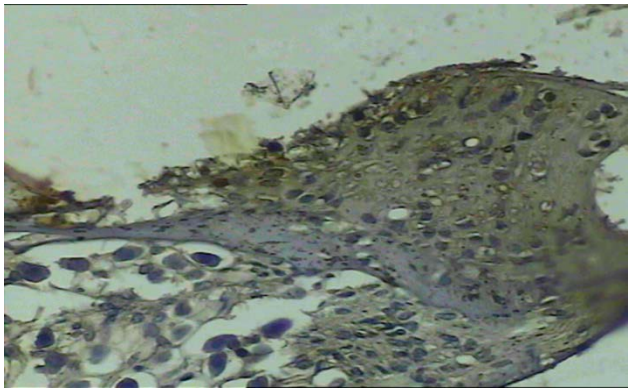
a. The organ of Corti in normal view in the DPO group (x200, HE)



b. The normal SGN in the DPO group (x200, HE)



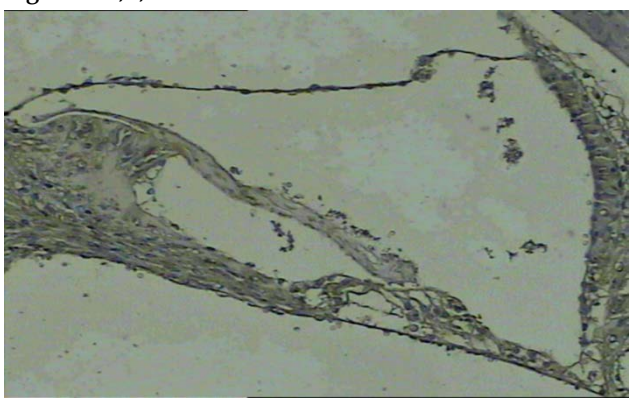
c. Caspase 8 positivity in the spiral ganglion in CDDP group (x400, DAB)



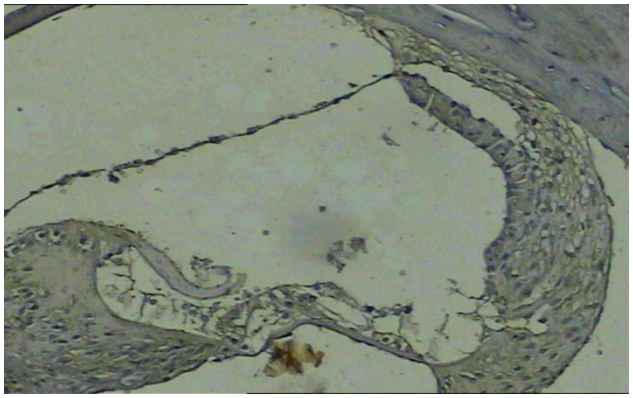
d, Caspase 9 positivity in the organ of Corti in the CDDP group (x400 DAB)

Figure 4 a-d: Immunohistochemical results were found to be normal in the control and DPO groups ($p>0.05$). It supports that DPO alone does not trigger apoptosis or oxidative stress (Figure 3 a,b). When the control group and CDDP group were compared, a significant increase was found in the expression of caspase-8 and caspase-9 in the cochlea ($p=0.002$, $p=0.046$, respectively) (Figure 3 c,d).

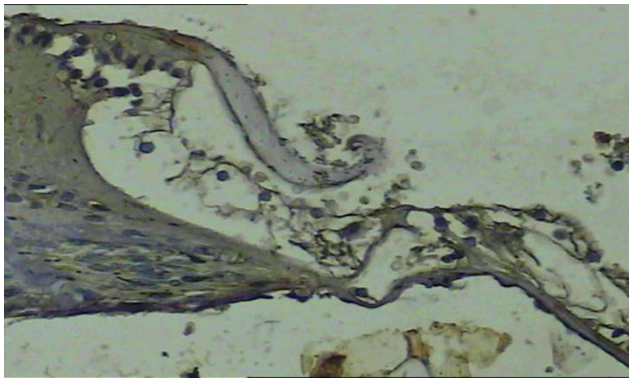
Figure- 5 a,b,c



a. HO-1 positivity in the organ of Corti in the CDDP-DPO group (x400, DAB



b. NQO1 positivity in the organ of Corti in the CDDP-DPO group (x400, DAB



c. nNOS positivity in the organ of Corti in the CDDP-DPO group (x400, DAB)

Figure 5 a-c. Immunohistochemistry staining images in the DPO-CDDP group. The expression of HO-1 and NQO1 supports that DPO activates the anti-oxidant system, and the nitric oxide system is altered against CDDP-induced oxidative stress ($p=0.002$) (Figure 5 a,b). The slight increase in nNOS expression, which supports CDDP damage in which SGN is seen, is not statistically significant ($p>0.05$) (Figure 5c).

When the control and the DPO-treated groups were compared, no statistical difference was found in the tissues, supporting that DPO alone does not trigger apoptosis or oxidative stress ($p>0.05$).

When the control and CDDP groups were compared, a significant increase was found in the expression of caspase-3, caspase-8, and caspase-9 in the cochlea ($p=0.003$, $p=0.002$, $p=0.046$, respectively). Although the slight increase in nNOS expression in the CDDP group was not statistically significant, it supports the CDDP damage seen in the spiral ganglion ($p>0.05$). There was no difference in the expression of iNOS, Nrf2, HO-1, and NQO1 in the CDDP group compared to the control group ($p>0.05$).

When the control and DPO-CDDP groups were compared, no statistical difference was found in caspase-3, caspase-8, and caspase-9 ($p>0.05$). This finding supports the apoptosis-reducing effects of DPO.

In the DPO-CDDP group, signs of increased expression were found in caspase-3, caspase-8, caspase-9, HO-1, NQO1, and nNOS, which indicate CDDP damage. Although the change in caspase-3 and caspase-8 expressions from moderately positive to slightly positive was not statistically significant, it supports that DPO reduces/alleviates CDDP-induced apoptosis ($p>0.05$).

While HO-1 and NQO1 were not expressed in the control, DPO, and CDDP groups, their expression in the DPO-CDDP group supports that DPO activates the antioxidant system against CDDP-induced oxidative stress the nitric oxide system changes ($p=0.002$).

When compared CDDP and CDDP-DPO groups, no difference was found in iNOS and nNOS expressions ($p>0.05$).

Caspase 3, caspase-8, caspase-9, iNOS, nNOS, and Nrf-2 expression were detected at similar levels between these groups (p>0.05) (Table 1).

Table-1

	Control	CDDP	DPO	DPO-CDDP
Caspase 3	-	+ +	-	+
Caspase 8	-	+ +	-	+
Caspase 9	-	+ +	-	+
iNOS	-	-	-	-
nNOS	-	+	-	+
Nrf-2	-	-	-	-
HO-1	-	-	-	+
NQO-1	-	-	-	+

Table 1: Scoring of immunohistochemical results. The primary antibodies administered are caspase 3,8, and 9, iNOS, nNOS, Nrf2, HO-1, NQO1. Ground staining with HE (hematoxylin-eosin), coloring with DAB (di amino benzidine), and grading as mild, moderate, and severe under the light microscope. (- : no expression, + : mild, ++ :moderate, +++:severe)

Correlation Results

When the correlation analysis between hearing measurements and histopathological findings was made with Spearman's test, hearing measurements between the control and CDDP groups on the 7th day showed a correlation with caspase-3 and caspase-8 expression at all frequencies ($p<0.05$). It was determined that hearing loss and caspase-3 and caspase-8 expression were correlated.
When correlation analysis was performed in CDDP and DPO-CDDP groups, a correlation was found between caspase 3 and caspase-8 expressions at 12, 16, 20, and 32 Hz in hearing tests ($p<0.05$). It was determined that there was a correlation with the change in HO-1, NQO1 at 12, 16, 20, and 32 Hz. ($p<0.05$). Nrf2 expression was correlated with the hearing test only at 20 Hz. ($p=0.025$).

DISCUSSION

CDDP is a chemotherapeutic agent widely used in many areas, especially in childhood cancers and adults. Ototoxicity that occurs mainly in childhood and the resulting hearing loss suggests a vital need to reduce the side effects of these agents, which are still used intensively. This study was created to elucidate the ototoxicity due to CDDP and DPO's biochemical and molecular mechanisms. In our study, first of all, it was shown experimentally that the significant change achieved the formation of ototoxicity due to CDDP in hearing tests. In the group in which DPO was applied together with CDDP, hearing loss was detected at all frequencies on the seventh day, but the difference was not statistically significant, suggesting that DPO alleviates the ototoxic effect of CDDP and has protective effects. In studies conducted, the positive protective effects of substances such as N-acetyl-cysteine (20), resveratrol (7), Korean red ginseng (8,21), acetyl L-carnitine (18) to prevent toxicities due to CDDP administration have been shown both in-vitro and in-vivo. Erythropoietin (EPO) is also used as an autoprotective against inner ear damage (10-11,22-28). Although EPO is an agent used to treat chronic renal failure and anemia due to cancer treatment, a study conducted in spiral ganglion neuron cell cultures determined that EPO had a significant regenerative effect on inner ear cells (12).

DPO is the active synthetic form of EPO with a longer half-life and is more effective in-vivo, and many studies have been conducted on its neuroprotective effects apart from its erythropoiesis-stimulating effects (17-18,23-24). A review reported that erythropoiesis-stimulating agents have neurodevelopmental improvement effects in preterm and term infants (22). In addition, it has been

reported to be protective against ethanol intoxication-induced neurotoxicity and CDDP nephrotoxicity (16-18).

Similar to the results of our study, in a study examining the effects of L-arginine against CDDP-induced ototoxicity, it was found that besides a decrease in TNF-α levels, an increase in antioxidant levels and an increase in Nrf2/HO-1 (25).

A recent study has features partially similar to ours, and it has been shown that zingerone polyphenol prevents cisplatin-induced cardiotoxicity (20 mg/kg, IP) by reducing lipid peroxidation, oxidative stress, and inflammation (26).

In some studies, the effects of EPO and DPO were compared in-vivo in terms of cerebral hemorrhage, and it was reported that better neuroprotective results were obtained with DPO than with EPO (17). In a study examining the effect of EPO on CDDP ototoxicity in the HEI-OC1 cell line, the regulatory effect of EPO on CDDP ototoxicity was shown in-vitro by acting through an increase in Nrf2, HO-1, and NQO1 expression concerning the Nrf2/antioxidant system, and it is compatible with the results of this in-vivo study we conducted with the use of DPO (27). However, in the in-vivo study of Doğan et al., following the results of our study, while CDDP-induced hearing loss occurred, the addition of EPO inhibited apoptosis and showed a preventive role against CDDP-induced ototoxicity (28). Although there are studies in the literature on the neuroprotective effects of DPO, no study investigates the effects on CDDP ototoxicity. Therefore, this study is the first in-vivo study to show the protective role of DPO in CDDP-induced ototoxicity and the relationship of the protective mechanism with Nrf2 and its target proteins.

Our study determined that DPO given before CDDP application significantly reduced the damage in the cochlea and brain tissues. These protective effects of DPO have been demonstrated by both apoptotic protein expressions and oxidative stress-related markers Nrf-2 and related HO-1 and NQO1. Again, our study revealed that antioxidant glutathione and anti-inflammatory TNF-α and IL-1β molecules also prevent CDDP-induced ototoxicity of DPO. In this way, the mitigating effect of DPO on the ototoxicity caused by CDDP has been demonstrated at functional, histochemical, and biochemical levels.

CONCLUSION AND RECOMMENDATIONS

It has been shown that the protective effect of darbepoetin against CDDP-induced inner ear and brain damage is mediated by a decrease in apoptosis, antioxidant system, nitric oxide, and anti-inflammatory system. Since our research is an experimental study, the autoprotective effect of CDDP and DPO application should be considered. However, in the presence of cancer, it is clinically undesirable for the anti-cancer activity of CDDP to decrease or interfere with possible protective agents such as DPO. For this reason, it will be possible to study our study results in further in-vivo experimental animal tumor models and then evaluate them in terms of clinical use.

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