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Original Article

The Protective Effect of Recombinant Human Erythropoietin against Cisplatin-Induced Ototoxicity

Ersoy Doğan, Yüksel Olgun, Günay Kırkım, Efsun Kolatan, Pınar Erçetin, Safiye Aktaş, Zekiye Altun, Hülya Ellidokuz, Müge Kıray, Alper Bağrıyanık, Osman Yılmaz, Enis Alpin Güneri

Department of Otorhinolaryngology, Dokuz Eylul University School of Medicine, İzmir, Turkey (ED, YO, GK, EAG)
Department of Laboratory of Animal Science, Dokuz Eylul University School of Medicine, İzmir, Turkey (EK, OY)
Department of Basic Oncology, Dokuz Eylul University School of Medicine, İzmir, Turkey (PE, SA, ZA)
Department of Biostatistics, Dokuz Eylul University School of Medicine, İzmir, Turkey (HE)
Department of Physiology, Dokuz Eylul University School of Medicine, İzmir, Turkey (MK)
Department of Histology, Dokuz Eylul University School of Medicine, İzmir, Turkey (AB)

OBJECTIVE: To investigate the potential protective effect of recombinant human erythropoietin (rhEPO) against cisplatin-induced ototoxicity.

MATERIALS and METHODS: Twenty-eight Wistar albino rats were divided randomly into four groups, and baseline distortion product otoacoustic emission (DPOAE) responses and auditory brainstem response (ABR) thresholds were obtained. Group I received intraperitoneal (i.p.) saline, Group II received a single dose of i.p. 2000 IU/kg rhEPO, and Group III and Group IV received a single dose of i.p. 16 mg/kg cisplatin injection. In Group IV, 2000 IU/kg rhEPO was also injected intraperitoneally two times: 24 hours before and 30 minutes after i.p. 16 mg/kg cisplatin injection. DPOAEs and ABR measurements were repeated 72 hours following cisplatin administration, and the animals were sacrificed. The cochleae of animals were evaluated histopathologically by light microscopy, and percentage of apoptotic cells in the spiral ganglion was then determined by immunofluorescence analysis.

RESULTS: Post-treatment auditory assessment revealed significant ABR threshold elevations at click and 6 kHz and 8 kHz frequencies in Group III compared to Groups I and II ($p<0.05$). In Group III, 8 kHz DPOAEs were also significantly deteriorated compared to Groups I and II ($p<0.05$). In Group IV, the ABR thresholds and DPOAEs were protected at click and 6 kHz; there was no statistically significant difference between Group IV and Group I at those frequencies. Concomitant administration of rhEPO and cisplatin significantly reduced apoptosis and cell damage. The apoptotic cell percentage of spiral ganglions in Group IV was significantly less than in Group III ($p=0.01$).

CONCLUSION: Our results showed that i.p. application of rhEPO inhibited apoptosis and prevented cisplatin-induced ototoxicity in rats.

KEY WORDS: Cisplatin, recombinant human erythropoietin, ototoxicity, apoptosis

INTRODUCTION

Cisplatin (CDDP) is one of the most effective chemotherapeutic drugs used in the treatment of pediatric and adult tumors. However, the clinical application of CDDP is often limited due to its adverse side effects, including ototoxicity [1]. Ototoxicity is a well-documented side effect of CDDP, which is characterized by bilateral and permanent high-frequency sensorineural hearing loss [1-3]. The process of CDDP ototoxicity was investigated with previous experimental studies. It has been reported that increased production of reactive oxygen species (ROS) induced by CDDP caused apoptotic cell death in several areas of the cochlea, resulting in permanent hearing loss [2-4]. Consequently, it was aimed to inhibit the production and harmful effects of ROS to prevent CDDP ototoxicity. For this purpose, a variety of free radical scavengers and antioxidants have been tested in experimental studies [5-9]. Numerous compounds have been shown to prevent CDDP ototoxicity when tested in experimental studies; however, no prophylactic treatment modality has been approved yet to use in clinical practice.

Erythropoietin (EPO) is a glycoprotein hormone produced mainly in the kidney and the liver that serves as the primary regulator of hematopoiesis [10]. Recently, EPO and its receptor have been shown to be present in several nonhematopoietic tissues, such as kidney, liver, brain, and heart [10, 11]. The demonstrated presence of EPO receptor in nonhematopoietic tissues has led researchers to examine the potential beneficial effects of EPO, apart from its hematopoietic function, and the cytoprotective effects of EPO have been demonstrated extensively in various tissues. In these studies, rhEPO (recombinant human erythropoietin) has been found to be protective against both ischemic and toxic injuries, such as ischemic brain and myocardial injury [12, 13] and CDDP-induced kidney and liver injury [14, 15]. In addition, it has also been demonstrated that EPO and its receptors are widely expressed in several cell types within the cochlea, and EPO was found to be protective against gentamicin-induced hair cell damage in rat cochlea cell lines [16].

Corresponding Address:
Ersoy Doğan, Department of Otorhinolaryngology Head and Neck Surgery, Dokuz Eylul University School of Medicine, İzmir, Turkey
Phone: +90 232 412 32 53; E-mail: ersoy.dogan@deu.edu.tr

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This study was designed to evaluate the potential protective effect of rhEPO on CDDP-induced ototoxicity in Wistar rats.

MATERIALS and METHODS

The study was approved by the Animal Experiments Local Ethic Committee of Dokuz Eylül University Medical School (decree no: 2013/15). Twenty-eight Wistar albino rats, reproduced in Dokuz Eylül University Multidisciplinary Research Laboratories, weighing 250-300 g, were included in the study. Animals with signs of any external and middle ear disorders were excluded from the study.

Experimental Design

The animals were randomly divided into four groups, each including 7 rats:

Group I (n: 7): Saline control group
Group II (n: 7): rhEPO group
Group III (n: 7): CDDP group
Group IV (n: 7): rhEPO + CDDP group

The animals were kept in ordinary cages with free access to food and water at a temperature of 23±3°C and a relative humidity of 50±10% with artificial lighting in a period of 12 h. All animal care and procedures were performed humanely. All animals were anesthetized with intraperitoneal ketamine hydrochloride (60 mg/kg) (Ketalar, Pfizer Warner Lambert, Istanbul, Turkey) and xylazine hydrochloride (5 mg/kg) (Ksilazol, Provet, Istanbul, Turkey) injections. Baseline distortion product otoacoustic emissions (DPOAEs) and auditory brainstem response (ABR) testing were performed for all animals on the first day of the study (before any medication). On the 4th day of the study, Groups III and IV received a single dose of 16 mg/kg CDDP(Cisplatin®, Kocak Farma İlac ve Kimya Sanayi A.Ş. İstanbul, Turkey) intraperitoneally. The same volume of saline was injected in Group I intraperitoneally, and a single dose of 2000 IU/kg rhEPO(NeoRecormon, Roche, Mannheim, Germany) was applied intraperitoneally in Group II. In Group IV, 2000 IU/kg rhEPO was injected intraperitoneally two times: 24 hours before and 30 minutes after intraperitoneal CDDP injection.

Although there was free access to water, all animals were also hydrated with daily intraperitoneal injections of 5 mg/kg/day saline between the 1st and 7th days of the study. The dose of CDDP was determined by previous research conducted at our laboratory. Seventy-two hours following CDDP administration, on the 7th day of the study, animals were anesthetized as described previously, and final auditory measurements were obtained. Then, animals were sacrificed, and cochleae were collected for histopathological examinations.

Auditory Assessment

All rats were tested with DPOAEs and ABR in a quiet room prior to any injection (baseline measurement) and 72 h following the intraperitoneal CDDP administration. The tympanic membranes and external auditory canals of all animals were inspected prior to the auditory assessment, and animals with either middle or external ear problems were excluded from study. DPOAEs and ABR testing were performed as previously described in the studies conducted at our laboratory.

DPOAEs Measurements

To test the integrity of the outer hair cells, DPOAE recordings were elicited from the right and left ear of each rat using an ILO-96 apparatus cochlear emission analyzer (Otodynamic Ltd, London, UK). The primary tones were introduced into the rats' outer ear canal through an inserted earphone using the newborn probe. Equilevel primary tones f1 (75 dB) and f2 (65 dB) were fixed at f1/f2=1.22, and DPOAEs were measured at frequencies ranging from 1000 to 8000 Hz. DPOAEs were determined as DP (distortion product) grams. The baseline hearing status of all rats was determined with DP gram, and the signal-to-noise ratio was recorded at 7 frequencies.

ABR Measurements

Auditory brainstem responses were recorded using ICS Medical Charter equipment via the insert earphones in a silent room. The insert earphones were placed directly into the rat's external auditory canals. A neonatal probe tip was used to seal the external auditory canal. Subdermal needle electrodes were placed over the vertex (active), right and left retroauricular regions (reference), and on the dorsum (ground). The stimuli used were alternating clicks (pulse duration 0.1 ms) at a rate of 21.1/s and tone bursts of 6 kHz and 8 kHz (1-ms plateau, 2-ms rise/fall times) at a rate of 31.1/s. EEG activity was preamplified (100,000x) and band-pass analog-filtered from 100 to 3000 Hz for click stimuli and from 50 to 1500 Hz for tone bursts. The number of averaged responses was 1024, and each averaged response was replicated. Each series of stimuli began from the supra-threshold level with subsequent measures using lower intensity levels. Wave II, the wave that had the largest amplitude in these animals, was used to define the threshold.

Sacrifice and Tissue Sampling

Immediately following the post-treatment DPOAE and ABR measurements, all rats were sacrificed under ether anesthesia, and the cochleae were dissected.

Histopathological Examination

The dissected cochleae of both ears per rat were fixed in 10% formalin for 48 hours. They were incubated in 5% glacial acetic acid for 72 hours and 24 hours more after being cut into two pieces. After washing in distilled water, routine tissue processing was applied, and tissues were embedded in paraffin. Hematoxylin and eosin-stained sections were evaluated by light microscopy (Olympus BH-2, Tokyo, Japan) for necrosis and tissue damage.

Apoptosis Detection

Cell death was assessed with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling) assay, which can detect fragmented DNA in the nucleus (GenScript Tunel Apoptosis Detection Kit. L00300, for paraffin-embedded tissue sections, FITC (fluorescein isothiocyanate) labeled POD). The kit was applied on slides according to the manufacturer's instruction. After deparaffinization and rehydration of sections, they were incubated with Proteinase K, and blocking was done by 3% H_2O_2; TUNEL reaction mix containing equilibration buffer, FITC-12-dUTP, and TdT was applied for 60 minutes at 37°C. The assay was done with an Olympus fluorescence microscope using an excitation wave of 450-500 nm and emission wave of 515-565 nm (green). All cells of both ears per rat in the spiral ganglion and the organ of Corti were evaluated and scored as % of apoptosis per all cells. All cell numbers were assessed by 4',6-diamidino-2-phenylindole (DAPI) counterstain.

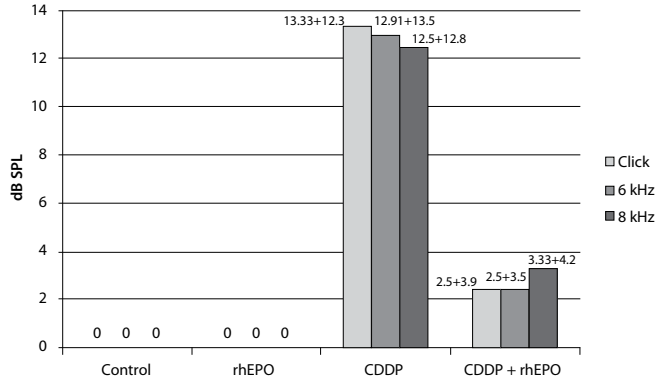

Figure 1. Mean and standard deviation auditory brainstem response (ABR) values in decibels sound pressure level (dB SPL) for each experimental group kHz: kilohertz; rhEPO: recombinant human erythropoietin; CDDP: cisplatin

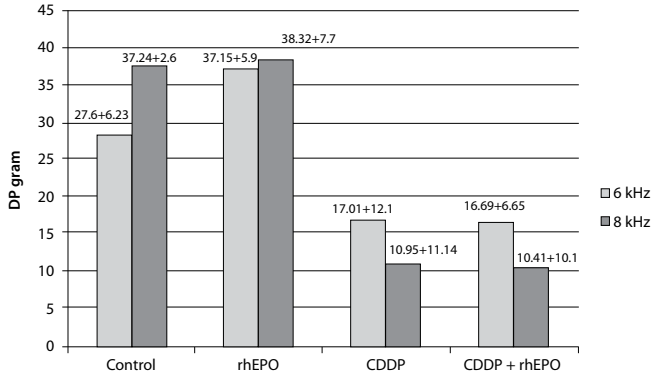

Figure 2. Mean and standard deviation distortion product otoacustic emission (DPOAE) values in distortion product (DP) gram for each experimental group kHz: kilohertz; rhEPO: recombinant human erythropoietin; CDDP: cisplatin

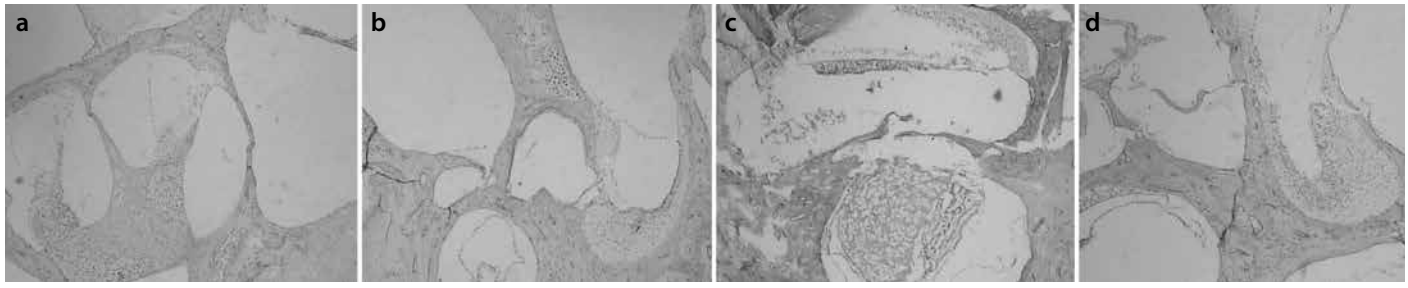

Figure 3. a-d. Light microscopic photographs of inner ear of rats (hematoxylin and eosin; original magnification 40x). Group I, normal morphology (a). Group II, well-preserved tissues (b). Group III, prominent tissue damage (c). Group IV, less pronounced damage (d)

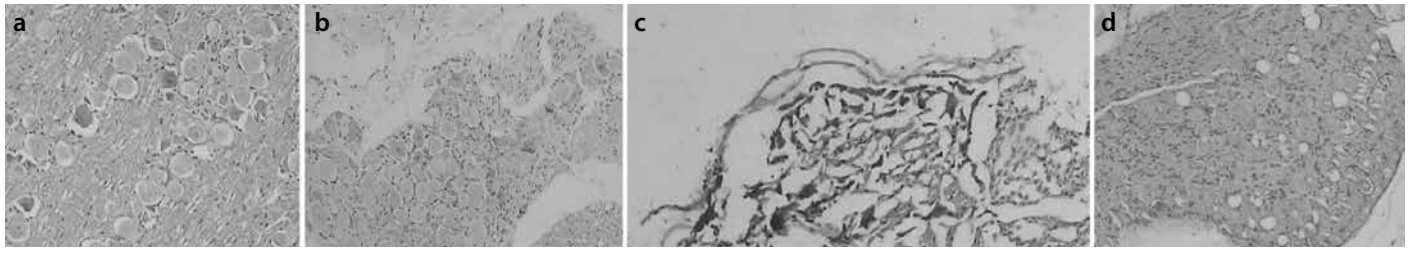

Figure 4. a-d. Light microscopic photographs of spiral ganglion neurons (hematoxylin and eosin; original magnification 200x). Group I, normal morphology (a). Group II, well-preserved neurons (b). Group III, prominent neuronal damage (c). Group IV, less neuronal damage (d)

Statistical Analysis

Statistical analyses were performed using the SPSS 15.0 software package for Windows (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL, USA), and p values less than 0.05 were considered statistically significant. Results are presented as means ± standard deviation. One-way analysis of variance (ANOVA) post hoc Bonferroni test was used to compare the DPOAE values and ABR thresholds in each group, while Mann-Whitney U-test was used to compare the apoptosis percentages of the groups.

RESULTS

Auditory Assessment

There was no statistically significant difference between groups regarding baseline ABR and DPOAE values ($p>0.05$).

Final auditory assessment revealed a significant click and 6 kHz and 8 kHz ABR threshold elevation in Group III compared to Groups I and II ($p<0.05$) (Figure 1). In Group III, 8 kHz DPOAE thresholds were also significantly deteriorated compared to controls ($p<0.05$) (Figure 2). The only statistically significant difference between Group IV and Group I was at the 8 kHz ABR and 8kHz DPOAE values ($p<0.05$). However, concomitant administration of rhEPO and CDDP was found to be otoprotective at click and 6 kHz ABR thresholds and DPOAEs; there was no statistically significant difference between Group IV and Group I at those frequencies. Group II animal DPOAE and ABR values also showed no difference from controls according to the final auditory measurements ($p>0.05$).

Histopathological Examination

In Group I and Group II, the cochlea and spiral ganglion structures showed normal morphology. Light microscopic findings of Group III revealed hair cell loss, disruption on the epithelium of the organ of Corti and necrosis and apoptosis on the spiral ligament. Moreover, necrotic and pyknotic spiral ganglion neurons were observed. In Group IV, these pathological changes were less prominent (Figure 3 and 4). The epithelium of the organ of Corti was intact, and there were no signs of necrosis. Necrotic and pyknotic spiral ganglion cells were lower in number. Many of the ganglion cells were well preserved.

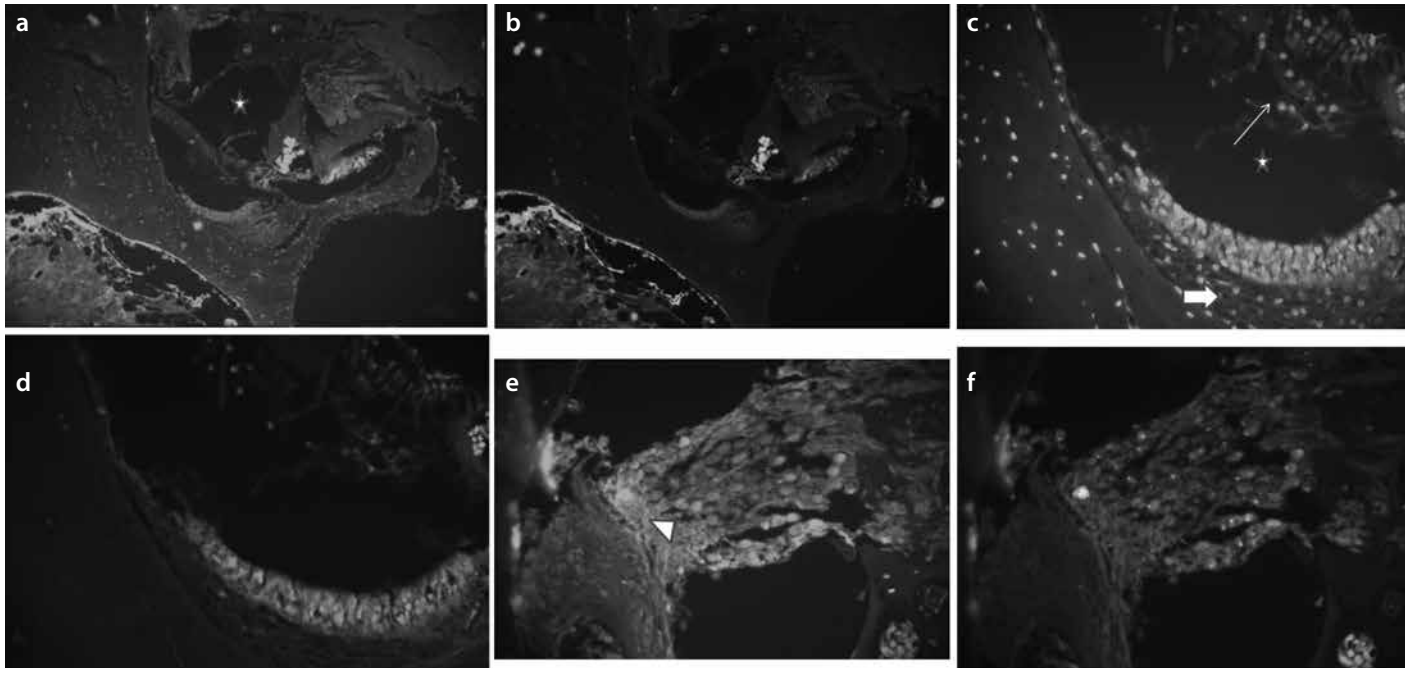

Figure 5. a-f. Immunofluorescence images of inner ear of rats in Group II. Tunel method showing very few to any apoptotic cells. DAPI (4′, 6-diamidino-2-phenylindole)-stained photos (a, c, e) showing the whole nuclei in bright blue. Apoptotic cells are shown in green FITC (fluorescein isothiocyanate)-labeled (b, d, f). The lower magnification of inner ear (a): stars showing scala vestibuli; arrowhead: spiral ganglion; thick arrow: vestibulary membrane; thin arrow: the organ of Corti

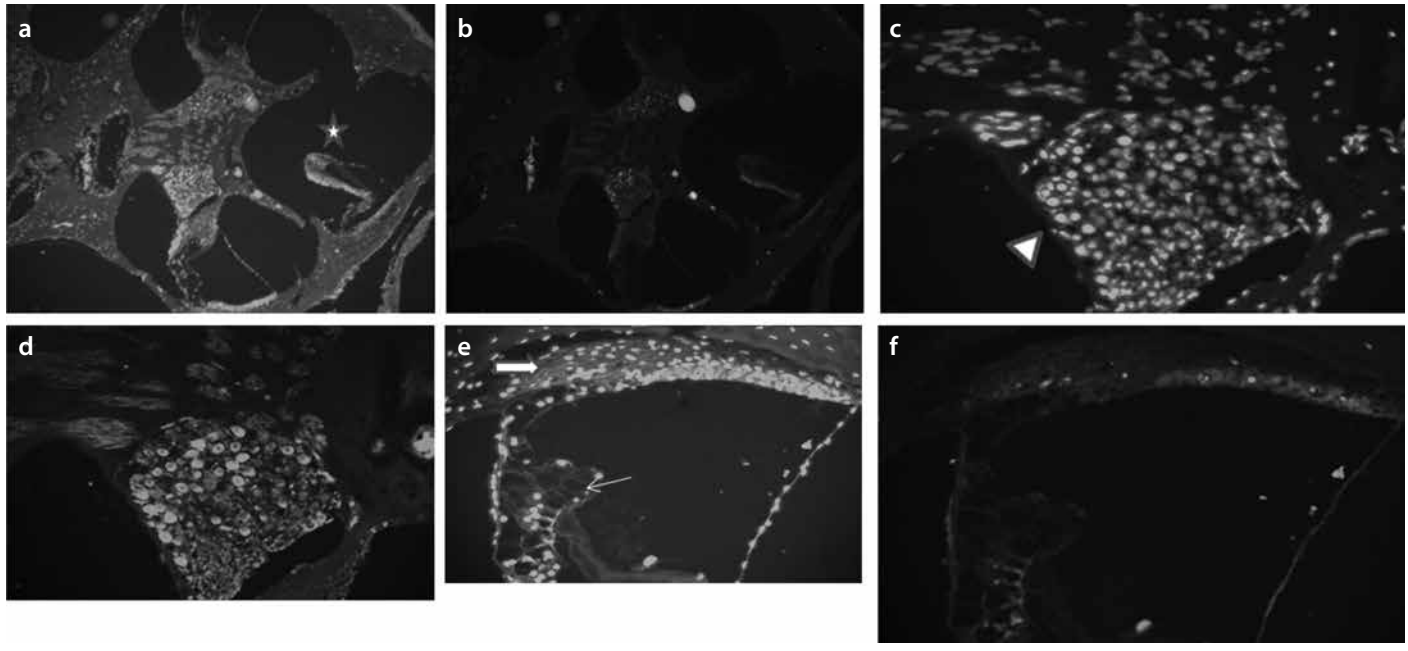

Figure 6. a-f. Immunofluorescence images of inner ear of rats in Group III. Tunel method showing apoptotic cells. DAPI (4′, 6-diamidino-2-phenylindole)-stained photos (a, c, e) showing the whole nuclei in bright blue . Apoptotic cells are shown in green FITC (fluorescein isothiocyanate)-labeled (b, d, f). The lower magnification of inner ear (a): stars showing scala vestibuli; arrowhead: spiral ganglion; thick arrow: vestibulary membrane; thin arrow: the organ of Corti

Apoptosis

The percentage of apoptotic cells in the spiral ganglion was 1%, 1%, 17%, and 5% in Group I, Group II, Group III, and Group IV, respectively. The spiral ganglion cell apoptosis was significantly higher in Group III, when compared with Groups I, II, and IV (p=0.0001). Although the apoptotic cell percentage of spiral ganglions in Group IV was high compared to Groups I and II, the percentage of apoptotic cells was significantly decreased in Group IV compared to Group III (p=0.01). Apoptotic spiral ganglion cells of the rats in Group II, Group III, and Group IV are shown in Figures 5, 6, and 7, respectively.

DISCUSSION

The present study showed that intraperitoneal application of rhEPO inhibited apoptosis and prevented CDDP-induced ototoxicity in rats. Although the cytoprotective effects of rhEPO were demonstrated in various tissues, to our knowledge, this is the first study

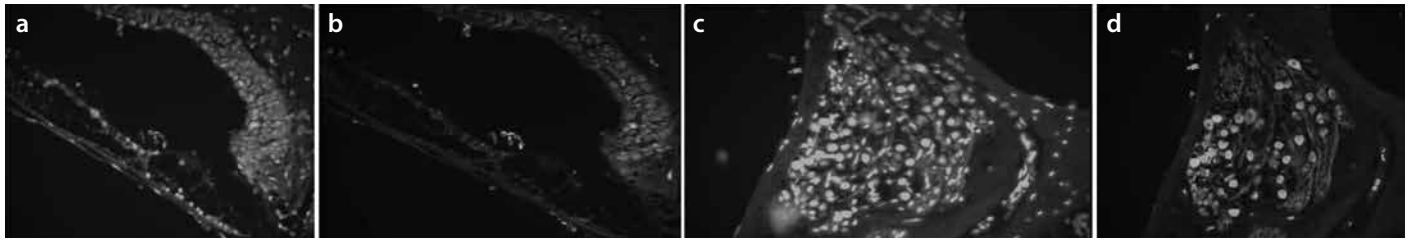

Figure 7. a-d. Immunofluorescence images of inner ear of rats in Group IV. Tunel method showing apoptotic cells. DAPI (4′, 6-diamidino-2-phenylindole)-stained photos (a, c) showing the whole nuclei in bright blue. Apoptotic cells are shown in green FITC (fluorescein isothiocyanate) labeled (b, d). Apoptotic cells in spiral ganglion (d) are less than in Group III

to evaluate the effects of rhEPO for the prevention of CDDP ototoxicity.

Cisplatin is an effective chemotherapeutic agent used in the treatment of various pediatric and adult cancers. Unfortunately, it has dose-limiting side effects, including ototoxicity [1-3]. Ototoxicity is a well-known side effect of CDDP that has been described already in both clinical and experimental trials [1, 3, 4]. It manifests as sensorineural hearing loss beginning at high frequencies and later progressing to speech frequencies [3, 17]. Early detection of CDDP ototoxicity is possible by high-frequency audiologic monitoring; however, once the ototoxic injury is occurred, inner ear damage can not be completely reversed by treatment, and hearing loss usually becomes permanent [3, 4, 17, 18]. For this reason, current research studies are far more focused on prevention of CDDP ototoxicity than its treatment. In the past, several clinical and experimental studies have been performed in an effort to clarify the mechanisms of CDDP ototoxicity and to improve regimens for preventing permanent hearing loss. In these studies, the molecular mechanism of CDDP ototoxicity was mainly based on the induction of apoptosis leading to cochlear cell death [19-21]. It has been hypothesized that increased production of ROS by CDDP caused oxidative stress, which results in apoptosis and cell death in several areas of the cochlea. Many compounds that exhibit antiapoptotic properties are currently used to prevent apoptotic cochlear cell death caused by CDDP [22, 23].

Erythropoietin is a glycoprotein that regulates the process of erythropoiesis [10]. It supports the survival, proliferation, and differentiation of erythroid progenitor cells. In clinical practice, EPO is used as rhEPO for the treatment of anemia associated with pathologic conditions, such as chronic renal failure and myelosuppressive chemotherapy [10, 24]. In addition to its erythropoietic effects, EPO has been found to be cytoprotective in various models of cellular injury by its antiapoptotic effects. Olgun et al. [25] reported that rhEPO decreased hypoxic-ischemic encephalopathy-induced apoptotic cell death in the cochlea, spiral ganglion, and central auditory pathways of newborn rats and prevented hypoxic-ischemic encephalopathy-induced hearing loss. Rjiba-Touati et al.[26] demonstrated that treatment with EPO decreased CDDP-induced apoptotic cell death and tubular injury in rat kidney. They also showed that the antiapoptotic action of EPO was more pronounced when administrated 24 hours before CDDP treatment. Besides, the neuroprotective effects of rhEPO by antiapoptosis have been documented in experimental models of ischemic brain, spinal cord, and retinal injury [11, 27-29].

Caye-Thomasen et al. [30] have reported that EPO receptor is widely expressed by several cell types within the guinea pig cochlea, and they argued that EPO could be used for the prevention of inner ear damage. Moreover, Monge et al. [16] demonstrated the presence of EPO and its receptors in the organ of Corti and the stria vascularis. In this study, EPO was found to be protective against gentamicin-induced hair cell damage in rat cochlea cell lines.

Regarding these studies, we thought that EPO may protect the cochlea from CDDP-induced apoptotic cell death by its antiapoptotic effects, and we treated the rats with rhEPO 24 hours before and 30 minutes after CDDP injection. The possible protective effect of rhEPO on CDDP-induced ototoxicity was evaluated using audiologic tests, as well as histopathological examination and immunofluorescence analysis of apoptotic cell death. Initially, hearing impairment caused by CDDP treatment was revealed by DPOAEs and ABR. Post-treatment ABR thresholds for click, 6 kHz, and 8 kHz stimuli in the rats that received CDDP alone were significantly higher compared to controls ($p<0.05$); also, DPOAE values for 8 kHz in the CDDP group were significantly decreased compared to the other groups ($p<0.05$). On the other hand, animals treated with rhEPO, in addition to CDDP, demonstrated significant otoprotection at click and 6 kHz ABR thresholds and DPOAEs; there was no statistically significant difference between Group IV and Group I in those frequencies ($p>0.05$). Immunofluorescence analysis showed that the percentage of apoptotic cells in the spiral ganglions of the rats treated with CDDP alone was significantly higher compared to control groups ($p=0.0001$). Concomitant administration of rhEPO and CDDP significantly reduced apoptosis and cell damage. The percentage of apoptotic cells in Group IV was significantly less than in Group III ($p=0.01$).

These results suggested that rhEPO ameliorates the ototoxic side effects of CDDP by its antiapoptotic effects. The main advantage of rhEPO over the compounds that were previously shown to be preventive against CDDP ototoxicity is that rhEPO is an FDA-approved drug and is already used clinically for a number of different indications, including chemotherapy-induced anemia in cancer patients. The otoprotective effect can be considered another beneficial effect of rhEPO for cancer patients, in addition to its hematopoietic effects.

It is concluded that intraperitoneal application of rhEPO prevented CDDP-induced ototoxicity by suppressing apoptotic cell death in the cochlea of rats. Application of rhEPO may serve as a potential therapy for the prevention of CDDP-induced ototoxicity.

Ethics Committee Approval: Ethics committee approval was received for this study from the Animal Experiments Local Ethic Committee of Dokuz Eylül University Medical School (decree no: 2013/15).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - E.D., Y.O.; Design - E.D., Y.O., S.A.; Supervision - E.A.G., O.Y.; Funding - E.D., S.A., E.A.G.; Materials - E.K., P.E., M.K., A.B.; Data Collection and/or Processing - E.D., E.K., G.K., P.E.; Analysis and/or Interpretation - G.K., S.A., H.E., M.K., A.B.; Literature Review - E.D., Y.O.; Writer - E.D., Y.O.; Critical Review - E.A.G., S.A., Z.A.

Conflict of Interest: None of the authors have any conflicts of interest that could inappropriately influence (bias) the work.

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