

Original Article

The Protective Effect of *Nigella sativa* Oil against Experimentally Induced Cisplatin Ototoxicity: An Animal Study

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OBJECTIVES: The objective of this study was to investigate the potential protective effect of *Nigella sativa* oil (NSO) against cis-diamminedichloroplatinum or cisplatin (CDDP)-induced ototoxicity.

MATERIALS and METHODS: Twenty-four Wistar albino rats were randomly and equally divided into four groups. Groups 1 and 2 were given a total of 15 mg/kg CDDP intraperitoneally, which was divided equally into three doses on days 1, 3, and 5. Group 2 was treated via gavage feeding with 15 ml NSO that was divided into five doses on days 1, 3, 5, 7, and 9. Groups 3 and 4 received only 15 ml of NSO and 15 ml of 0.9% saline solution, respectively, which were orally administered and divided into five doses on days 1, 3, 5, 7, and 9. Baseline high-frequency (8, 12, 16, and 32 kHz) auditory brainstem response (ABR) measurements were collected in all the groups before the medical administrations and were repeated on the 14th day before sacrifice. Afterward, a histopathological evaluation of the cochlea was performed.

RESULTS: There was a significant difference in the histopathological changes between group 1 and the other groups ($p < 0.01$). Changes in the spiral ganglion cells, the stria vascularis, and the external ciliated cells were significantly different between groups 1 and 2 ($p = 0.019, 0.039,$ and 0.045 , respectively). The ABR results revealed significant differences in the 16 and 32 kHz measurements between groups 1 and 2 ($p = 0.013$ and $p < 0.01$, respectively).

CONCLUSION: According to the results, NSO may have a protective effect on cochlear function against the disruptive effects of CDDP in rats.

KEYWORDS: *Nigella sativa*, cisplatin, ototoxicity, rat

INTRODUCTION

Cis-diamminedichloroplatinum or cisplatin (CDDP) is a chemotherapeutic agent that has been used for the treatment of many types of solid tumors, including breast, testicular, ovarian, bladder, lung, uterine, cervix, and head and neck carcinomas for more than 30 years^[1-3]. Even though newer platinum compounds have been developed, CDDP is still considered to be the most adaptable platinum containing antineoplastic drug^[3,4]. CDDP is known to cause some side effects including myelotoxicity, gastrointestinal toxicity, peripheral neuropathy, nephrotoxicity, and ototoxicity^[5-7]. Some of these complications can be prevented, or reduced, such as in the case of nephrotoxicity, by the use of forced diuresis and hydration. However, there is still no accepted preventive treatment for ototoxic side effects^[8-10]. Because ototoxicity is an irreversible side effect of CDDP usage, studies that examine the prevention of this side effect have been going on.

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As a side effect of CDDP, hearing loss is progressive, bilateral, sensorineural, usually irreversible, and of a high frequency [9, 11, 12]. The main cause of hearing loss is the destruction of the outer hair cells and, sporadically, the destruction of the inner hair cells, which are initiated with apoptosis in the organ of Corti [1–4]. Destruction starts from the base and progresses to the apex. The atrophy of the stria vascularis, the collapse of Reissner's membrane, the significant decrease in spiral ganglion cells, and damage to the cells that support the organ of Corti can also be observed during the destruction period [1–9].

The main cytotoxic effects of CDDP are known to be mediated by the monohydrated CDDP complex, which forms platinum–DNA adducts after the reaction with nuclear DNA. It is also known to increase oxidative stress and production of reactive oxygen species (ROS). Cell damage and death after ROS exposure may be caused by lipid peroxidation. This can then lead to a calcium influx within the cochlear cells, which ultimately leads to apoptosis [3,9].

By preventing the cytotoxic effects of CDDP-based chemotherapeutic agents on organ systems such as the cochlea, safer treatments can be provided. Recently, antioxidant compounds that diminish the oxidative stress–induced damage have been used to limit the nephrotoxic effects in animal models [1,12]. In accordance with these findings, the prevention of CDDP effects in the inner ear has been studied and resulted in varying degrees of success [2,9]. Studies on animal models have reported that the local inner ear administration of cytoprotective antioxidants such as D- or L-methionine, Ginkgo biloba extract, vitamin E, thiourea and the systemic administration of salicylate and vitamin L may reduce ototoxicity caused by CDDP [2, 3, 8–10, 12].

Nigella sativa L. (NS) belongs to the *Ranunculaceae* family that is native to the Middle East and Southwest of Asia. It is a herbaceous plant that possesses several therapeutic effects, including anti-inflammatory, antibacterial, and antioxidant properties [13–15]. Thymoquinone (2-methyl-5-isopropyl-1, 4-benzoquinone) (TQ) is the major con-

stituent of oil of NS seeds. TQ has been studied for its effectiveness against several health problems [16]. TQ regulates many biological activities and had protective effects against free radical–induced tissue damage in experimental animals [14, 16].

The aim of this study was to examine the protective effects of the NS oil (NSO) against CDDP-induced ototoxicity with histopathological and high frequency (8, 12, 16, and 32 kHz) auditory brain stem response (ABR) testing in Wistar albino rats.

MATERIALS AND METHODS

Ethical Approval

Our study was approved by the Yeditepe University Ethics Committee for animal studies (February 2nd, 2017-584).

Animals

Twenty-four 2.5–3-month-old healthy adult male Wistar albino rats (weighing between 250 g and 300 g) with normal tympanic membranes were used in this study. Rats were housed in cages that were in a temperature-controlled room (21°C±2) with a 12-hour dark/light cycle and a relative humidity of 55%±10%. Food and water were freely accessible to animals. Rats with ear problems or having no recorded ABR waves in any of the tested frequencies were excluded from the study.

Chemicals

Nigella sativa oil (Zade Vital Natural Supplements, Konya, Turkey) was administered via gavage feeding in two groups of animals. NSO was produced using the cold pressing method.

Cisplatin (cis-diamminedichloroplatinum, Liba Drug Company, Istanbul, Turkey) was administered intraperitoneally (i.p.) to the two groups of animals.

Experimental Design

The animals were randomly divided into four groups of seven rats each (group 1, CDDP; group 2, CDDP plus NSO; group 3, NSO; group 4, control).

Group 1 was injected with a total of 15 mg/kg body weight of CDDP i.p. that was divided equally into three doses and administered on days 1, 3, and 5. Group 2 received 15 ml of NSO that was divided into five doses and administered on days 1, 3, 5, 7, and 9, plus they received 15 mg/kg body weight of CDDP i.p. that was divided equally into three doses and administered on days 1, 3, and 5. Group 3 received 15 mL of NSO that was divided into five doses and administered on days 1, 3, 5, 7 and 9. Group 4 received only 15 mL of 0.9% saline solution (via gavage feeding) that was divided into five doses administered on days 1, 3, 5, 7, and 9.

Anesthesia

Animals were sedated with 7.5 mg/kg xylazine (Rompun, Bayer, Berlin, Germany) and 50 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey), which was given i.p.

One animal from group 1 and one animal from group 2 (two rats in total) died and were excluded from the study.

MAIN POINTS

- Hearing loss, as a side effect of cisplatin (CDDP), is progressive, bilateral, sensorineural, usually irreversible, and of a high frequency.
- The protective effects of *Nigella sativa* oil (NSO) against CDDP-induced ototoxicity was investigated with the use of high-frequency ABR testing (8, 12, 16, and 32 kHz) and also histopathologically.
- The results of the hearing thresholds were significantly different at the 16 and 32 kHz measurements between CDDP only and CDDP + *Nigella* groups ($p=0,013$ and $p<0,01$, respectively) suggesting that NSO may have a protective effect against CDDP ototoxicity at the 16 and 32 kHz hearing levels.
- The histopathological findings supported the ABR results and showed that the CDDP + *Nigella* group was less affected by CDDP ototoxicity.

ABR

Auditory brain stem response recording was performed under anesthesia for all animals on day 0 of the study and on the 14th day (before the decapitation). A calibrated warming blanket was used to maintain body temperatures at 35°C during anesthesia. Before the placement of earphones, an otoscopic examination was performed and only healthy rats were included in the study. ABR measurements were performed using a Smart EP software system (Intelligent Hearing System, Miami, FL, USA) with Etymotic Research 2 high-frequency insert earphones being placed into both ears of each rat. The responses were recorded by using subdermal needle electrodes, which were placed at vertex (positive), ipsilateral (negative), and contralateral (ground) positions with the impedances being kept at below 3 Ω (Figure 1). The Smart EP system created 8, 12, 16, and 32 kHz tone pip stimuli by using a Blackman window, with a 0.03–3 kHz bandpass filter at a repeat rate of 37.1/sec; this pip served as the auditory stimulant. Hearing thresholds were determined to begin from 80 dB Sound Pressure Level with 10 dB decrements. When we approached the threshold level, 5 dB intensity levels were preferred to determine the threshold. The averaging was fixed as being 1,024 samples in all the recordings. Behavior reproducibility was tested by repeating measurements at least twice. The ABR threshold was defined as being the lowest level of intensity at which a wave II (the most prominent wave observed in rats) was observed. The ABR thresholds that were determined at pre- and post-drug administrations were compared.

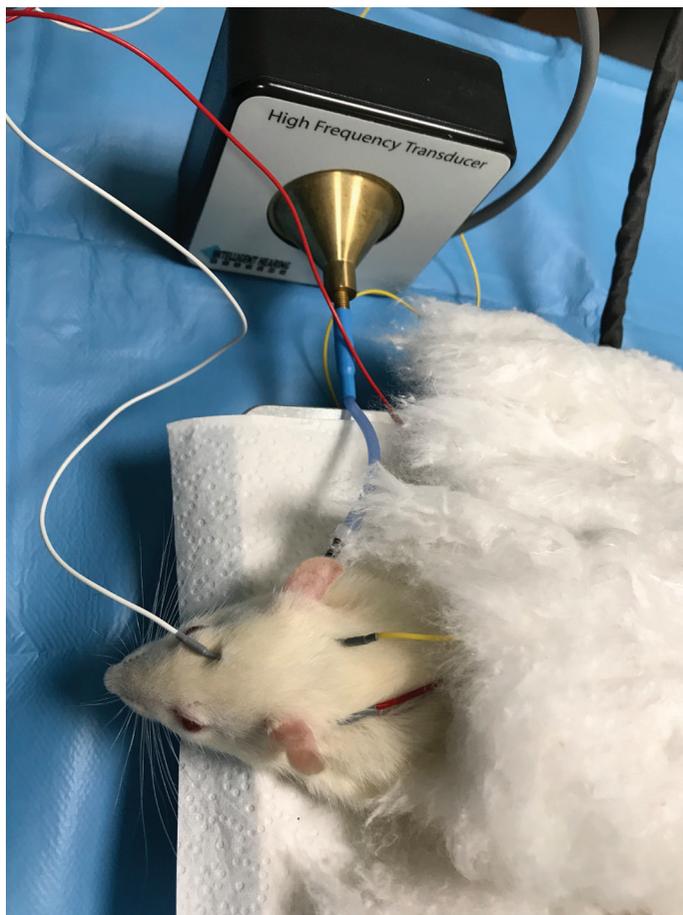


Figure 1. Applications of electrodes, high-frequency transducer, and insert earphone during ABR testing. ABR: auditory brainstem response.

Histopathological Analysis

On the 14th day, all animals were sacrificed under deep anesthesia by decapitation after final recordings were performed. The temporal bones were immediately removed by exposing the tympanic bulla under a dissecting microscope. The cochleae were removed and perfused through the apex and through the oval and round windows with 10% buffered formaldehyde. They were then postfixed in same fixative for 3 days, after which 10% ethylenediaminetetraacetic acid disodium salt (pH 7.4) was used for decalcification. After 1 week, the decalcification solution was washed away with distilled water, and tissues were fixed for 2 more days in formaldehyde. The specimens were embedded in paraffin, sectioned at a 5 μ m thickness passing parallel to the modiolus and stained with hematoxylin and eosin staining. The tissues were evaluated by a single pathologist who was blind to the study groups by using a light microscope (Olympus BX53, Olympus Corporation, Tokyo, Japan), with photographs taken at \times 400 magnification. All histological measurements were quantitatively recorded using an Olympus DP73 camera (Olympus Corporation, Tokyo, Japan) and digital microscopy software (Olympus BX53, Olympus Corporation, Tokyo, Japan), and all the parameters were individually classified.

The grading for CDDP-induced injury was performed with a four-point scoring system that was previously described by Freitas et al.^[17]. In this scoring system, the number of external ciliated cells (ECCs) of the organ of Corti was assessed. The expected number of hair cells was determined, and the apical, mid, and basal cells were counted together in each section. The categorization was as follows: the presence of three ECCs with intact nuclei was given a score of 0; a cochlea with an injury to one ECC was given a score of 1; a cochlea with injuries to two ECCs was given a score of 2; and a cochlea with injuries to three ECCs was given a score of 3.

For the evaluations of the stria vascularis; cytoplasmic vacuolization, marginal cell blebbing, and shrinkage of the intermediate cells were analyzed and tissues were categorized as follows: a normal thickness of stria vascularis and no indications of marginal cytoplasmic vacuolization, cell blebbing, or atrophy was given a score of 0; slight thickness was given a score of 1; mild thickness was given a score of 2; moderate thickness was given a score of 3; and severe thickness was given a score of 4.

Vacuolization and nuclear degeneration in the spiral ganglion cells were also evaluated, and the categorization, which was based on the severity of changes, was as follows: no change=0, mild change=1, moderate change=2, and severe change=3.

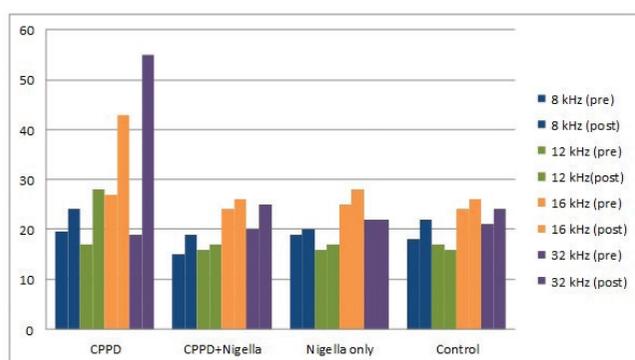
Statistical Analysis

All the data are stated as the mean \pm standard deviations. To compare the differences between the experimental groups, the Kruskal–Wallis and Mann–Whitney U tests were utilized. To differentiate the data with a normal distribution in the groups, the Wilcoxon test was used. Furthermore, the Wilcoxon sign test was used to compare the parameters within the groups. A p-value of less than 0.05 was considered to be statistically significant in all the tests. The Statistical Packages for the Social Sciences (SPSS) 21.0 software (IBM Corp.; Armonk, NY, USA) was used to perform the statistical analyses.

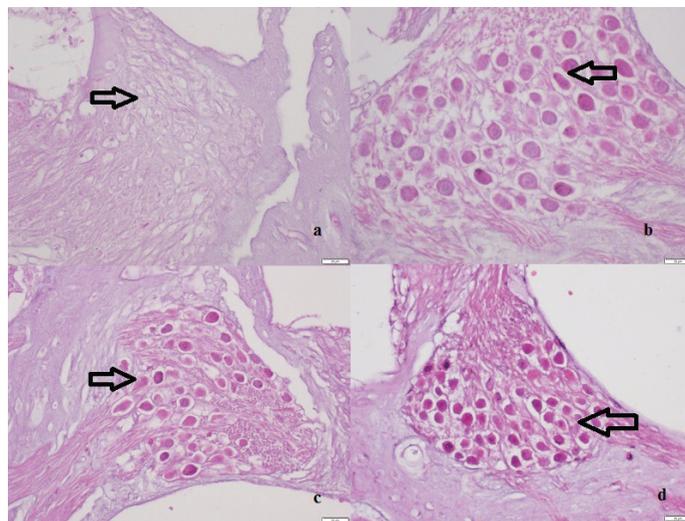
Table 1. Mean hearing levels of all groups (dB)±SD, results of the first (0) and the last (14) day of the experiment

ABR test frequencies		CDDP group	CDDP+Nigella group	Nigella only group	Control group
8 kHz	0 day	19.5±9.26	15±6.66	19.16±3.58	18.75±5.27
	14 th day	24±3.94	19.5±6.43	20.41±6.55	22.5±3.98
12 kHz	0 day	17±6.32	16±3.16	16.66±4.92	17.08±3.34
	14 th day	28.5±14.91	17.5±4.24	17.5±4.52	16.25±4.33
16 kHz	0 day	27±4.83	24±3.16	25.41±3.34	24.58±4.98
	14 th day	43±17.02	26.5±4.11	28.33±3.25	26.25±3.76
32 kHz	0 day	19±2.10	20±4.08	22.08±3.34	21.25±4.82
	14 th day	55.5±17.70	25±4.71	22.5±3.37	24.58±3.34

ABR: auditory brainstem response; CDDP: cis-diamminedichloroplatinum or cisplatin; SD: standard deviation

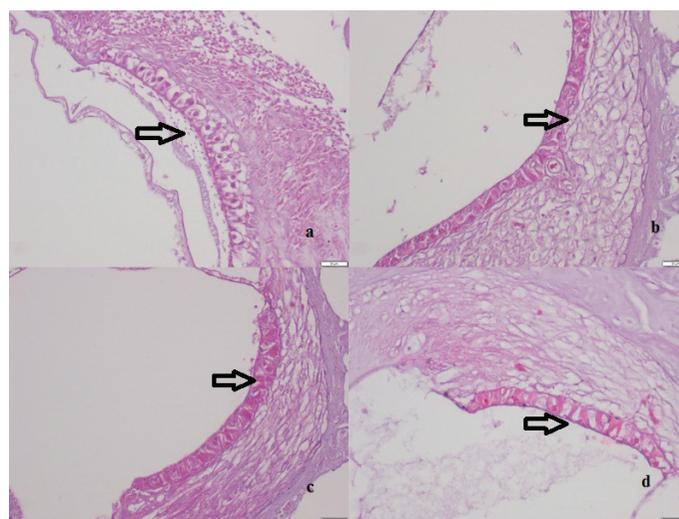


CPPD: Cisplatin

Figure 2. Mean hearing levels (dB) before (pre) and after (post) drug administration.**Figure 3. a–d.** Cross-sectional area of spiral ganglion cells: (a) severe degeneration and spiral ganglion cells' nuclei seen in the cisplatin group; (b) arrows showing eosinophilic cytoplasm of spiral ganglion cells, vacuolization, and nuclear degeneration also seen in the cisplatin+Nigella group; (c) no degeneration in spiral ganglion cells of the Nigella only group; and (d) normal appearance of spiral ganglion cells in the control group (Hematoxylin & Eosin staining, ×400).

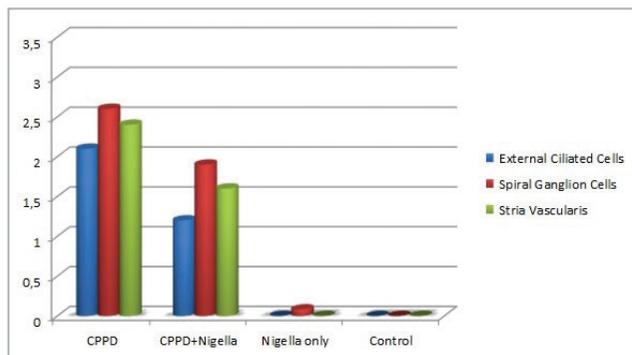
RESULTS

The ABR test results of the groups on days 0 and 14 are shown in Table 1. The ABR results for the groups on day 0, which was the time

**Figure 4. a–d.** Stria vascularis: (a) in the cisplatin group, arrow showing highly fragmented irregular cytoplasm and nuclei-degenerating cells; (b) in the cisplatin+Nigella group, arrow showing slightly thickened stria vascularis, marginal cell blebbing, and cytoplasmic vacuolization; (c) arrow showing close to normal, no degeneration in cells; and (d) normal cells (Hematoxylin & Eosin staining, ×400).

point immediately before any drug administration, were similar for all of the tested frequencies ($p>0.05$). In group 1, the ABR results for all frequencies between days 0 and 14 exhibited a significant difference ($p<0.05$). In group 2, whereas there were significant differences at the 8 and 32 kHz frequencies ($p=0.014$ and $p=0.015$, respectively), the differences between the 12 and 16 kHz frequencies were not significant ($p=0.18$ and $p=0.132$, respectively). In groups 3 and 4, the differences in the ABR results for all the frequencies between days 0 and 14 were not significant ($p>0.05$). Furthermore, for groups 1 and 2, the difference in the hearing thresholds was significantly different for the 16 and 32 kHz frequencies ($p=0.013$ and $p<0.01$, respectively), which suggests that NSO may have a protective effect against CDDP ototoxicity at the 16 and 32 kHz frequencies (Figure 2). The differences were not significant for the 8 and 12 kHz frequencies ($p>0.05$).

In terms of the ECC grading, there were significant differences between groups 1 and 2 ($p=0.045$), groups 1 and 3 ($p<0.01$), groups 2 and 3 ($p<0.01$), groups 1 and 4 ($p<0.01$), and groups 2 and 4 ($p<0.01$). The difference was not significant between groups 3 and 4 ($p>0.05$). When we focused on the changes in the spiral ganglion



CPPD: Cisplatin

Figure 5. Comparison of the grading scores of histopathological values of all groups.

cells and stria vascularis, the differences were significant between groups 1 and 2 ($p=0.019$ and $p=0.039$, respectively) (Figures 3, a-d and 4, a-d). The median scores of the changes were significantly lower in group 4 and significantly higher in group 1 ($p<0.05$) (Figure 5).

DISCUSSION

The mechanism of CDDP ototoxicity is still not fully understood. The formation of ROS, the reduction of antioxidant enzymes in the cochlea, the additive effect of nicotinamide adenine dinucleotide phosphate oxidase 3 isoform and ROS production, and the activation of the transient receptor potential vanilloid 1 channel are considered to be the causes of CDDP ototoxicity. As a result, the lipid peroxidation rate increases, proteins and nucleic acids are damaged by the activated caspase system, and cochlear proteins are exposed to S-nitrosylation. All of these undesired changes can result in cochlear cell injury [18]. Antioxidant agents were thought to inhibit the effects of ROS and to reduce CDDP ototoxicity. Many antioxidants that act as protective agents for CDDP ototoxicity have been previously studied. These agents include bilberry extract, ginkgo biloba, thiourea, alpha-tocopherol and tiopronin, coenzyme Q10, trolox, acetyl-L-carnitine, vitamin E, L-methionine, aminoguanidine, erdosteine, chrysin, lycopene, and TQ [1-5,7-9, 11, 12, 18-23].

Nigella sativa oil has been used worldwide in the field of herbal medicine. NSO seeds contain volatile oil, fixed oil, proteins, alkaloids, and saponins. It has been reported that NSO components have antioxidant effects. NSO has been used to treat liver fibrosis, carbon tetrachloride hepatotoxicity, liver damage caused by *Schistosoma mansoni* infection, and cirrhosis [24]. The components of the volatile oil include p-cymene (37.3%), carvacrol (11.77%), TQ (13.7%), carvone (0.9%), thymol (0.33%), 4-terpineol, longifoline, and t-anethole [25, 26]. The fixed oil consists of unsaturated fatty acids, which are composed of C20:2 arachidic acid and eicosadienoic acids. Houghton et al. [27] have demonstrated that nonenzymatic lipid peroxidation in liposomes is inhibited by both TQ and the fixed oil of NSO as an antioxidant agent. TQ, t-anethole, carvacrol, and 4-terpineol have been reported to have free radical scavenger characteristics [26]. Free radical scavengers are believed to suppress the generation of free radicals and ROS to aid in repairing cell damage. The anti-inflammatory effects of NSO have also been examined in previous studies. TQ was observed to inhibit the production of

eicosanoids such as thromboxane B2 and leukotriene B4 because of the inhibition of both lipooxygenase and cyclooxygenase. The fixed oil of NSO had greater antioxidant and antieicosanoid effects than TQ [27]. Al-Ghamdi et al. [28] reported that the aqueous extract of NSO was observed to have significant anti-inflammatory, analgesic, and antipyretic activities.

Sagit et al. [23] have reported that TQ had a protective effect against CDDP-induced ototoxicity in their experimental study that was performed on 30 healthy female Sprague-Dawley rats. In contrast to this study and its use of TQ, we used cold pressed NSO, which has many effective components such as fixed oil, proteins, alkaloids, and saponins in addition to the volatile oil. To the best of our knowledge, our study is the first study that has investigated the potential protective effect of cold pressed NSO in CDDP-induced ototoxicity.

It was reported that the extract of NSO seeds can be safely used [24]. Zaoui et al. [29] have reported that 10 ml/kg of NS fixed oil, which was orally administered to rats for 12 weeks, did not cause any alterations in hepatic enzymes or mortality. Khanna et al. [30] did not observe any toxicity or mortality while using 10 mL/kg of NS seed oil in rats and mice during a 48-hour time period. The intraperitoneal administration of 50 mg/kg NSO extract to the rats for 5 days did not cause any significant effects on renal and hepatic functions [31]. However, in the literature, two side effects (maculopapular eczema and allergic contact dermatitis) have been reported after using topical pure NSO [24]. We used 15 mL of NSO that was divided into five doses at days 1, 3, 5, 7, and 9, which was approximately equivalent to 10 mL/kg of NSO; this dose represents the maximum nontoxic dose. We did not observe any signs of toxicity in the group that only received NSO (group 3).

In this study, the protective effects of NSO against CDDP-induced ototoxicity in Wistar albino rats was investigated with the use of high-frequency tonal ABR measurements rather than click stimuli. Because the click stimulus has a wide frequency spectrum, it cannot stimulate the whole cochlea in a synchronous way, and it reflects the response of 2-4 kHz in humans [32]. However, owing to anatomical differences in rats, the click stimulus primarily stimulates the 8-10 kHz range [33]. Rats can hear between 0.2 kHz and 80 kHz, and their most sensitive frequency range is between 8 kHz and 30 kHz [34, 35]. Therefore, use of the click stimulus would not be enough to detect and investigate high-frequency hearing loss. With the use of high-frequency tonal stimuli, it was possible to determine high-frequency-specific hearing loss and to establish a hearing curve. According to the results of our study, when we compared groups 1 and 2, the results of the hearing thresholds were significantly different at the 16 and 32 kHz measurements ($p=0.013$ and $p<0.01$, respectively). These results suggest that NSO may have a protective effect against CDDP ototoxicity at the 16 and 32 kHz hearing levels. According to the results of the histopathological findings, there was a significant difference in the means of the ECC grading when we compared group 1 with other groups ($p<0.01$). The changes in the spiral ganglion cells, stria vascularis, and ECCs were statistically significant between groups 1 and 2 ($p=0.019$, $p=0.039$, and $p=0.045$, respectively). These histopathological findings suggest that group 2 (the CDDP+Nigella group) was less affected by CDDP ototoxicity, which supported the ABR results.

Limitations

Animal experiments may not exactly reflect the results that will occur in humans due to the reasons such as having different genetic background, different metabolic pathways from humans and the small sample size. Therefore, extensive clinical trials are needed.

CONCLUSION

As per our knowledge, this is the first study that has investigated the possible protective effects of cold pressed NSO in CDDP-induced ototoxicity. On the basis of the high-frequency ABR results and histopathological evaluation, we can conclude that NSO may have a protective effect on cochlear function against the disruptive effects of CDDP in rats. However, further detailed studies are required on the effects of NSO to determine the optimal dose and use of longer durations of administration, which are required before it can be used in clinical practice.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Yeditepe University Ethics Committee for animal studies (February 2nd, 2017-584).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – N.K., O.K.E., E.Y.; Design – N.K., O.K.E., S.Ş.; Supervision – M.T.K., E.Y.; Resource – N.K., O.K.E., M.E.; Materials – N.K., O.K.E., M.E., S.Ş.; Data Collection and/or Processing – N.K., O.K.E., M.E., A.I.D.E.; Analysis and/or Interpretation – M.E., A.I.D.E., E.Y.; Literature Search – N.K., O.K.E., S.Ş.; Writing – N.K., O.K.E., M.E., A.I.D.E., S.Ş., E.Y., M.T.K.; Critical Reviews – M.T.K., E.Y., A.I.D.E.

Conflict of Interest: The authors have no conflict of interest to declare.

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