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Protective Effect of Spirulina on Cisplatin-Induced Ototoxicity: A Functional and Histopathological Study

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ABSTRACT

Objective: The purpose of this study was to evaluate the protective effect of an antioxidant and anti-inflammatory agent, "spirulina," against cisplatin-induced ototoxicity in rats.

Methods: Twenty-eight adult Sprague–Dawley rats were divided into 4 groups. Before drug administration, distortion product otoacoustic emission and auditory brainstem response tests were performed. Group 1 (n=7) received 1 mg of intraperitoneal saline. Group 2 (n=7) received a single dose of intraperitoneal cisplatin at 15 mg/kg/day. Group 3 (n=7) received oral spirulina at 1000 mg/kg/day for 10 days. Group 4 (n=7) received a single i.p. dose of cisplatin at 15 mg/kg/day, followed by 10 days of oral spirulina at 1000 mg/kg/day. The final distortion product otoacoustic emission and auditory brainstem response measurements were provided 10 days after the initial drug administration. Cochleas were removed, the histochemical examination was performed by caspase-3, caspase-9, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling methods.

Results: Initially, there were no significant differences in distortion product otoacoustic emission and auditory brainstem response measurements between groups. Following cisplatin treatment, the mean difference in signal to noise ratio values was lower in the cisplatin + spirulina group compared to the cisplatin-only group. The increase in auditory brainstem response thresholds was more significant in the cisplatin-only group than in the cisplatin + spirulina group. Posttreatment auditory brainstem response latencies were prolonged in cisplatin and cisplatin + spirulina groups; however, a significant difference was obtained between these 2 groups. The cisplatin + spirulina group had a lower density of apoptotic cells than the cisplatin-only group.

Conclusion: Spirulina has no adverse effects on cochlear functions and may provide some protection against cisplatin-induced ototoxicity.

Keywords: cisplatin, ototoxicity, spirulina, antioxidants, hearing loss, auditory brain stem evoked responses, distortion product otoacoustic emissions, apoptosis.

Introduction

Cisplatin is a highly effective antineoplastic agent that is frequently used for treating pediatric and adult tumors. It has side effects such as neurotoxicity, nephrotoxicity, bone marrow toxicity, gastrointestinal toxicity, and ototoxicity.[1] Cisplatin ototoxicity is characterized by bilateral and persistent hearing loss, especially in high frequencies. Although the detailed molecular mechanisms of cisplatin ototoxicity are still not fully elucidated, it is documented that oxidative stress and reactive oxygen species play an essential role in pathogenesis.[2,3] If the endogenous antioxidant mechanisms of the cochlea are insufficient to eliminate oxidative stress, planned cell death (apoptosis) and necrosis start in the cochlea.[4-6]

An ideal otoprotective agent should be nontoxic, capable of reaching high concentrations in the inner ear, and not inhibiting the antitumoral effect of cisplatin.[5] Among the otoprotective agents against cisplatin shown in animal studies, there are many agents such as N-acetylcysteine, sodium thiosulfate, acetylsalicylic acid, resveratrol, quercetin, and amifostine.[6-9] Although the effects of several drugs have been demonstrated, there is no commonly accepted otoprotective drug that is approved in treatment guidelines.

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Spirulina is a type of cyanobacteria that has a high nutritional value and immunomodulatory and antioxidant properties.[10] The antioxidant activity of spirulina extract was documented in vitro and in vivo.[11] It contains C-picocyanine, an antioxidant substance that eliminates reactive oxygen radicals from the tissues.[12] Previous research revealed that spirulina has a protective effect against cisplatin-related nephrotoxicity and neurotoxicity.[13,14] The current experimental study investigated the otoprotective effect of spirulina using audiological and histopathological methods. To the best of our knowledge, no previous studies have evaluated the potential protective properties of spirulina against cisplatin ototoxicity. Therefore, this study aimed to investigate the efficacy of spirulina in cisplatin-induced ototoxicity.

Methods

The study protocol was approved by Başkent University Ethics Committee on Animal Research (project no: DA19/32), and all experiments were conducted at the Animal Researcl Laboratory of Başkent University.

Animals and Study Design

In this study, 28 adult (12 months old) male Sprague–Dawley rats weighing between 250 g and 350 g were used. Rats were housed under standard laboratory conditions (12 hour light and 12 hour dark) in a room at a constant temperature of 21°C ± 1°C with free access to food and water. This study included 56 ears of 28 rats with normal auditory brainstem response (ABR) and distortion product otoacoustic emission thresholds (DPOAE). Initially, the rats were anesthetized with an intraperitoneal (i.p,) injection of 60 mg/kg ketamine hydrochloride (Ketalar adhesive, Pfizer Pharmaceuticals, Istanbul, Turkey) and 6 mg/kg xylazine (Rompun, Bayer, Germany). In a quiet room, 40 rats (80 ears) were subjected to ABR evaluation and DPOAE measurements. The rats were anesthetized with an intravenous injection of 60 mg/kg ketamine hydrochloride (Ketalar adhesive, Pfizer Pharmaceuticals) and 6 mg/kg xylazine (Rompun). Before the audiological measurements, the rats were laid on a warm cover, and otoscopic examination was performed under general anesthesia. Animals with the pathology of the external auditory canal, middle ear, or tympanic membrane were excluded from the study. On days 0 and 11, following the anesthesia, all ears were subjected to ABR evaluation and DPOAE measurements.

Main Points

- Spirulina has a protective role against cisplatin ototoxicity.
- Pathologically, spirulina reduced cochlear apoptosis caused by cisplatin ototoxicity.
- When spirulina is used in addition to cisplatin, the magnitude of auditory brainstem response (ABR) latency elongation decreases and ABR thresholds are less affected.
- According to otoacoustic emission measurements, spirulina reduces the dysfunction of outer hair cells by cisplatin.
- In addition to its antioxidant activity, spirulina is also otoprotective.

The rats were randomly assigned and treated into 4 groups as follows:

Group 1 (n = 7) received 1 mL/kg i.p. saline. Group 2 (n = 7) received a single dose of 15 mg/kg ip cisplatin (Kocak Farma, Istanbul, Turkey). Group 3 (n = 7) received 1000 mg/kg/day peroral spirulina (Ege University EGERT Spirulina capsule, İzmir, Turkey) dissolved in distilled water and divided into 2 equal doses administered by gavage daily for 10 days. Group 4 (n = 7) received 1000 mg/kg/day spirulina for 10 days following the administration of a single dose of 15 mg/kg ip cisplatin.

After the second DPOAE and ABR measurements were accomplished (day 11), rats were euthanized, temporal bullas were removed, and stored in a 10% formaldehyde solution for histopathological examination.

Distortion Product Otoacoustic Emission Measurements

Distortion product otoacoustic emission measurements were conducted with a Neuro-Audio (Neurosoft, Ivanovo, Russia) in a silent room where the noise level did not exceed 50 dB. Infant hearing screening probes were used. Distortion product otoacoustic emission values were obtained using 2 speakers for 2 stimuli (f1 and f2) in the outer ear canal. The ratio (f2/f1) between f2 and f1 frequencies was kept to be 1.22. The stimulus intensity was taken as L1 for the f1 frequency and L2 for the f2 frequency and kept at the level of L1 = 55 and L2 = 55. Distortion product otoacoustic emissions were measured at a frequency of 2f1 – f2. Signal-to-noise ratios (SNR) values were recorded from both ears on days 0 and 11 at 988 Hz, 1270 Hz, 1778 Hz, 2222 Hz, 2500 Hz, 3200 Hz, 4444 Hz, 5000 Hz, 6154 Hz, 8000 Hz, 8889 Hz, 10 000 Hz, and 11 429 Hz.

Auditory brainstem response Measurements

Auditory brainstem response responses were recorded by subcutaneous needle electrodes placed on vertex, ipsilateral, and contralateral mastoid areas. Click stimulus was given with insertion of headphones. Filter was 100-3000 Hz, repetition rate was 21.1/s, the time window was set to 15 millisecond. Two thousand samples were taken for signal averaging. Hearing thresholds were obtained beginning from 80 dB SPL with 10 dB decrements. When the threshold was approached, the exact threshold was determined by 5 dB adjustments. The ABR thresholds were defined as the lowest level of intensity where wave II was obtained. The latencies of wave II were also recorded.

Histopathological Evaluation

The cochlea of each animal was fixed in 10% formaldehyde for 24 hours, and then a decalcification process was performed by ethylenediaminetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA) solution for 3 weeks. The sections were then washed in a water flow for the night. After being dehydrated in a graded ethanol series, the sections were cleared in xylene and sent for paraffin wax embedding using standardized methods. Tissue paraffin blocks were cut into 5 mm sections for immunohistochemistry, and the slides were stained with primary antibodies caspase-3 (LabVision, Bucharest, Romania) and caspase-9 (1:100, LabVision). A light microscope (Olympus CX41, Tokyo, Japan) was used for all evaluations, and the pictures were obtained from the basal turn. Two pathologists who were blinded to the experimental data independently

assessed the immunolabeling scores. The intensity of the staining on the slides was graded semi-quantitatively, and the Histostain Score (HSCORE) was calculated using the formula: HSCORE = Pi I + 1, where I is the staining strength, with values of 1, 2, or 3 (weak, moderate, or high, respectively), and Pi is the percentage of stained cells for each intensity, ranging from 0% to 100%. The abovementioned deparaffinized and rehydrated sections were reassessed using a commercial kit for TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining (Apoptag, S7101, Chemicon, Calif, USA). The number of positive immunoreactive cells was identified and analyzed in all subjects from apical to the basal turn. Terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells were counted in randomly selected fields, and the percentage of TUNEL-positive cells was calculated. Cells in necrotic areas with poor morphology were excluded. To standardize the apoptosis count, we performed counts and pictures at the basal turn.

Statistical Analysis

R Studio software (Boston, MA) was used for statistical analysis and data visualization.[15] Kolmogorov–Smirnoff, Shapiro–Wilk, and Andersen–Darling tests were used to test whether the variables fit the normal distribution. The homogeneity of variances was evaluated by the Levene test. The parameters were expressed as mean ± standard deviation or median and 25-75% quartiles. Parametric tests were applied if the variables were normally distributed, and non-parametric tests were used if they were not distributed. Groups were compared by 1-way analysis of variance (ANOVA) and Kruskal–Wallis tests. The post hoc pairwise comparisons were performed via the Games–Howell test and Dunn test. The paired sample *t*-test and wilcoxon signed-rank tests were used to compare the difference between DPOAE values and ABR thresholds at the beginning of the study and after 10 days. A value of $P < .05$ was considered statistically significant.

Results

Distortion Product Otoacoustic Emission Results

Baseline SNR values were not statistically different among the groups in all frequencies (1-way ANOVA or Kruskal–Wallis test, *P*-values > .05). Pretreatment and posttreatment SNR values did not differ between the control and spirulina groups. The cisplatin treatment markedly decreased SNR values in 2222 Hz, 2500 Hz, 3200 Hz, 4444 Hz, 5000 Hz, 8000 Hz, 8889 Hz, 10 000 Hz, and 11 429 Hz frequencies.

There were also statistically meaningful changes in the cisplatin plus spirulina group. However, the mean difference values before and after treatment were lower than the cisplatin group. Table 1 represents SNR measurements and mean difference values before and after treatment in all groups. In terms of posttreatment results, no significant difference was observed between the control, spirulina, and CS (Cisplatin+Spirulina) groups as lower SNR values were obtained in the cisplatin group in pairwise comparisons (Figure 1A).

ABR Results

There was no significant difference between groups in terms of ABR threshold and latencies before drug administration. In control and spirulina groups, no significant difference was found between the latencies and thresholds before and after drug administration. While ABR posttreatment thresholds were significantly higher than pretreatment ABR thresholds in the cisplatin + spirulina group, pretreatment and posttreatment latencies did not differ statistically (Table 2). Posttreatment ABR thresholds were markedly higher in the cisplatin-only group than in other groups (Figure 1B). Posttreatment ABR latencies were significantly prolonged in cisplatin and cisplatin + spirulina groups (Figure 1C).

Histopathological Evaluation Results

A significant difference was found between the groups when the ratio of apoptotic cells was compared using caspase-3, caspase-9, and TUNEL methods.

All histopathological indices of apoptosis were markedly increased in the cisplatin group compared to other groups. At the same time, weak staining was observed in the Corti organ in the control and spirulina groups in the caspase-3 immunostaining (Figure 2A-D). A pretty severe expression was noted in the inner and outer hair and support cells of the cisplatin group (Figure 2B). In the cisplatin + spirulina group, caspase-3 immunoreaction was significantly decreased in the Corti organ (Figure 2C). In the caspase-9 immunostaining, a markedly weak expression was observed in the Corti organ of the control group (Figure 3A). In the cisplatin group, a strong reaction was evident in the inner and outer hair and support cells of the Corti organ (Figure 3B). In the cisplatin + spirulina and spirulina groups, it was observed that the immunoreaction decreased in the Corti organ similar to the control group (Figure 3C, D). For the TUNEL method, few TUNEL-positive cells were observed in the inner and outer hair and support cells in the control group (Figure 3A). In the cisplatin-applied group, a markedly strong TUNEL reaction was noted in all cells, especially in the outer hair cells (Figure 3B). In the cisplatin + spirulina group, the TUNEL-positive reaction continued in the support cells, while the TUNNEL-positive reaction cells in the inner and outer hair cells were significantly reduced (Figure 3C). A similar and close TUNEL reaction was noted as the control in the spirulina group. Terminal deoxynucleotidyl transferase dUTP nick end labeling reaction was low, especially in the inner and outer hair cells (Figure 3D). There was no statistically significant difference between the control, spirulina, and cisplatin + spirulina groups in caspase-3, caspase-9, and TUNEL staining. The mean number of apoptotic cells was lower in the cisplatin + spirulina group compared to the cisplatin group (post hoc Games–Howell test, $P < .001$). Table 3 represents histopathological findings and intergroup comparisons.

Discussion

Cisplatin ototoxicity causes cumulative, bilateral, dose-dependent, and commonly permanent hearing loss, affecting higher frequencies, in up to 75% of patients.c Since the discovery that cisplatin causes hearing loss, research into cisplatin ototoxicity and prevention has been ongoing. The exact mechanism of cisplatin ototoxicity is not fully understood. However, increasing reactive oxygen species and pro-apoptotic factors lead to cell death via caspase activation.[17] This has strengthened the assumption that antioxidant and anti-inflammatory

Table 1. Pretreatment and Posttreatment DPOAE Responses in All Frequencies

		Control			Spirulina			Cisplatin			Cisplatin + Spirulina		
		Mean ± SD	MD	*P**	Mean ± SD	MD	*P**	Mean ± SD	MD	*P**	Mean ± SD	MD	*P**
2222 Hz	Pretreatment	3.8 ± 4.6	0.91	.668	3.3 ± 4	0.03	.984	3.2 ± 5.1	6.37	**.006**[A]	−0.3 ± 7.2	1.60	.492
	Posttreatment	2.9 ± 5.8			3.3 ± 6.2			−3.2 ± 6.7			1.4 ± 4.3		
2500 Hz	Pretreatment	7.8 ± 6.6	0.60	.817	5.6 ± 5.9	2.07	.290	4.8 ± 2.5	7.60	**.007**[A]	5.5 ± 2.9	1.09	.430
	Posttreatment	7.2 ± 4.9			7.6 ± 4.4			-2.8 ± 8.4			4.4 ± 3.9		
3200 Hz	Pretreatment	12.8 ± 8.2	3.62	.216	10.3 ± 7.3	1.42	.549	7.9 ± 4.7	7.17	**.003**[A]	7.3 ± 3	1.08	.562
	Posttreatment	9.2 ± 5.7			11.7 ± 6.9			0.7 ± 6.5			8.4 ± 5.8		
4444 Hz	Pretreatment	14.8 ± 8.6	3.25	.413	14.3 ± 7.8	1.47	.507	15.8 ± 8.4	10.4	**.004**[A]	17.1 ± 7.8	5.86	**.046**
	Posttreatment	11.5 ± 8.3			12.8 ± 5.9			5.3 ± 6.8			11.2 ± 6.5		
5000 Hz	Pretreatment	13.1 ± 9	0.94	.790	11.8 ± 9	0.46	.874	14.3 ± 8.3	9.99	**.005**[A]	17.9 ± 7.3	8.94	**.001**
	Posttreatment	12.1 ± 6.6			12.3 ± 7.6			4.4 ± 6.2			9 ± 5.3		
6151 Hz	Pretreatment	14.9 ± 7.6	0.07	.978	15.3 ± 9.9	0.60	.874	14.4 ± 9.4	7.74	**.071**[A]	20.5 ± 9.6	10.9	**<.001**
	Posttreatment	14.9 ± 6.1			14.7 ± 7.6			6.6 ± 10.3			9.6 ± 5.3		
8000 Hz	Pretreatment	19 ± 12.1	3.20	.526	24.1 ± 11.8	2.43	.580	19.8 ± 10.7	11.6	**.025**[A]	23.8 ± 11.1	9.32	**.014**
	Posttreatment	22.2 ± 10.4			21.6 ± 12.4			8.1 ± 11.9			14.5 ± 8		
8889 Hz	Pretreatment	22.2 ± 11	0.98	.823	26.4 ± 10.7	0.79	.851	22.8 ± 11.5	14.4	**.005**[A]	23.5 ± 11.1	8.84	**.037**
	Posttreatment	23.2 ± 10.1			25.6 ± 10.8			8.3 ± 10.5			14.6 ± 9.6		
10 000 Hz	Pretreatment	19.9 ± 13	3.11	.525	23 ± 11.6	0.27	.953	20.6 ± 11.6	14.7	**.008**[A]	26.1 ± 9.5	13.5	**.002**
	Posttreatment	23 ± 9			23.3 ± 12.8			5.8 ± 14.4			12.7 ± 8.6		
11 429 Hz	Pretreatment	13.7 ± 8.1	0.20	.947	15.6 ± 10.9	1.08	.779	14.4 ± 5.9	13.4	**.004**[A]	14.4 ± 5.6	5.69	**.002**
	Posttreatment	13.5 ± 5.2			14.5 ± 6.7			1.1 ± 11.3					

*Paired samples *t*-test.

[A]Posttreatment comparisons between groups were also statistically significant (1-way ANOVA with Games–Howell and Kruskal–Wallis with Dunn test). Bold prints in *P*-value column indicates statistically significant difference.

DPOAE, distortion product otoacoustic emissions; SNR, signal-to-noise ratio; SD, standard deviation; MD, mean difference.

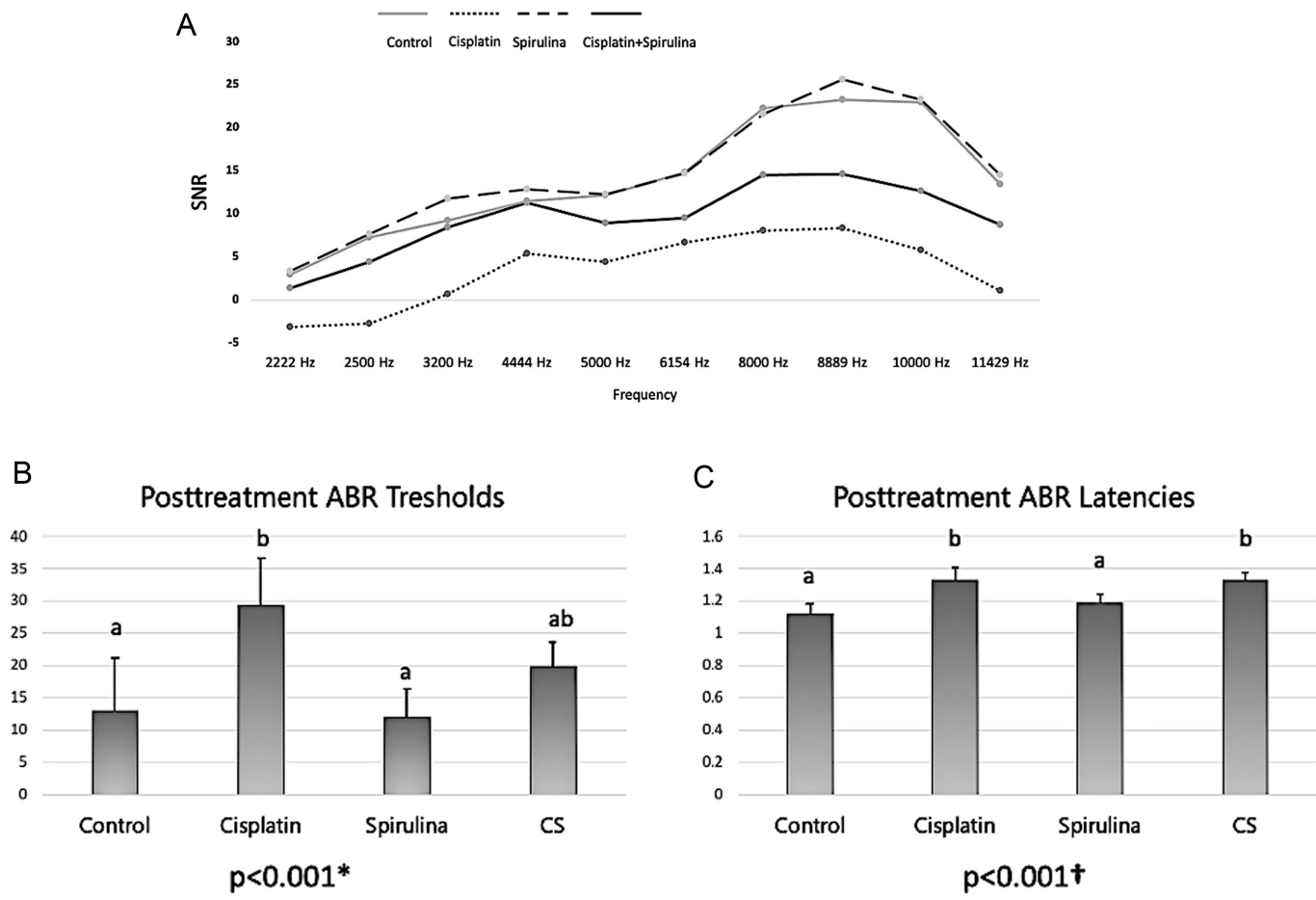

Figure 1. Posttreatment audiological evaluation results. (A) DPOAE responses (S/N ratios) in the groups. S/N ratios in cisplatin group were significantly lower than the other groups at 2222 Hz, 2500 Hz, 3200 Hz, 4444 Hz, 5000 Hz, 8000 Hz, 8889 Hz, 10 000 Hz, and 11 429 Hz frequencies (1-way ANOVA and Kruskal–Wallis tests). (B) Comparison of posttreatment ABR thresholds. Each different superscript letter (a-b) indicates a significant difference in inter-group comparions (Kruskal–Wallis test and post hoc Dunnett's T3 test). (C) Comparison of posttreatment ABR latencies. Each different superscript letter (a,b) indicates a significant difference in inter-group comparions (1-way ANOVA and post hoc Games–Howell test).

agents are effective in preventing cisplatin ototoxicity, and research on the subject has progressed. Many antioxidant and anti-inflammatory agents have been studied in recent publications, including beta-glucan, resveratrol, vitamin E, caffeic acid, etanercept, and astaxanthin.[18-23]

In this study, the preventive effect of spirulina with known antioxidant activity on cisplatin-induced ototoxicity was investigated audiologically and histopathologically. According to the audiological findings, spirulina did not completely prevent the effect of cisplatin, but it reduced its ototoxic effect.

Caspase-3- and caspase-9-mediated pathways play an essential role in cisplatin-induced ototoxicity, and members of the intrinsic apoptosis caspase cascade are triggered following cisplatin treatment.[24] Histopathologically, spirulina reduced the number of apoptotic cells in the Corti organ in our study. Otoacoustic emission and ABR are preferred in ototoxicity studies in experimental animals. A single method is used in many publications in the abovementioned literature. Distortion product otoacoustic emission is the most commonly used audiological examination method in experimental studies on cisplatin ototoxicity as it is sensitive to outer hair cell damage by detecting high-frequency loss at an early stage. In addition, we also applied ABR to better demonstrate possible sensorineural loss due to the known neurotoxic effect of cisplatin.

Posttreatment SNR values in the cisplatin-only and cisplatin + spirulina groups were substantially lower than pretreatment values, according to our findings. The mean difference between before and after drug administration, however, was more significant in the cisplatin-only group. Posttreatment SNR values were also significantly lower in the cisplatin-only group than in the other groups. When the DPOAE findings were analyzed, it was clear that the protective effect of spirulina in combination with cisplatin is most prominent in the frequency range 2222-3200 Hz. At other frequencies, we observe a partial protective effect as well. Although the otoprotective effect of an agent is more important at high frequencies, our study found that spirulina is more effective at low frequencies.

After drug administration, ABR thresholds were significantly higher than the pretreatment values in cisplatin and cisplatin + spirulina groups. Again, the mean difference between pre- and posttreatment thresholds was greater in the cisplatin-only group. Auditory brainstem response latencies were markedly prolonged in the cisplatin-only group, while there was no statistically significant prolongation in the other groups.

Table 2. Pretreatment and Posttreatment ABR Responses in the Groups

ABR Threshold		Mean ± SD	Median (25-75%)	MD	*t*	*P*
Control	Pretreatment	11.4 ± 3.6	10 (10-10)	−1.43	1.00	1.0**
	Postreatment	12.9 ± 8.3	10 (10-10)			
Cisplatin	Pretreatment	12.1 ± 4.3	10 (10-10)	−17.1	8.83	**.001***
	Postreatment	29.3 ± 7.3	30 (22.5-30)			
Spirulina	Pretreatment	11.4 ± 3.6	10 (10-10)	−1.72	2.00	.773†
	Postreatment	12.1 ± 4.3	10 (10-10)			
Cisplatin + Spirulina	Pretreatment	11.1 ± 2.9	10 (10-10)	−6.79	−6.03	**<.001***
	Postreatment	17.9 ± 3.8	20 (20-20)			
ABR Latency						
Control	Pretreatment	1.18 ± 0.04	1.17 (1.14-1.21)	0.057	1.83	.118*
	Postreatment	1.12 ± 0.04	1.13 (1.10-1.13)			
Cisplatin	Pretreatment	1.16 ± 0.04	1.14 (1.13-1.17)	−0.178	−5.15	**.002***
	Postreatment	1.33 ± 0.07	1.35 (1.27-1.27)			
Spirulina	Pretreatment	1.14 ± 0.01	1.14 (1.13-1.15)	−0.048	−.170	.140*
	Postreatment	1.19 ± 0.07	1.17 (1.15-1.25)			
Cisplatin + Spirulina	Pretreatment	1.28 ± 0.01	1.28 (1.12-1.30)	−0.072	3.00	.078†
	Postreatment	1.33 ± 0.06	1.36 (1.29-1.37)			

*Paired samples *t*-test; †Wilcoxon signed rank test. Bold prints in *P*-value column indicates statistically significant difference.
ABR, auditory brainstem response; SD, standard deviation; MD, mean difference; *t*, test statistics.

Histopathological analyses of caspase-3, caspase-9, and TUNEL immunoreactivity yielded similar findings. Compared to the other groups, histopathological indices of apoptosis that could be associated with ototoxicity were considerably higher in the cisplatin-only group. The cisplatin + spirulina group, showed less deterioration in DPOAE and ABR values and fewer histopathological abnormalities in the cochlea compared to the cisplatin-only group. As a result, we suggest that spirulina could have a partial protective effect in cochlear cells against cisplatin-induced apoptosis.

The effects of drugs such as etanercept, riluzole, and levosimendan on cisplatin ototoxicity were studied in the literature.[23,25,26] It is not always appropriate to use an off-label drug to minimize the side effects of another drug. Since spirulina is a food supplement and is excluded from the drug category, it can be safer in terms of side effects. Spirulina is a type of cyanobacteria that has a high nutritional value as well as immunomodulatory and antioxidant properties. Youn et al[14] demonstrated that tempol, a compound that acts as superoxide dismutase or catalase, removing oxygen radicals from the environment and reducing oxidative stress, can be effective in preventing cisplatin ototoxicity. It reduces the toxic effects of chemotherapy in the liver, kidneys, and testicles.[14] Kuhad et al[27] discovered that Spirulina fusiformis increased superoxide dismutase and catalase activity, which was deteriorated by cisplatin.[27] Ototoxicity involves similar antioxidant mechanisms. Lee et al discovered that spirulina reduces apoptosis in nerve cells by lowering the production of reactive oxygen radicals.[28] The degeneration of the spiral ganglion cells and vestibulocochlear nerve caused by reactive oxygen production and neurotoxicity are the most critical steps in the formation of ototoxicity. Reactive oxygen compounds formed due to cisplatin damage the renal tubular epithelium and show nephrotoxic effects.[29] Kuhad et al[27] Showed that the superoxide dismutase and catalase activity reduced by cisplatin was increased by Spirulina fusiformis. Although similar antioxidant mechanisms are involved in ototoxicity, there is no up-to-date publication on the otoprotective effect of spirulina in the literature. Spirulina contains the C-phycocyanin pigment. It acts as an antioxidant by increasing the activity of the enzymes superoxide dismutase, catalase, and glutathione peroxidase. C-phycocyanin was found to have an anti-apoptotic effect in Corti organ cell culture.[30] Although a positive effect was observed in cell culture, no combined audiological and histopathological studies regarding the otoprotective effect of spirulina have been previously published. Further ototoxicity studies can be planned by isolating substances such as C-phycocyanin or chlorophyll-a contained in the spirulina.

Besides cisplatin and drug-induced ototoxicity, various antioxidant molecules have been studied in inner ear damage caused by acoustic trauma. Many molecules are effective in acoustic trauma, including black seed oil, metformin, oxytocin, thymoquinone, and curcumin.[31-35] Inspired by our study, the effect of spirulina on acoustic trauma may be a topic of a new study. Chan et al[36] revealed that spirulina can help prevent hearing loss in senescence-accelerated prone 8 mice by raising superoxide dismutase, catalase, and glutathione peroxidase gene expression and decreasing oxidative stress damage in the cochlea and brainstem. According to Chen et al's study, dietary

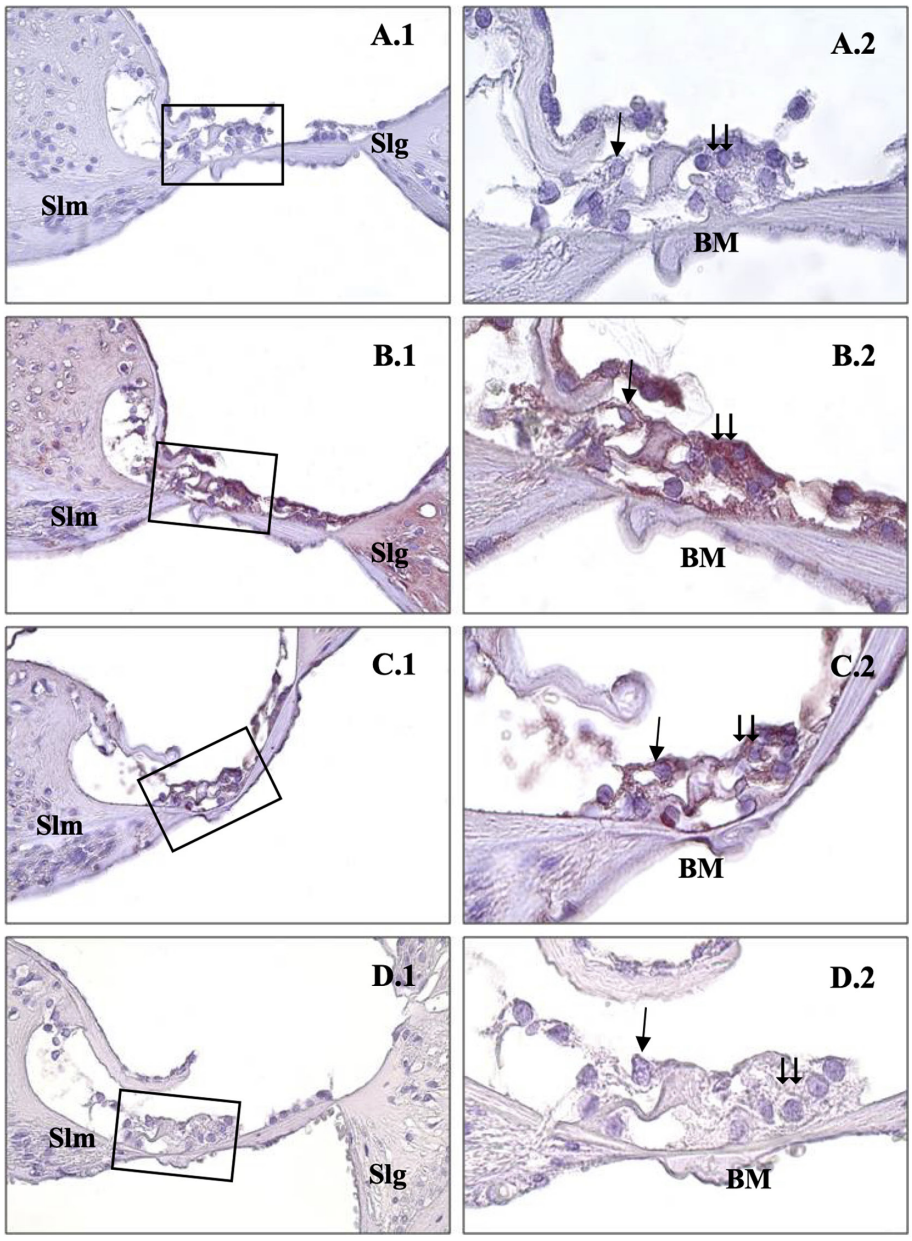

Figure 2. Immunohistochemical staining of cochlea with caspase-3. Control (A); cisplatin (B); cisplatin + spirulina (C); spirulina (D). Corti organ ×40 (1), Corti organ ×40 (1), Corti organ ×100 (2). Mayers hematoxylin base staining. →, inner hair cells; ⇉, outer hair cells; Slm, spiral limbus; Slg, spiral ligament; BM, basillary membrane.

supplement with spirulina may help prevent agerelated hearing loss by lowering the oxidative stress in the inner ear.[35] Spirulina was also effective in reducing oxidative damage caused by ototoxicity according to our findings.

Gingko biloba and lycopene are 2 well-known antioxidant substances that Esen et al[35] investigated in cisplatin ototoxicity. In this study, where DPOAE was used up to 8000 Hz, it was discovered that the protective effect of ginko biloba was more effective at low frequencies. In contrast, lycopene was more effective at higher frequencies. Failure to examine higher frequencies by DPOAE is also the weakness of our study.

There are some limitations to the current study. During ABR and DPOAE tests, investigating other higher frequencies, such as 16-32 kHz, can provide additional data. Higher frequency research is essential since cisplatin-induced ototoxicity begins with high frequencies. The fact that we could not exceed 11 420 Hz was a limitation in our study, and this limitation was attempted to be partially overcome with ABR. Another restriction of our study is the lack of analysis of oxidative and antioxidative parameters in plasma. In future research, oxidative and antioxidative parameters, as well as electrophysiological and electron microscopy studies, may confirm our findings regarding spirulina's protective effect against cisplatin ototoxicity.

Conclusion

There is no completely agreed drug or food supplement to prevent cisplatin ototoxicity. Studies are currently being conducted to prevent the damage caused by chemotherapeutic

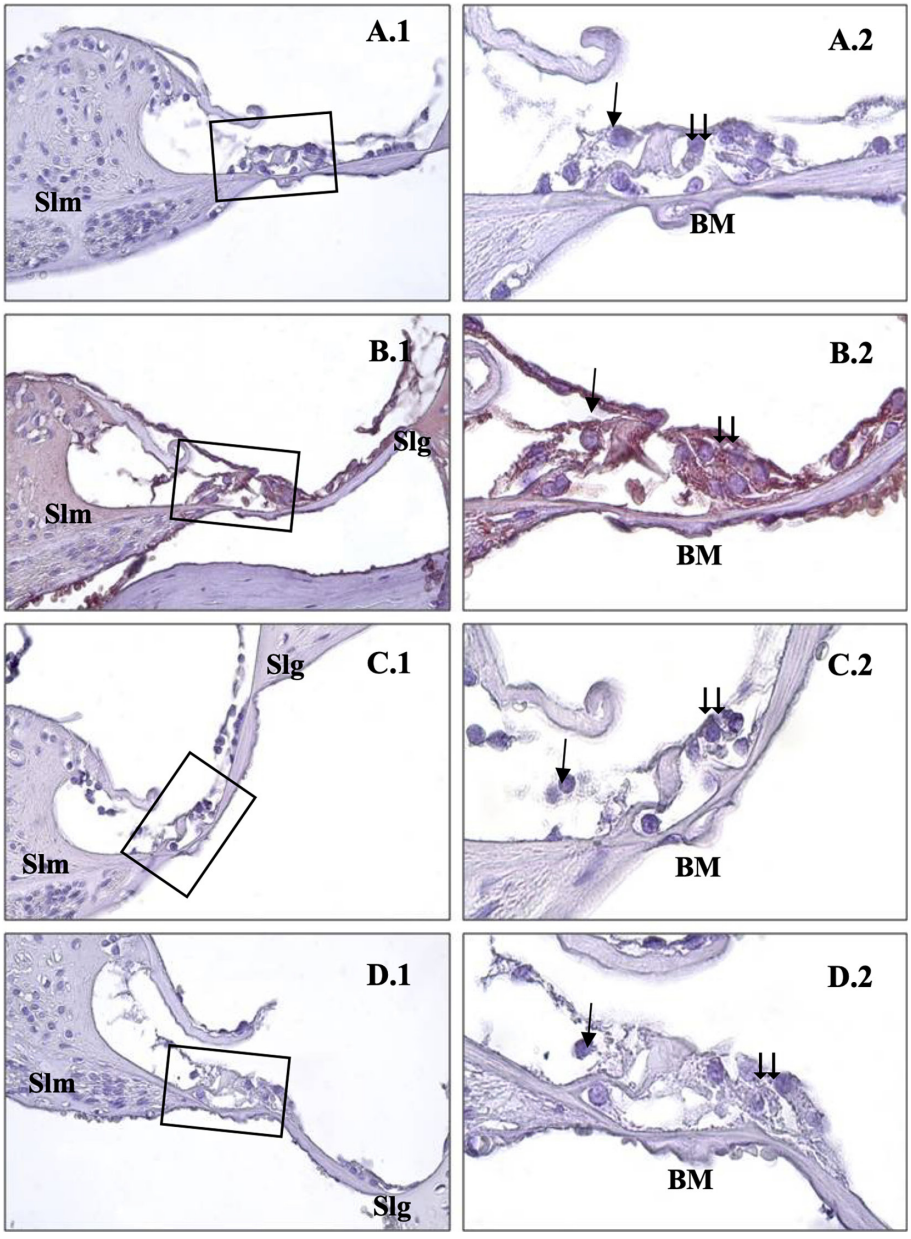

Figure 3. Immunohistochemical staining of cochlea with caspase-9. Control (A); cisplatin (B); cisplatin + spirulina (C); spirulina (D). Corti organ ×40 (1). Corti organ ×40 (1), Corti organ ×100 (2). Mayers hematoxylin base staining. →, inner hair cells; ⇉, outer hair cells; Slm, spiral limbus; Slg, spiral ligament; BM, basillary membrane.

Table 3. Comparison of Caspase-3, Caspase-9, and TUNEL-Stained Apoptotic Cells Between Groups

	Control Mean ± SD	Cisplatin Mean ± SD	Spirulina Mean ± SD	Cisplatin + Spirulina Mean ± SD	F	P*
Caspase-3	33.5 ± 3.5[a]	178.7 ± 12.9[b]	45.0 ± 12.1[a]	47.8 ± 8.8[ac]	488	**<.001**
Caspase-9	33.2 ± 4.7[a]	174.0 ± 10.1[b]	40.1 ± 7.9[a]	44.3 ± 7.0[ac]	617	**<.001**
TUNEL	2.6 ± 0.7[a]	21.1 ± 2.8[b]	4.1 ± 1.9[a]	2.9 ± 1.3[ac]	164	**<.001**

*One-way analysis of variance.
[a,b,ac]There is no significant difference between groups with the same superscript letter for each row after post hoc pairwise comparisons (Games–Howell test).
TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; SD, standard deviation; F, test statistics.

drugs in the inner ear. This study is the first study investigating the effectiveness of spirulina on cisplatin-induced ototoxicity in rats. In addition to audiological evidence, we examined cochlear cell apoptosis using caspase-3 immunoreactivity. Spirulina has been shown electrophysiologically and immunohistochemically to have a protective effect in this study. Since spirulina is a safe agent that can be added to the daily eating regimen, it may be a feasible option for preventing

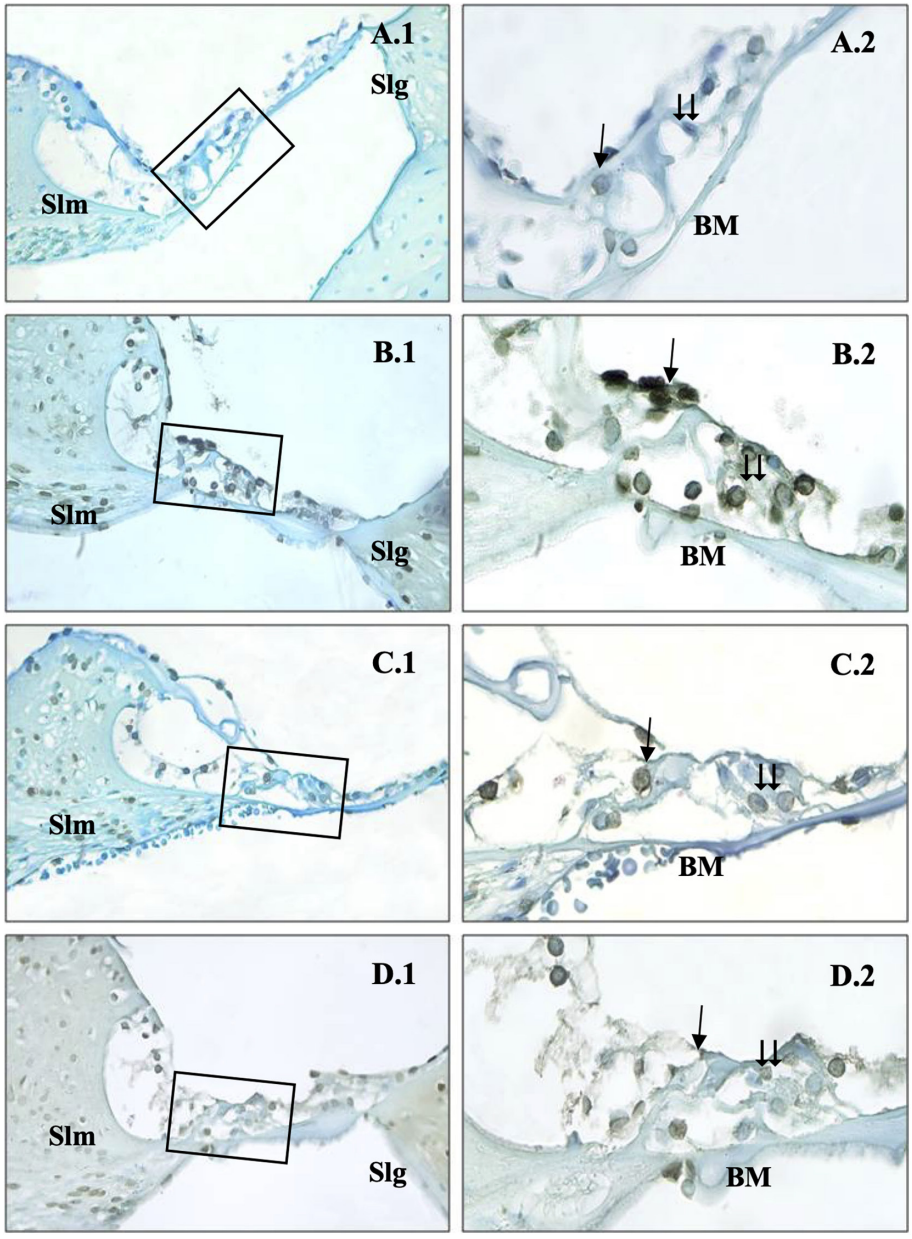

Figure 4. Immunohistochemical staining of cochlea with TUNEL. Control (A), cisplatin (B), cisplatin + spirulina (C), spirulina (D). Corti organ ×40 (1), Corti organ ×40 (1), Corti organ ×100 (2). Mayers hematoxylin base staining. →, inner hair cells; ⇉, outer hair cells; Slm, spiral limbus; Slg, spiral ligament; BM, basillary membrane.

cisplatin-induced hearing loss. Further clinical studies are necessary to demonstrate its efficacy in humans.

Ethics Committee Approval: This study was approved by the ethical commitee of Başkent University (Approval No: 19/32).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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