Isotretinoin's action against cisplatin-induced ototoxicity in rats

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Abstract: Current data do not support the routine use of any agent to prevent cisplatin ototoxicity. Although there are various diseases in which derivatives of vitamin A are used due to their antioxidant effects, there is no study for prevention from ototoxicity. In this study, the protective effect of isotretinoin was investigated on cisplatin ototoxicity in rats. 21 Wistar Albino rats were divided randomly into 3 groups. Group I: cisplatin, Group II: cisplatin + isotretinoin and Group III was the control group. Hearing assessment of all rats was done with ABR and DPOAE tests before and after the procedure. After the procedure, cochleas were resected and transmission electron microscopic examination was performed. Our DPOAE and ABR findings showed that isotretinoin has protective effects on cisplatin ototoxicity. According to transmission electron microscopic findings, isotretinoin has protective effects on cell integrity. We think that new experimental and clinical studies to be carried out in this regard may give us a new option on prevention of cochlea from ototoxicity.

Keywords: Isotretinoin, carotenoids, cisplatin, ototoxicity.

INTRODUCTION

The ototoxic acceptance of any drug should result in bilateral, at least 10 dB sensorineural type loss between frequencies of 20-8000 Hz (Brock, 2012). The main drug groups that lead to ototoxicity are aminoglycosides, macrolides, loop diuretics, cisplatin and salicylates. (Cummings, 2010). The mechanism in the cisplatin ototoxicity is that reactive nitrogen molecules such as superoxide anion and hydrogen peroxide and reactive nitrogen molecules such as nitrous oxide react with cellular lipids, proteins and DNA to cause cellular damage. Reduction in glutathione and antioxidant enzymes after cisplatin ototoxicity has been shown in rats (Cummings, 2010).

Current data do not support the routine use of any agent to prevent cisplatin ototoxicity. Although studies on the use of "amifostine" against cisplatin ototoxicity have been conducted, American Society of Clinical Oncology (ASCO) 2008 rulings have shown that amifostine is inadequate for routine use against cisplatin ototoxicity. Animal models emphasize the potential protective effects of vitamin E. There are animal and human studies on the protective effect of N-acetylcysteine and sodium thiosulfate against ototoxicity of platinum compounds (Doolittle, 2001; Dickey, 2005). It has also been shown that intratympanic dexamethasone administration may have beneficial effects (Marshak, 2014). Although these results are promising, a major obstacle to the development of otoprotective agents is the lack of precise criteria for the assessment of ototoxicity (Brock, 2012; Chang, 2010).

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All-trans retinoic acid has been suggested to reduce chemotherapy-induced neuropathy in experimental studies but there is a need for evidence level studies (Arrieta, 2011). Studies on vitamin A in cerebral ischemia, neuropsychiatric and neuroprotective effects have been carried out (Choi, 2011). Although there are various diseases in which derivatives of vitamin A are used due to their antioxidant effects, there is no study for prevention from ototoxicity.

In our study, the protective effect of isotretinoin -a synthetic oxidative vitamin A derivative- was investigated on cisplatin-induced ototoxicity in rat models.

MATERIALS AND METHODS

During the study, all aspects of experimental animal studies in the Declaration of Helsinki were adhered to. Necmettin Erbakan University, Animal Experiments Local Ethics Committee granted for our work (No.2015-007 dated 28-02-2015).

Study design

Our study was carried out on 21 adult female and male healthy Wistar Albino rats ranging in weight from 250-300 g. Rats were kept in an environment where free food and water were available and at the temperature of 22 ± 2 °C and the moisture 45-65% and 12 hours light-12 hours darkness cycle.

The animals were divided randomized into three groups;
Group I (n=7) (cisplatin): rats were treated with cisplatin at 10 mg/kg single dose, intraperitoneally (i.p.) on day 7.

Table 1: Mean SNR and standard deviations before and after cisplatin administration for each of the three groups.

SNR		1khz before	1khz after	1,4khz before	1,4khz after	2khz before	2khz after	2,8khz before	2,8khz after	4khz before	4khz after	6khz before	6khz after	8khz before	8khz after
I	Mean	5,264	1,507	7,386	4,2	10,79	7,943	14,38	8,429	19,9	6,279	20,44	-2,46	23,06	7,107
	SD	3,174	4,331	2,084	2,891	6,954	5,123	9,67	7,679	7,627	8,938	7,938	11,98	8,304	5,065
II	Mean	7,136	3,336	4,443	1,943	4,336	2,864	6,264	4,2	9,136	7,036	15,35	12,71	17,01	15,44
	SD	3,494	1,929	4,721	3,85	1,623	1,978	1,265	2,122	2,129	3,422	2,954	5,666	5,754	5,873
III	Mean	3,8929	4,4429	3,7643	4,5571	6,6643	5,8571	10,8143	10,0643	16,228	17,335	18,071	19,071	16,907	15,221
	SD	3,2044	5,471	2,96145	3,11737	5,1909	5,1123	4,5978	6,42323	6,1594	5,4963	7,7587	6,3349	5,2367	5,5629

SNR: Signal Noise Ratio, SD: Standard deviation.

Group II (n=7) (cisplatin + isotretinoin): Rats were treated with isotretinoin at 7,5 mg/kg by oral gavage for once every two days for 7 days. On day 7 rats were treated with cisplatin at 10 mg/kg (i.p.) single dose. After, rats were treated with isotretinoin at 7,5 mg/kg by oral gavage for once every two days for 7 days again.
Group III (n=7) (control): Nothing was applied.

Anesthesia
Thiopental sodium (Pental, I.E. Ulagay, Istanbul, Turkey) at a dose 40 mg/kg (i.m.). and 7,5 mg/kg xylazine (i.m.)(Rompun, Bayer, Leverkusen, Germany) were administered to all rats for anesthesia before distortion product otoacoustic emissions (DPOAE), auditory brainstem responses and surgery.

Distortion product otoacoustic emissions (DPOAE)
In our study, the DPOAE test was used to measure emissions. Emission measurements were made with Otodynamics Echoport ILO292 USB II device by using premature newborn probe. At the beginning of study and after 3 days observation at the end of the study, DPOAE test was applied to both ears of all rats. The ratio of frequencies f2 and f1 (f2 / f1) was held to be 1.22. The stimulus intensity was taken as L1 for frequency f1 and L2 for frequency f2 and the difference between levels L1-L2 was kept at 10 dB SPL (L1 = 65 dB SPL, L2 = 55 dB SPL). DPOAEs were measured at 2f1-f2 frequency. The values that 3 dB above the noise threshold of the DPOAE amplitudes were considered significant. The signal-to-noise ratios (SNR) at 1000, 1400, 2000, 2800, 4000, 6000 and 8000 Hz were recorded in the geometric averages of f1 and f2. SNR ratios were used in evaluating DPOAE results.

Auditory brainstem response (ABR)
ABR measurements were performed on all rats bilaterally, pre-study and post-study after 3 days observation. ABR measurements were made with the Medelec Synergy ABR Device. Subdermal needles (Reusable Tip300 Insert Phones, 13 mm, Natus / 041-704000) and premature newborn ear probes (Eartips for use with Intra Auricular Headset and Ear Phone Type: Premature Natus/51023) were used for ABR measurements. The negative electrode was placed on the mastoid, the positive electrode on the test side, and the ground electrode on the opposite side to the mastoid. The suitability of the electrodes was checked with an impedancemeter.
ABR potentials were measured using 11.00 rate click stimuli, 10 msec measurement time, 100-1500 Hz filtering rarefaction polarity. Averages of 1500 click stimuli were received. The test was started with 70 dB SPL, the threshold was obtained by reducing the SPL in 10 dB steps, then the fine tuning level in 5 dB steps up and down to identify the ABR pattern could be recognized. The hearing level at which all waves disappeared was taken as the hearing threshold.

Transmission electron microscopy (TEM)
After detection in 2% glutaraldehyde at +4 [deg.] C. overnight, the cochleas were decalcified with EDTA solution, the second fixation was carried out for 60 minutes in 1% osmium tetroxide. Afterwards, the tissues were buried into plastic for routine electron microscope examination and it was expected to polymerize the material for 2 days. Semi-thin sections were taken from plastic blocks. Findings obtained in the semi-thin section with methylene blue - azur Z were also included in the study. After obtaining the desired region from the semi-thin sections, it was trimmed and thin sections were taken. Thin sections were stained with lead citrate and uranyl acetate and then examined by TEM and images were taken.

STATISTICAL ANALYSIS

Statistical evaluations were performed in SPSS for Windows Version 16.0. The Kolmogorov-Smirnov Test was used to show that the numerical values in all groups were normally distributed. The Kruskal-Wallis test was used to show that there was no significant difference between the right-left ears of all rats. Mean SNR and mean ABR thresholds for all frequencies were analyzed by Paired T-Test before and after the study. The differences in mean SNR and mean ABR thresholds across all frequencies were compared between the groups with Oneway ANOVA Test. Post hoc test was used in multiple comparisons. $P < 0.05$ was used as the statistical significance criterion.

RESULTS

DPOAE findings
The DPOAE test was applied to all rats in the study at 1 kHz, 1.4 kHz, 2 kHz, 2.8 kHz, 4 kHz, 6 kHz and 8 kHz

Table 2: DPOAE results of group I(cisplatin) and group II (cisplatin+isotretinoin).

SNR (before)	Group I-Group II SNR Mean Difference	Std. Error Mean	P	95% Confidence Interval of The Difference	
				Lower	Upper
1kHz	-1,87143	1,08911	0,211	-4,5248	0,782
1,4kHz	2,94286	1,32502	0,08	-0,2853	6,171
2kHz	6,45	1,65253	<0,01*	2,4239	10,4761
2,8kHz	8,11429	2,20021	<0,01*	2,7539	13,4747
4kHz	10,76429	1,86196	<0,01*	6,228	15,3006
6kHz	5,08571	2,0835	0,049**	0,0097	10,1618
8kHz	6,05	2,33734	0,035**	0,3555	11,7445
SNR (after)	Group I-Group II SNR Mean Difference	Std. Error Mean	P	95% Confidence Interval of The Difference	
				Lower	Upper
1kHz	-1,82857	1,14342	0,258	-4,6143	0,9571
1,4kHz	2,25714	1,30449	0,207	-0,921	5,4353
2kHz	5,07857	1,34591	<0,01*	1,7995	8,3576
2,8kHz	4,22857	1,79536	0,06	-0,1455	8,6026
4kHz	-0,75714	2,24209	0,939	-6,2196	4,7053
6kHz	-15,1714	3,00042	<0,01*	-22,4814	-7,8615
8kHz	-8,32857	1,85271	<0,01*	-12,8424	-3,8148

Table 3: DPOAE results of group III(control) and group I (cisplatin).

SNR (before)	Group III-Group I SNR Mean Difference	Std. Error Mean	P	95% Confidence Interval of The Difference	
				Lower	Upper
1kHz	-1,37143	1,15158	0,635	-4,4278	1,685
1,4kHz	-3,62143	1,11535	0,011**	-6,5817	-0,6612
2kHz	-4,12143	1,84228	0,127	-9,011	0,7682
2,8kHz	-3,56429	2,42848	0,464	-10,0097	2,8811
4kHz	-3,67143	2,59263	0,495	-10,5525	3,2097
6kHz	-2,36429	2,795	0,832	-9,7825	5,0539
8kHz	-6,15	2,83492	0,145	-13,6742	1,3742
SNR (after)	Group III-Group I SNR Mean Difference	Std. Error Mean	P	95% Confidence Interval of The Difference	
				Lower	Upper
1kHz	2,93571	1,42249	0,179	-0,8397	6,7111
1,4kHz	0,35714	1,33137	0,993	-3,1764	3,8907
2kHz	-2,08571	1,73032	0,626	-6,6781	2,5067
2,8kHz	1,63571	2,18202	0,876	-4,1556	7,427
4kHz	11,05714	2,83897	<0,01*	3,5222	18,592
6kHz	21,52857	3,555	<0,01*	12,0933	30,9639
8kHz	8,11429	2,09399	<0,01*	2,5566	13,6719

* P< 0,01, ** P< 0,05. SNR: Signal Noise Ratio.

frequencies before and after the study (after 3 days of observation). The measurements were made bilaterally on all rats in three groups and the number of ears was used instead of the number of subjects in the sampling. SNR (signal noise ratios) were used for statistical analysis.

Mean SNR and standard deviations before and after cisplatin administration for each of the three groups are given in table 1 for each frequency. Also, Before cisplatin administration; Mean SNR were compared between different groups. It was observed that there was no significant difference between group I and II at 1 and 1.4 kHz. At other frequencies, mean SNR were higher in Group I than in Group II (table 2). Group III and Group I were compared (table 3). In Group I, mean SNR was found higher than in control group at 1.4 kHz. There was no statistically significant difference in mean SNR between Group I and Group III at other frequencies.

After cisplatin administration

Mean SNR were compared between different groups. It was observed that mean SNR were higher in Group II than in Group I at 6 and 8 kHz. And there was no difference between them in 1 kHz, 1,4 kHz, 2,8 kHz and 4 kHz (table 2). Mean SNR at 4, 6 and 8 kHz were higher in group III than in group I. There was no statistically significant difference in other frequencies (table 3).

ABR findings

Mean ABR thresholds of all groups before and after cisplatin administration are shown in table 4. There was

no statistically significant difference between the thresholds of the three groups before the study.

After the study, cisplatin+isotretinoin group's (Group II) ABR thresholds were lower and that was statistically significant.

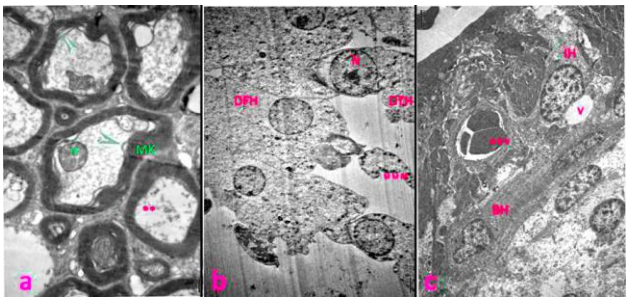

Fig. 1: (a) This electron micrograph shows cochlear section of a rat in Group I (cisplatin). Myelinated axon sections of the cochlear nerve, myelin sheath (MK) and mitochondria (M) in the axon cytoplasm are seen. Lamellar separation of the myelin sheath towards the axon cytoplasm (→) and loss of neurofilament in the cytoplasm (*). (uranyl acetate- lead citrate, original magnification ×20.000). (b) This electron micrograph shows cochlear section of a rat in Group II (cisplatin+isotretinoin). It is seen that outer hair cells (DTH) and outer phalangial cells which supports them (DFH). Normal mitochondrion (M) and euchromatic nuclei (N) of cells are seen in outer hair cells (uranyl acetate- lead citrate, original magnification ×5000). (c) This electron micrograph shows another cochlear section of a rat in Group II(cisplatin+isotretinoin). Stria vascularis is seen. Intraepithelial capillaries (*), intermediate cells (IH), basal cells (BH) are seen. Vacuol (V) is seen in the intermedate cell (uranyl acetate- lead citrate, original magnification ×6000).

Transmission electron microscopy (TEM) findings

Fig. 1 shows electron micrographs of randomized rats in group I and II. In our study, the scoring system of Karaer *et al*. (Karaer, 2015) which was used for the level of inner ear damage was modified and used.

According to this, the sections obtained from 6 rats for each group were included in the scoring. table 5 summarizes the scores of all groups according to this scoring.

DISCUSSION

Major side effects of cisplatin are nausea, vomiting, nephrotoxicity, neurotoxicity and ototoxicity. Hearing loss due to cisplatin treatment usually occurs bilaterally, persistently, accompanied by tinnitus, which also affects low frequencies as well as continuation of treatment. According to McKeage *et al*., 75% to 100% of patients getting cisplatin treatment have elevated hearing threshold levels (McKeage, 1995).

Ototoxic side effects are closely related to the duration and dose of treatment and are influenced by certain risk factors that can be determined. Dietary and nutritional status may be risk factors that affect ototoxicity for both cisplatin and aminoglycosides (Garetz, 1994). There are also studies supporting the development of more severe hearing loss in patients with hypoalbuminemia and anemia (Blakley, 1997). Cumulative dose of cisplatin in ototoxicity is important. In rat models of cisplatin-induced ototoxicity, two or three doses were administered or single dose. In a cisplatin ototoxicity study performed by Paksoy *et al*., single dose of 20 mg/kg cisplatin intraperitoneally was administered to Wistar Albino rats and a significant increase in hearing thresholds was observed (Paksoy, 2011).

According to Demir *et al*., Wistar Albino rats were given a single intraperitoneal dose of 12 mg / kg cisplatin and a significant increase in hearing thresholds was observed (Demir, 2015). In our study, a single dose of cisplatin 10 mg/kg intraperitoneally was administered to achieve ototoxic effect with a minimum dose of the drug and to reduce the loss of rats so we did not observe any animal loss. In group I, SNR before and after cisplatin administration were compared, it was observed that there were statistically significant decreases in all frequencies after the procedure. After administration, control group's mean SNR were compared to the cisplatin-only group (group I) and lower in the cisplatin group at 4, 6 and 8 kHz. There was no difference between the control group and the cisplatin group in terms of mean ABR threshold before the procedure. In the cisplatin group ABR thresholds were statistically higher than control group. Both DPOAE and ABR results were associated with cisplatin ototoxicity. According to our DPOAE findings, this ototoxicity appears at 4, 6 and 8 kHz, which seems to be consistent with cisplatin ototoxicity, which affects higher frequencies more.

Many studies on cisplatin and aminoglycoside ototoxicity have focused on the initiation of necrosis and apoptosis through the formation of reactive oxygen metabolites and their elevated levels in the cochlea. For this reason, various antioxidants have been evaluated for their protective efficacy against cisplatin and aminoglycoside ototoxicity. It is known that carotenoids have antioxidant activity and have been used in some clinical situations for this purpose. In the literature, there are very limited study of the use of vitamin A and carotenoids on prevention of ototoxicity. According to Le Prell *et al*., use of vitamins A, C, E and magnesium is protective against noise-induced neurodegenerative cell death (Le Prell, 2007a). According to Le Prell *et al*., vitamin A is a cleaner of reactive oxygen species and vitamin C is a free radical detoxifier (Le Prell, 2007b). Vitamin E has a preventive

Table 4: Mean ABR thresholds of all groups and mean differences before and after the procedure.

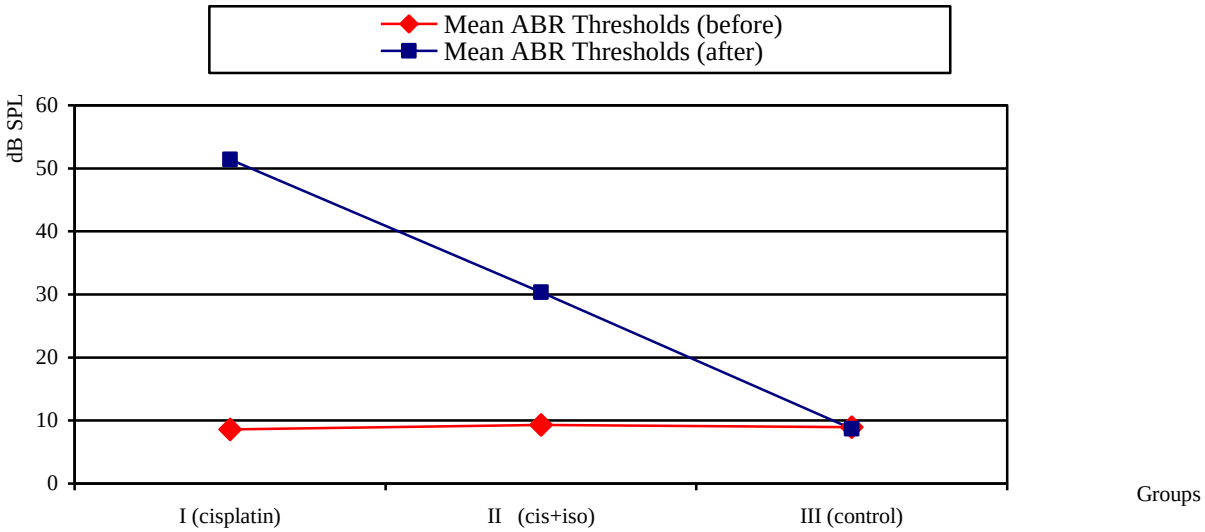

Table 5: Scoring of degenerative changes in the spiral ganglion, stria vascularis and organ of corti in all groups.

	Control	I (cisplatin)	II (cisplatin+isotretinoin)
Edema in the spiral ganglion	0	2	1
Epithelial degeneration in the stria vascularis	1	2	1
Edema in the stria vascularis	0	2	1
Degenerative changes in the organ of corti	1	3	2

0: no degeneration; 1: mild degeneration; 2: moderate degeneration; 3: severe degeneration

role for lipid peroxidation. These studies also show that antioxidant drugs are synergistic effects of combined use in exposure to noise. Based on these informations, we studied the protective activity of isotretinoin, a synthetic vitamin A derivative, against cisplatin-induced ototoxicity. According to our ABR and DPOAE findings, isotretinoin has protective effect on cisplatin-induced ototoxicity. According to our DPOAE findings, this protective effect has seen especially at the high frequencies that cisplatin affects.

In our study, according to TEM findings, "*severe degeneration*" was observed only in the cisplatin group (group I) whereas "*moderate degeneration*" was observed in the cisplatin + isotretinoin group (group II) in the scoring of the degenerative changes in the organ of corti. These findings suggest that isotretinoin has protective effects on cell integrity in the outer hair, inner hair and supporting cells.

The targets of drugs are not just hair cells. Aminoglycosides thinning the stria vascularis and decrease marginal cell count (Ruedi, 1952; Hawkins, 1973). Degeneration of spiral ganglion cells after aminoglycoside therapy was linked to the loss of hair cells that was innervated by the ganglion (Hawkins, 1976; Johnsson, 1981) However, due to the pattern and complexity of the damage, it has been suggested in some studies that spiral ganglion may be affected without hair cell damage (Hinojosa, 1987, Sone, 1998). Damage after cisplatin treatment also begins in hair cells and after stria vascularis and spiral ganglion have also been effected (Ravi, 1995; Klis, 2000; Tsukasaki, 2000).

In our study, the scoring of TEM findings; edema of the spiral ganglion, epithelial degeneration of the stria vasculature, and edema of the stria vascularis were observed at the "moderate" level in the cisplatin group, and "mild" in the isotretinoin+cisplatin group. These findings suggest that isotretinoin may have protective effect against ototoxic effects of cisplatin in stria vascularis and spiral ganglion.The most sensitive part of organ of corti in cisplatin ototoxicity is the outer hair cells and damage progresses from baseline to apex (Hawkins, 1976). This cause a hearing loss, in which low frequencies are also affected primarily. Cell death begins with the first row of outer hair cells and then the second and third rows are affected. Inner hair cells are very resistant to damage, and usually damage in the inner hair cells begins after all of the outer hair cells have been damaged.

Currently, antioxidant therapies are being updated and popular in the prevention of ototoxicity. There is a limited number of studies of isotretinoin which we have shown protective effects in this study. New studies and clinical

experiences on the protective effect of isotretinoin or other vitamin A derivatives against ototoxicity are necessary. It is not known that how isotretinoin -given as a protective- affects the chemotherapeutic effect of cisplatin. This is the most important limitation of our study. We think, there is a need for new studies on this and on the administration time of isotretinoin.

CONCLUSION

As a result, in this study the protective effects of isotretinoin on cisplatin-induced ototoxicity has been shown by auditory brainstem responses, otoacoustic emissions and transmission electron microscopy. We think that new experimental and clinical studies to be carried out in this issue may give us a new option to prevent cochlea from ototoxicity.

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