Protective Role of Bilberry Extract Against Cisplatin Induced Ototoxicity in Rats

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Abstract To investigate the potential preventive effect of bilberry extract in cisplatin-induced ototoxicity. Thirty-five 3–3.5-month healthy adult female Sprague–Dawley rats were randomly divided into three groups and treated as follows: Both, group 1 ($n = 10$) and group 2 ($n = 15$) subjects received a single dose of 12 mg/kg cisplatin intraperitoneally; while in group 2, bilberry extract was also administered via gavage feeding for 15 days. Group 3 ($n = 10$), received no cisplatin or bilberry extract. Baseline distortion product otoacoustic emissions testing were performed in all subjects prior to administration of any medication. The test was repeated at 15th day following administration of any medication. The distortion product otoacoustic emissions were evaluated at 1.5, 2, 3, 4, 5, 6, 7, 8, 10 and 12 kHz. Histopathological changes in the cochlea of rats were observed by light microscopy. There was no statistically significant difference in apical turn between three groups but there was a statistically significant difference in basal and mid turn external ciliated cells number. Stria vascularis changes were statistically significant between three groups. The median score for stria vascularis injury and spiral ganglion cells changes were significantly greater in group 1 than in group 2. The initial distortion product otoacoustic emissions measurement results gave similar statistically insignificant values in the three groups ($p > 0.05$). In contrast to initial measurements statistically

significant differences were recorded between day 0 and 15 otoacoustic thresholds ($p < 0.05$). Bilberry extract group had a significantly higher DP-gram except for 1.5 and 2 kHz frequencies when compared to cisplatin group. The analyses of the results revealed statistically significant differences between two groups ($p < 0.05$), suggesting that bilberry extract had shown a protective effect against cisplatin ototoxicity. The results of our study revealed that treatment with bilberry extract affords significant protection to the cochlea from cisplatin toxicity and thus, oral experimental dose of bilberry extract administration may have a protective effect against cisplatin ototoxicity in rats.

Keywords Cisplatin · Ototoxicity · Bilberry extract

Introduction

Cis-diamminechloroplatinum (cisplatin) is a chemotherapeutic agent frequently used for the treatment of many cancers. Its dose-limiting side effects are nephrotoxicity, ototoxicity and neurotoxicity [1, 2]. While nephrotoxicity can be prevented by increased hydration and forced diuresis, there is still no cure or preventive treatment for ototoxicity. Reports indicate that an elevation in hearing thresholds can occur in up to 75–100 % of patients. The rate of this adverse effect is even higher in children [2, 3].

The cellular and molecular mechanisms of cisplatin-induced ototoxicity are not well understood. It has been postulated that cisplatin destroys primarily the cochlea especially outer hair cells, leading to excessive production of free oxygen radicals in the organ of Corti, stria vascularis, spiral ligament, and spiral ganglionic cells [4, 5]. The main cytotoxic effects of cisplatin are believed to be mediated by the monohydrated cisplatin complex, which

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reacts with nuclear DNA, forming platinum–DNA adducts [6]. The cytotoxicity includes the generation of reactive oxygen species, and therefore, antioxidants have been in focus in the search for protection against cisplatin-induced ototoxicity for many years [7]. To date, there is no FDA-approved product that has demonstrated efficacy in preventing or reducing cisplatin ototoxicity [8].

Berry fruits, such as blueberries, bilberries, or blackcurrants, contain large amounts of anthocyanins, making them one of the major sources for dietary anthocyanin intake [9, 10]. Bilberries (*Vaccinium myrtillus*) are widely consumed as fresh and dried fruits, jams, or juices in diets. Bilberry extract (BE) has shown to have a wide spectrum of physiological effects, such as vision improvement, anticancer, anti-inflammatory activities, neuroprotective effects in rats but effects against ototoxicity is unknown [11–15].

This study is the first demonstration of bilberry extract's attenuating effects against cisplatin ototoxicity in rat models as shown by distortion product otoacoustic emissions (DPOAEs).

Materials and Methods

Animals and Study Design

The study was approved by the Institutional Ethics Committee of Animal Experiments. Thirty-five 3–3.5-month healthy adult female Sprague–Dawley rats (weight 220–280 g) with normal tympanic membranes were used in this study. Rats were individually housed in wire-bottomed cages in a temperature-controlled room ($21^{\circ}\text{C} \pm 2$) with a 12-h light–dark cycle and a relative humidity of $55 \pm 10\%$ and had free access to food and water. Institutional and governmental guidelines for the care and use of laboratory animals were followed. Animals with signs of external and middle ear disorders or animals without DPOAEs in any of the frequencies investigated, before the experiment were all taken off the study. Rats were randomly divided into three study groups and treated as follows: Both, group 1 ($n = 10$) and group 2 ($n = 15$) subjects received a single dose of 12 mg/kg cisplatin intraperitoneally; while in group 2, BE was also administered via gavage feeding for 15 days. Group 3 ($n = 10$) received single intraperitoneal injection of 1 ml of saline solution and served as a control group. Group 3, received no cisplatin or BE. The BE (standardized bilberry ethanolic extract containing 36 % anthocyanins, supplied by Indena, Milan, Italy.) which was dissolved in saline solution before use and orally administered to animals at 0.1 mL/10 g body weight for 15 days at dosage of 200 mg/kg following cisplatin injection. Oral saline delivered by a similar route in control group.

The doses of cisplatin and BE were selected based on previously published data [3, 13].

In study groups, baseline DPOAE testing preceded the administration of the drugs. The subjects which treated with cisplatin plus saline and cisplatin plus BE were tested 15 days after the beginning of the experiment. Animals were anesthetized with 40 mg/kg of ketamine hydrochloride (Ketalar, Eczacıbasi Ilac Sanayi ve Ticaret A.Ş., Luleburgaz, Turkey) and 5 mg/kg of xylazine (Alfazyne 2 %, Alfasan International B.V., Woerden, The Netherlands) cocktail during DPOAE measurement.

Distortion Product-Evoked Otoacoustic Emission Measurements

Only rats with normal replicable DPOAEs before the administration of any substance on day 0 were included in this study. DPOAEs recordings were performed in a quiet room. The DPOAEs were elicited from the right ear as controls. A standard commercial cochlear emission analyzer (MI 34 OAE apparatus MAICON Ltd., Berlin, Germany) was utilized for testing procedure. An infant size screening probe was attached to the external auditory canal. The stimulus consisted of two pure tones (F1 and F2; F1/F2 ratio = 1.22) at 70 dB SPL. In total, 1,000 acquisitions were analyzed. The resulting otoacoustic emissions were evaluated at 1.5, 2, 3, 4, 5, 6, 7, 8, 10 and 12 kHz. DPOAE testing was considered positive for signal-to-noise ratios of 6 dB SPL, as specified by the manufacturer.

Histopathological Analysis

All animals were sacrificed by decapitation under deep anesthesia after the last DPOEA test (at 15th day) to observe damage. The temporal bones were immediately removed under a dissecting microscope. The round and oval windows and the bone near the apex were opened, followed by gentle local perfusion from the apex. Samples were fixed with 10 % buffered formaldehyde for 3 days, and decalcification was performed using 10 % EDTA 2Na (pH 7.4). After 3 h, distilled water was used to wash away the decalcification solution, and the tissue was fixed for 2 more days in formaldehyde. An automated tissue tracking device was used for routine processing 13 h post-fixation with two containers of xylene, two containers of formaldehyde, four containers of alcohol, and two vessels in paraffin. After washing with PBS, specimens were processed to form paraffin blocks. Serial longitudinal sections passing parallel to the modiolus were cut at sections of 5 mm thickness and subjected to haematoxylin and eosin stain the organ of Corti was dissected. The tissues were observed under fluorescence microscopy (Olympus CX 31) at X400 magnification and photographed using an Olympus

E330 digital camera by a pathologist (MK) blinded to the study groups. Missing inner and outer hair cells were determined in 0.19 mm sections from apex to base.

A four-point scoring system described by Freitas et al. [16] was used to grade for cisplatin induced injury. Scores were based on an assessment of external ciliated cells (ECC) number for the organ of Corti. Basal, mid, and apical cells were counted and expected number of hair cells for each section was determined by using the control group. The mid-modiolar sections of two basal and middle turns and one apical turn were evaluated and 15 ECCs were counted (basal turns 6 cells, middle turns 6 cells, and apical turn 3 cells). Based on stria vascularis assessment, which was subjective and included marginal cell blebbing, cytoplasmic vacuolization, and atrophy of intermediate cells (shrinkage), tissues were categorized as follows: normal thickness of the stria vascularis and no marginal cell blebbing, cytoplasmic vacuolization, or shrinkage 0, slight 1, mild 2, moderate 3, and severe 4. Spiral ganglion cells, vacuolization, and nuclear degeneration were also evaluated, subjectively, and categorized based on the severity of the changes (i.e., no change 0, mild 1, moderate 2, and severe changes 3).

Statistical Analyses

The statistical analysis was performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). All data are expressed as mean $\pm$ SD. The Wilcoxon test was used to compare data with a normal distribution in the experimental groups, and the Kruskal–Wallis and Mann–Whitney *U* tests were used to compare differences between experimental groups. Within group comparisons of parameters were made using the Wilcoxon sign test. In all tests, a *p* value of less than 0.05 was considered statistically significant.

Results

Functional Hearing Evaluation

The DP-gram results of all the groups before and after the drug administration are presented in Table 1. On day 0, prior to any medications, the initial DPOAEs measurement results gave similar values in the three groups ($p > 0.05$). In all groups statistically significant differences were recorded for all frequencies between day 0 and 15 ($p < 0.05$) measurements (Table 1). BE group had a significantly higher DP-grams except for 1.5 and 2 kHz frequencies when compared with the cisplatin group ($p < 0.05$). The analyses of the results as the median amplitudes of DP-grams revealed statistically significant differences between two groups ($p < 0.05$), suggesting that BE had a protective effect against cisplatin ototoxicity at 15th day (Fig. 1).

Histopathological Evaluation Results

A four-point scoring system described by Freitas et al. [16] was used to grade degeneration and injury to the ECC, as indicated by the number of cells absent from the basal turn of the cochlear duct (0: presence of 3 ECCs with intact nuclei; 1: cochlea with injury to one ECC; 2: cochlea with injury to two ECCs; 3: cochlea with injury to three ECCs). ECCs cells were separated according to basal, mid, and apical turns (basal turn ECC, BECC; mid turn ECC, MECC; and apical turn, AECC, respectively), counted, and compared between three groups (Fig. 2).

There was statistically significant difference in BECC numbers between three groups ($p < 0.05$). The BECC numbers in the control group were significantly higher ($p < 0.05$) than the cisplatin and BE groups ($p < 0.05$). BECC numbers in the BE group were significantly higher

Table 1 Distortion product-evoked otoacoustic emission amplitudes of study groups

kHz	Cisplatin group			Bilberry extract group			Control group			<i>p</i> values
	0 day	15 day	<i>p</i>	0 day	15 day	<i>p</i>	0 day	15 day	<i>p</i>	
1.5	13.2 $\pm$ 1.13	8.9 $\pm$ 1.22*	0.000	13.5 $\pm$ 1.83	9.5 $\pm$ 1.29*	0.002	13.5 $\pm$ 1.26	13.8 $\pm$ 1.59	0.158	0.545*
2	13.6 $\pm$ 1.25	9.1 $\pm$ 1.31*	0.000	14.2 $\pm$ 1.37	9.3 $\pm$ 1.17*	0.000	14.3 $\pm$ 1.36	14.1 $\pm$ 1.72	0.176	0.423*
3	14.8 $\pm$ 1.58	9.6 $\pm$ 1.64	0.000	15.1 $\pm$ 1.87	13.1 $\pm$ 1.47	0.000	15.7 $\pm$ 1.35	15.9 $\pm$ 1.72	0.430	<0.001
4	16.2 $\pm$ 1.37	11.3 $\pm$ 1.41	0.000	16.5 $\pm$ 1.84	12.2 $\pm$ 1.62	0.000	17.2 $\pm$ 1.38	17.3 $\pm$ 1.31	0.257	<0.001
5	25.2 $\pm$ 2.54	12.5 $\pm$ 2.42	0.000	24.8 $\pm$ 3.04	15.7 $\pm$ 2.17	0.000	26.1 $\pm$ 2.49	26.2 $\pm$ 2.45	0.241	<0.001
6	28.2 $\pm$ 2.43	11.9 $\pm$ 2.41	0.001	29.1 $\pm$ 2.01	15.4 $\pm$ 1.73	0.000	29.0 $\pm$ 3.54	29.1 $\pm$ 2.05	0.293	<0.001
7	30.6 $\pm$ 1.91	23.9 $\pm$ 1.89	0.000	31.2 $\pm$ 1.97	26.7 $\pm$ 2.05	0.000	31.2 $\pm$ 1.71	31.3 $\pm$ 1.74	0.298	<0.001
8	32.4 $\pm$ 2.62	24.7 $\pm$ 2.17	0.000	31.9 $\pm$ 1.93	27.4 $\pm$ 2.24	0.000	31.1 $\pm$ 2.46	30.7 $\pm$ 2.51	0.192	<0.001
10	31.5 $\pm$ 1.92	23.9 $\pm$ 1.39	0.000	31.9 $\pm$ 2.52	25.8 $\pm$ 1.28	0.000	31.5 $\pm$ 1.97	31.4 $\pm$ 2.31	0.156	<0.001
12	33.1 $\pm$ 2.11	24.2 $\pm$ 1.53	0.000	31.7 $\pm$ 1.83	26.5 $\pm$ 1.57	0.000	32.7 $\pm$ 2.17	32.3 $\pm$ 2.15	0.278	<0.001

* Bilberry extract group had a significantly higher DP-grams except for 1.5 and 2 kHz frequency when compared with Cisplatin group

Fig. 1 Comparison of distortion product-evoked otoacoustic emission measurements amplitudes for study groups at 15th day

than cisplatin group ($p < 0.05$). There was statistically significant difference in MECC numbers between three groups ($p < 0.05$). MECC numbers of the control group were significantly higher compared with the other two groups ($p < 0.05$). BECC numbers in the BE group were significantly higher than cisplatin group ($p < 0.05$). The difference in AECC numbers were not statistically significant between three groups ($p > 0.05$) (Fig. 3).

Damage in Stria Vascularis

We used a four-score system to grade stria vascularis injury. Scores indicated the degree of blebbing in marginal cells, cytoplasmic vacuolization, and atrophy of intermediate cells (shrinkage, 0: normal thickness of the stria vascularis and no marginal cell blebbing, cytoplasmic vacuolization, or shrinkage, 1: slight, 2: mild, 3: moderate, and 4: severe) (Fig. 4). There was statistically significant difference in changes of stria vascularis between three

Fig. 3 Comparison of external ciliated cells numbers in study groups

groups ($p < 0.05$). The median score for stria vascularis injury was significantly lowest in control group ($p < 0.05$).

Damage in Spiral Ganglion Cells

Vacuolization, and nuclear degeneration in spiral ganglion cells were evaluated subjectively according to the severity of the changes (0: no change, 1: mild, 2: moderate, 3: severe changes) (Fig. 5). There was statistically significant difference in spiral ganglion cells changes between three groups ($p < 0.05$). The differences in control group were significantly lower compared with the BE and cisplatin group ($p < 0.05$). The median change scores for spiral ganglion cells were significantly higher in cisplatin group than in BE group ($p < 0.05$).

Discussion

Cisplatin, containing the heavy metal platinum, is an effective therapeutic agent for a number of human cancers, including carcinoma of the head and neck, testicle, ovary, bladder, uterus, bone and lung [1]. Cisplatin administration may lead to hearing problems due to functional and morphological changes in the cochlea. Cisplatin ototoxicity

Fig. 2 Fluorescence microscopy sections of external ciliated cells of study groups crosssectional area through the basal turn from **a** control group, **b** bilberry group, **c** cisplatin group. Greatest epithelial degeneration was observed in cisplatin group (hematoxylin & eosin, $\times 400$)

Fig. 4 Cross-sectional area of stria vascularis in **a** control group, **b** bilberry group showing stria vascularis slightly thickened, marginal cell blebbing and cytoplasmic vacuolization, **c** cisplatin group

showing highly fragmented irregular cytoplasm and nuclei-degenerating cells (hematoxylin & eosin, $\times 400$)

Fig. 5 **a** Normal appearance of spiral ganglion cells in *control group*, **b** vacuolization and nuclear degeneration are seen in bilberry group, **c** showing eosinophilic cytoplasm of spiral ganglion cells; spiral

ganglion cells nuclei are not seen even in areas that can be viewed with the severe degeneration in cisplatin group (hematoxylin & eosin, $\times 400$)

may be persistent or temporary. The age of the patient, the dosage of cisplatin, chemotherapy cycle, acoustic trauma history and renal insufficiency are important factors in development of cisplatin ototoxicity [1, 2].

Mechanisms of cisplatin ototoxicity are not fully understood, but reactive oxygen species have been implicated in its pathogenesis. Reactive oxygen species reacts with cell membrane lipids to produce toxic aldehydes such as 4-hydroxynonenal. These are implicated in apoptosis and cell death in the organ of Corti explants and spiral ganglion cell cultures [2, 17]. In rats with documented cisplatin ototoxicity, loss of inner and outer hair cells, degeneration of the stria vascularis, and significant decrease in the number of spiral ganglion cells have been reported [18]. Morphologically, cisplatin targets three important tissues in the cochlea, including organ of Corti, spiral ganglion, and stria vascularis which has been well documented in experimental animal models [19]. Shredding of the myelin sheaths has been observed in spiral

ganglion cells. Apoptosis, indicating DNA damage, has been demonstrated in the outer hair cells and basal fold spiral ganglion cells in the organ of Corti of gerbil cochleas by staining methods [20].

Otoacoustic emissions are the voices that are produced spontaneously or by a stimulus in the normal cochlea. David Kemp discovered that the inner ear produce mechanical vibration energy (otoacoustic emission) [21].

Protection against cisplatin's toxic effects on cellular structures may be provided by endogenous molecules, or by sole or combined exogenous agents, that trigger endogenous protective mechanisms [22]. Recently, considerable attention has focused on dietary exogenous constituents that may be beneficial for the prevention and treatment of cisplatin ototoxicity [22]. Thus, antioxidant therapy may serve as a promising treatment approach for inhibiting the progression of oxidative stress-associated diseases. There is a growing interest in plant-based dietary antioxidants to counteract with oxidative stress-induced

diseases. Anthocyanins are common components of some fruits and vegetables, in particular edible berries, which provide pigmentation and serve as natural antioxidants [23, 24]. Principal therapeutic benefits attributable to anthocyanins include antioxidant protection and maintenance of DNA integrity. Anthocyanins also serve as anti-inflammatory and anti-mutagenic agents and provide cardioprotection by maintaining vascular permeability [10, 11]. Bilberry (*V. myrtillus* L.), commonly referred to as the European blueberry, is one of the richest sources of anthocyanins, with an anthocyanin glycoside content of 300–600 mg/100 g of fresh weight [25]. Dietary intake of anthocyanin rich foods has been associated with a reduced risk of coronary heart disease in the Iowa Women's Health Study, a prospective study of postmenopausal women [26]. A protective effect of BE on ototoxicity is unknown.

In this study, statistically significant reductions in DP-gram amplitudes were noted at all frequencies in the cisplatin group, strongly suggesting cisplatin-related ototoxicity. In the BE and cisplatin groups, a statistically significant difference was found between days 0 and 15 measurements except at 1.5 and 2 kHz.

To our knowledge, this is the first study to investigate the protective effects of BE against cisplatin-induced ototoxicity in animals. Based on the data in this study, it is our opinion that BE might have played a protective role against cisplatin-induced ototoxicity in rats as shown by means of DPOAEs. Finally, further studies to determine the appropriate indications and dosages of BE before its clinical use against cisplatin induced ototoxicity is warranted.

Conflict of interest None.

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