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ORIGINAL ARTICLE

The Protective Role of Molsidomine on the Cisplatin-Induced Ototoxicity

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Abstract This experimental study was designed to investigate the protective effects of molsidomine (MOL) on against cisplatin-induced ototoxicity (CIO). To examine this effect, distortion product otoacoustic emissions (DPOAEs) measurements and serum levels of oxidative and antioxidant status [including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPX), total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI)] were evaluated. Thirty-two female wistar albino rats were divided into four groups including; control (Group K), cisplatin (Group C), cisplatin plus MOL group (Group CM), and MOL group (Group M). DPOAEs measurements between 0.9961 and 8.0003 Hz as DP-gram and input/output (I/O) functions were performed in the same (left) ear of all rats on days 0, 1st, 5th and 12th. Prior to death, the last DPOAEs measurements and blood samples were taken. In the C group, statistically significant DPOAE amplitude reductions were detected at 2.5195, 3.1758, 3.9961, 5.0391, 6.3516 and 8.0039 Hz frequencies ($p < 0.05$) between 0th and 1st, 0th and 5th and 0th and 12th days' measurements ($p < 0.05$). Serum level of MDA, TAC and OSI levels were significantly higher in the C group versus K group ($p < 0.05$). In the CM group, there were no significant differences at all frequencies between 0th and other days' measurements ($p > 0.05$) and the serum levels of all biochemical parameters were shifted toward normal values, similar to the K group ($p < 0.05$). No significant differences were detected in the either M or K group's measurements. According to these results, cisplatin-related ototoxicity has been significantly prevented by MOL.

Keywords Cisplatin · Ototoxicity · Molsidomine · Otoacoustic emissions · Oxidative status

Introduction

Cisplatin is a potent chemotherapeutic drug that commonly used to treat many types of cancer including head and neck, breast, lung, bladder, testis, over cancer and hematologic tumors [1]. Although cisplatin treatment is highly effective, significant adverse effects appear such as vomiting, nausea, bone marrow depression, nephrotoxicity, hepatotoxicity, and ototoxicity [2].

The mechanisms of cisplatin-induced ototoxicity (CIO) are not well understood. The suggested mechanism of ototoxicity is the production of reactive oxygen species (ROS) that main cause of cell injury or death [2, 3]. Cisplatin administration also decreases antioxidant enzymes in the cochlea [4]. When the balance between the production of ROS and antioxidative defense is damaged oxidative stress can occur which can lead to cochlear cell injury or

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death. The use of antioxidant agents may prevent these effects of ROS [5].

Many antioxidant agents have been studied to prevent CIO [6–10]. MOL is a prodrug, belonging to the class of sydnonimines, has been used widely as an antianginal agent as a vasodilator. MOL is transformed into the metabolite 3-morpholinosydnonimine (SIN-1), which spontaneously liberates nitric oxide (NO). Apart from its anti-anginal effects, it has been more recently reported that MOL exerts potent antioxidant, antiapoptotic and anti-inflammatory effects [11–13]. Therefore, MOL treatment can exert beneficial effects against CIO which regarding with oxidative stress and production of excessive reactive radicals.

In the literature, there is no any investigation of MOL against CIO. Therefore, this study was designed to explore the possible protective and therapeutic effects of MOL on CIO. To accomplish this purpose DPOAEs measurements and serum levels of oxidative stress parameters malondialdehyde: MDA; total oxidant status: TOS; and oxidative stress index: OSI and antioxidant contents superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), reduced glutathione (GSH), and total antioxidant status (TAS) were studied.

Materials and Methods

Study Design

This study was approved by Animal Ethics Committee (Reference Number: 2013/A-82) and was conducted in accordance with the "Animal Welfare Act and the Guide for the Care and Use of Laboratory Animal (NIH publication No. 5377-3, 1996), Animal Ethics Committee." Thirty-two Wistar Albino rats, female, 3-month-old, weighing 230–280 g obtained from the Laboratory Animal Production Unit were used. Rats were monitored in the laboratory at a constant temperature (22 ± 2 °C) and humidity (50 ± 5 %) in a controlled room for acclimatization with exposure to 12 h of light and 12 h of darkness. Rats were fed with tap water and standard rodent feed ad libitum.

Rats anesthetizing were performed with 50 mg/kg intramuscular (i.m.) ketamine and 5 mg/kg i.m. xylazine. The external ear and tympanic membranes of all the rats were examined carefully. Hearing screening was performed with DPOAEs before the study.

The rats were randomly assigned to 4 groups (n = 32) as follows: (1) K group (n:8) rats applied only intraperitoneal (i.p.) saline; (2) C group (n:8) rats received a single i.p. dose injection of 16 mg/kg cisplatin (Cisplatin DBL, 50 mg, Orna Corp., Istanbul, Turkey); (3) CM group rats (n:8) received single i.p. dose of MOL (4 mg/kg/day) (Molsidomine, Sigma Chemical Co., St Louis, MO, USA) on the day before and on 5 consecutive days, following a single-dose 16 mg/kg i.p. injection of cisplatin; (4) M group rats (n:8) were treated for 6 consecutive days by i.p. with 4 mg/kg/day MOL.

The dosage of cisplatin and MOL were chosen depend on the previous dose–response studies that have been reported to cause ototoxicity and marked antioxidative and anti-inflammatory effects in rats respectively [6, 12].

Following, the rats were brought into a silent cabin (sound pressure level under 45 dB) for the DPOAEs measurements. DPOAE (by using Grason Stadler, Madison, USA) measurements were performed in the same ear of all rats (left ear). Probe calibration was done using an automated measurement system before each test and the measurements were made in the soundless cabin. Primer stimulus levels were equalized at 65 dB (L1 = L2) for measurements. Two different frequencies (f1 and f2) were made at a f2/f1 = 1.22, for taking the most powerful responses. DPOAEs measurements, between 996, and 8,004 (996, 1,078, 1,266, 1,582, 2,004, 2,519, 3,176, 3,996, 5,039, 6,351, and 8,004) Hz. Hz as DP-gram and input/output (I/O) functions, were performed in all groups on days 0, 1st, 5th and 12th. All animals were killed under the general anesthesia, on day 12. Before death, the last otoacoustic emission measurements and blood samples which were taken by vena cava inferior (for serum levels of MDA, SOD, CAT, GPX, GSH, TOS, TAC, and OSI) were obtained.

Biochemical Analyses

After the serum samples homogenization, MDA contents were determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances [14]. The results were expressed in nmol/ml, according to a prepared standard graph.

Total SOD activity was determined according to the method of Sun et al. [15]. The principle of the method is the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. SOD activity was expressed in U/mg protein.

CAT activity was measured by the method of Aebi [16]. The principle of the assay is based on the determination of the rate constant (k, s^{-1}) or the H_2O_2 decomposition rate at 240 nm. Results were expressed as k/g protein.

GPX activity was determined according to a method developed by Paglia and Valentine [17]. An enzymatic reaction in a tube containing NADPH, GSH, sodium azide and glutathione reductase was initiated by adding H_2O_2; the change in absorbance at 340 nm was observed using a

spectrophotometer. Activity was expressed as U/mg protein.

The GSH content in the serum as non-protein sulfhydryls was analyzed following a previously described method [18]. The absorbance values were extrapolated from a glutathione standard curve and expressed as GSH (μmol/L).

Serum TAC levels were determined using a novel automated measurement method, developed by Erel [19]. The results are expressed in μmol Trolox equiv/L.

Serum TOS levels were determined using a novel automated measurement method, developed by [20]. The assay is calibrated H_2O_2 and the results are expressed in μmol H_2O_2 Trolox equiv/L.

Measurement of OSI was calculated by the TOS to TAC ratio [21]. The results are expressed in arbitrary unit.

Statistical Analysis

For detecting even minor effects, the required sample sizes used in this experiment were identified using statistical power analysis. The sample sizes necessary for a power of 0.80 were estimated using NCSS software. SPSS for Windows Version 15 program was used. Data of DP-gram amplitudes were expressed as mean ± SD, assessed within a 95 % reliance and at a level of $p < 0.05$ significance. The Wilcoxon test was used to compare data with a normal distribution in the study groups, and the Kruskal–Wallis and Mann–Whitney *U* tests were used to compare differences between three groups. Within group comparisons of parameters were made using the Wilcoxon sign test. ANOVA and Post-Hoc Tukey tests were used to compare differences among the biochemical parameters. The results are expressed as mean ± SD. *p* values less than 0.05 were regarded as statistically significant.

Results

The DP-gram results of all the groups before and after the drug administrations are presented in Figs. 1, 2, 3 and 4. Briefly, in the control rats there is no any statistically significant difference among the DPOAE measurements performed on 0, 1st, 5th and 12th days ($p > 0.05$).

In the C group, one of the 8 rats died because of enteritis and weight loss that possibly second to cisplatin toxicity on the 10th day. Seven rats were able to complete the study and evaluated for further analyses. Statistically significant DPOAE amplitude reductions were detected at 2.519, 3.176, 3.996, 5.039, 6.352 and 8.004 Hz frequencies ($p < 0.05$) between 0 and 1st, 0 and 5th, 0 and 12th days' measurements ($p < 0.05$) However, there were no statistically significant difference between 1st and 5th, 1st and 12th, 5th and 12th days' measurements ($p > 0.05$).

In the CM group, one rat died because of anesthesia during the measurements of the 5th day, finally seven rats were evaluated. Between DPOAE measurements of C and CM groups, there was statistically significant DPOAE amplitude reductions at 1th, 5th, and 12th days' measurements at 5.0391, 6.3516 and 8.0039 Hz frequencies ($p < 0.05$). When compared to the M and CM groups, there were no statistically significant difference among the DPOAE amplitudes obtained from on day 0, 1, 5 and 12 measurements ($p > 0.05$).

In the M group, similar to K group, DPOAE measurements were performed on 0, 1st, 5th and 12th days, and there was no statistically significant difference at all frequencies ($p > 0.05$).

The serum levels of biochemical parameters are presented in the Table 1. In brief, MDA, TAC and OSI levels were significantly higher in the C group when compared to the K group. Also, serum SOD, CAT, GPX, GSH and TAC contents were significantly reduced in the C group whereas MOL treatment significantly ameliorated all of these parameters. In CM group all biochemical parameters were shifted toward normal values, similar to the K group.

Discussion

Although the pathogenesis of CIO has not been clarified clearly yet, however, it is considered to be due to the formation of ROS that initiates the inflammatory reactions and oxidative processes. These events can cause apoptosis and consequently to a decrease the cochlear cells of the inner ear [2–5].

In vitro studies with laboratory animals show that cisplatin-treatment leads to hearing loss by affecting many regions of the cochlea, including the cochlear outer hair cells, spiral ganglion, and stria vascularis. Cisplatin progressively destroys outer hair cells, from the base to the apex and ototoxicity may occur within hours. Hearing loss appears to be bilateral, usually permanent, dose-related, and initially at higher frequencies [2]. Recently it was reported that an increase in hearing thresholds may occur in up to 75–100 % of patients [22]. Many antioxidant agents have been studied to prevent this adverse effect. In the literature, as soon as our knowledge, there is no any investigation regarding with MOL against CIO. This study was designed to explore the possible protective and therapeutic effects of MOL on CIO.

MOL decarboxylases enzymatically to form 3 morpholinosyndnonimine (SIN-1) which spontaneously release

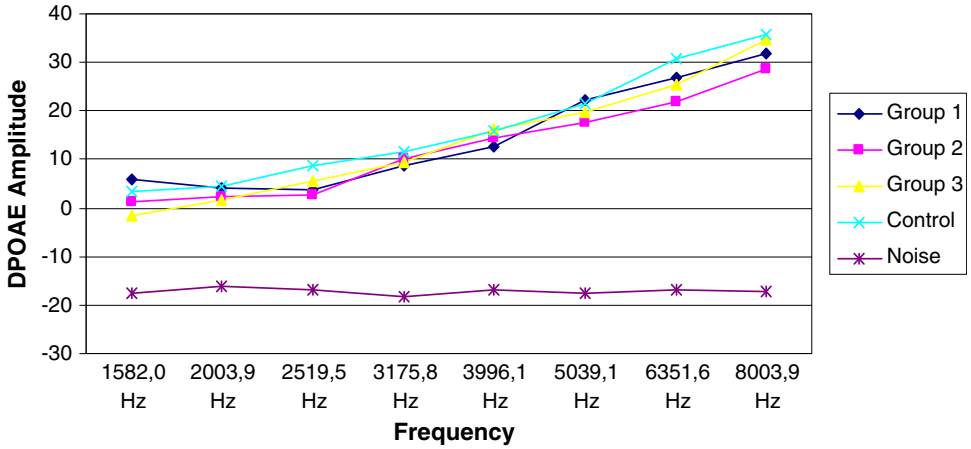

Fig. 1 DPOE amplitudes obtained from on day zero. *Group 1* cisplatin group (C), *Group 2* molsidomin group (M), *Group 3* molsidomin + cisplatin-treated group (CM)

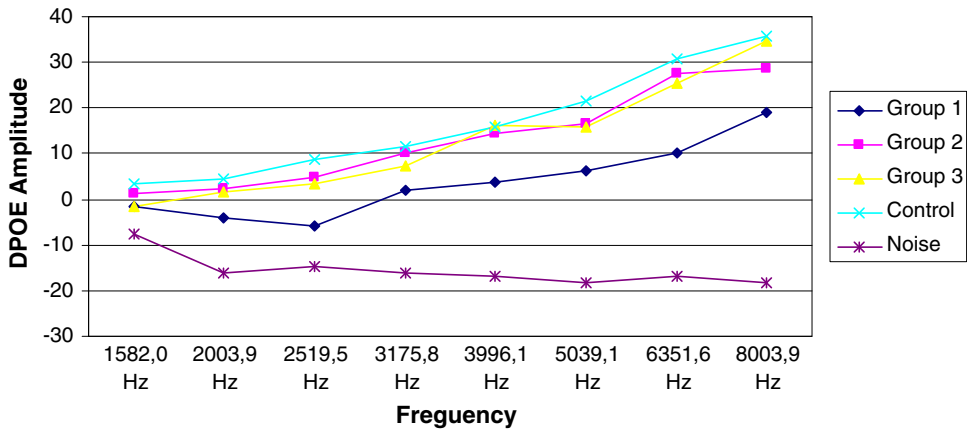

Fig. 2 DPOE amplitudes obtained from on day 1

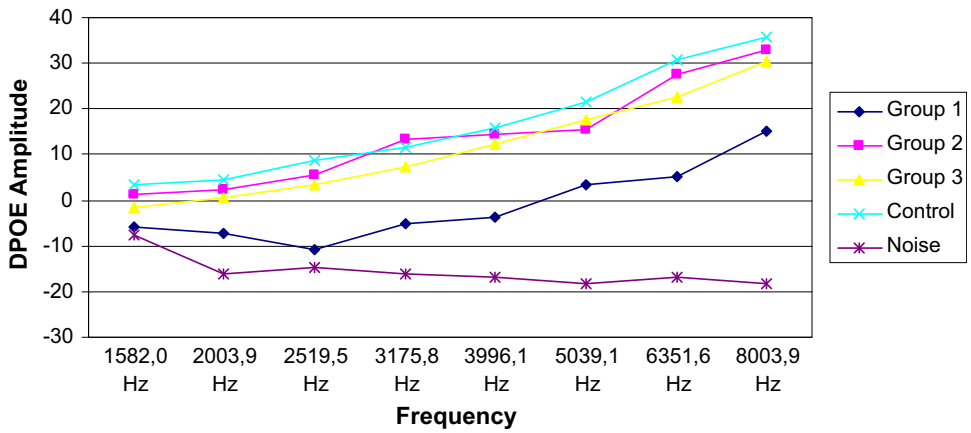

Fig. 3 DPOE amplitudes obtained from on day 5

NO in the liver [23]. NO is a substantial endogenous regulatory particle, contained in both pro-inflammatory and anti-inflammatory processes. NO may also prevent the release of cytotoxic products and cause the progression of inflammation [24]. Chander et al. has recently reported that MOL treatment prevents lipid peroxidation and the severe depletion of the antioxidant enzyme pool in rats [25]. They suggested that the beneficial effects of MOL may be due to the decreasing of neutrophil infiltration. Rodriguez-Pena et al. showed MOL reduced plasma levels of pro-inflammatory cytokines however it increases the levels of anti-inflammatory cytokines in rats [26]. Taking into consideration the reduced oxidative damage caused by MOL treatment, all investigators attributed the protective actions of MOL to its antioxidative and anti-inflammatory activities [27].

In this study we used DPOAEs, which is a highly selective tool for detecting cochlear hearing loss. DPOAEs

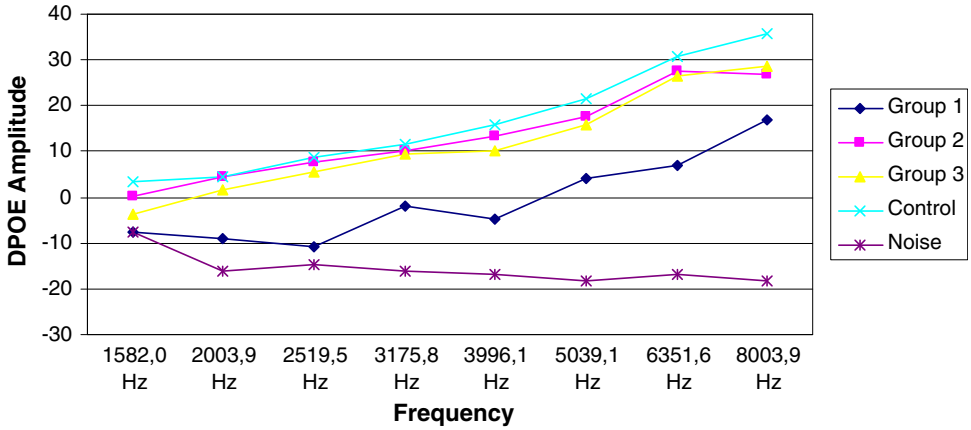

Fig. 4 DPOE amplitudes obtained from on day 12

Table 1 Comparison of serum oxidative stress parameters and antioxidant status

Groups	MDA (nmol/ml)	SOD (U/mg protein)	CAT (k/g protein)	GPX (U/mg protein)	GSH (μmol/L)	TOS (μmol H_2O_2 Eqv/L)	TAC (μmol Trolox Eq/L)	OSI (Arbitrary unit)
Control	1.29 ± 0.20	27.04 ± 6.23	1.74 ± 0.42	6.92 ± 1.45	0.51 ± 0.20	5.91 ± 1.25	1.08 ± 0.16	0.56 ± 0.15
Molsidomine	1.10 ± 0.21[b]	26.28 ± 4.74[b]	1.79 ± 0.38[b]	5.94 ± 1.40[b]	0.47 ± 0.15[b]	5.17 ± 1.42[b]	1.10 ± 0.17[b]	0.46 ± 0.09[b]
Cisplatin	2.32 ± 0.42[a]	12.30 ± 2.96[a]	1.00 ± 0.31[a]	2.50 ± 0.86[a]	0.17 ± 0.04[a]	7.80 ± 1.39[a]	0.88 ± 0.14[a]	0.94 ± 0.26[a]
Cisplatin + molsidomine	1.14 ± 0.27[b]	29.10 ± 2.92[b]	1.90 ± 0.61[b]	6.96 ± 0.76[b]	0.37 ± 0.10[b]	4.82 ± 1.00[b]	1.16 ± 0.11[b]	0.44 ± 0.10[b]

[a] $p < 0.05$ versus control group

[b] $p < 0.05$ versus cisplatin group

measurement is non-invasive and objective to define the early stages of sound processing and assess the biomechanical activity of the outer hair cells [28]. DPOAE measurement is a clear method for identifying the effects of cisplatin on the cochlea before the changes that are identified by pure-tone audiometry [29]. Statistically significant reductions in DP-gram amplitudes were noted at frequencies of 2.519, 3.176, 3.996, 5.039, 6.352 and 8.004 Hz frequencies in the C group, strongly seems to be related CIO. MOL supplementation with cisplatin (CM group) significantly reduced all of these detrimental changes. It is well established that cisplatin treatment can cause a decrease in the antioxidant enzymes such as SOD, CAT and GPX and an increase in the level of end product of lipid peroxidation such as MDA [30]. In accordance with the literature we found administration of cisplatin was resulted in a decrease in the level of antioxidant enzymes, SOD, CAT, GPX, and GSH, TAS contents whereas an increase in the level of MDA, TOS and OSI. MOL treatment significantly ameliorated all of these hazardous changes (Table 1). Thus it is seems to be related that, reduction of antioxidant enzyme activities and elevated lipid peroxidation and oxidative stress parameters are responsible for cochlear injury and ototoxicity [5]. Our biochemical results were found as in accordance with DPOAE measurements.

In this study, the CIO was shown by measuring of DPOAEs and biochemical parameters. The limitation of the current study is lack of the histologic analyses of inner ear. In the current study differently from other the ototoxicity studies on the same subject, we also assessed serum biochemical parameters including new oxidant and antioxidant contents such as TOS, TAC and OSI. According to those new parameters which were evaluated on, we showed that MOL is effective against CIO in rats. We propose that MOL acts in the body including ear as a potent scavenger of free radicals and anti-inflammatory effects to prevent the toxic effects of cisplatin in the light of biochemical and DPOAE measurements. Thus, we believe that it could be effectively combined with cisplatin.

In conclusion, finding of the present study showed for the first time that MOL, with its potent free radical scavenging, antioxidant and anti-inflammatory properties, seems to be a highly promising agent for preventing CIO. After a few animal and human studies, MOL which was widely used as anti-anginal and no important side-effects may be considered in treatment of CIO in the future. However, further studies are required to evaluate the role of different dose of MOL in the prevention and the treatment of CIO.

Conflict of interest None declared.

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