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Protective Effects of Molsidomine Against Cisplatin-Induced Nephrotoxicity*

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Abstract

Background. Cisplatin, an effective chemotherapeutic agent, is used for the treatment of several types of cancers. However, cisplatin has some severe side effects such as nephrotoxicity. On the other hand, molsidomine, a NO donor, has anti-oxidative and vasodilator effects.

Objectives. The aim of this study was to estimate the protective effects of molsidomine on cisplatin-induced nephrotoxicity.

Material and Methods. Thirty-two rats were randomly divided into 4 groups as follows: (1) control; (2) received a single-dose intraperitoneal (*i.p.*) injection of 5 mg/kg cisplatin; (3) received single *i.p.* dose of molsidomine (4 mg/kg/day) for 3 consecutive days before cisplatin treatment; (4) received single *i.p.* dose of molsidomine (4 mg/kg/day) for 3 consecutive days. The specific biochemical markers, including antioxidants, and the histopathological alterations were evaluated.

Results. Cisplatin significantly increased malondialdehyde (MDA) and myeloperoxidase (MPO) levels and decreased glutathione peroxidase (GPX) level. Molsidomine significantly decreased MPO level nearly to control level; however, its ameliorating effects on MDA, SOD, CAT and GPX did not reach to significant levels. Cisplatin-induced elevation of blood-urea-nitrogen and serum-creatinine were diminished after molsidomine administration. Cisplatin also induced severe tubular degeneration, nuclear condensation, apoptosis and scattered patchy inflammation in the histological examination. Molsidomine improved all of these histological damages.

Conclusions. In this study, the beneficial effect of molsidomine against cisplatin nephrotoxicity has been evaluated for the first time (Adv Clin Exp Med 2015, 24, 4, 585–593).

Key words: cisplatin, molsidomine, kidney, rat.

Cisplatin, a member of the platinum group, is a highly effective chemotherapeutic agent. It is mostly used for the treatment of several types of cancer, such as head and neck cancer, esophageal cancer, testicular cancer, ovarian cancer, bladder cancer and non-small lung cancer [1]. Because of the resistance developed during treatment and various side effects, including nephrotoxicity, neurotoxicity, hepatotoxicity and ototoxicity, the usage

of this drug is limited [2–4]. Although the exact mechanism(s) underlying nephrotoxicity is not clear, besides changing nitric oxide (NO) level and apoptosis, the generations of reactive oxygen species (ROS), which are extremely reactive and not stable, have been thought to play the major role [5–8]. In organism, ROS can give rise to harmful consequences, which can lead to cell death, *via* lipid peroxidation and DNA damage [9]. Many studies

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showed that ROS scavengers could prevent cisplatin-induced cell injury [10–12].

Molsidomine, which is a NO donor and an efficient vasodilator agent, has been used in the patients with stable *angina pectoris*. It is a pro-drug and enzymatically decarboxylated to the active form SIN-1, which provides NO formation, by liver [13, 14]. Nitric oxide, an intercellular messenger molecule, is part of the process of vasodilatation and neurotransmission. Additionally, it has an important role in the process of inflammation, tissue injury and cell defense [15]. Moreover, NO prevents the adhesion of neutrophils to endothelium and the generation of ROS *via* inhibition of leukocyte activation [16–18]. NO may also prevent the release of leukotrienes, cytokines and prostaglandins, which are cytotoxic and vasoconstrictor products, and cause the progression of inflammation [19].

In the previous studies, it has been revealed that molsidomine has the beneficial effect on nephrotoxicity, which was caused by different factors, preventing oxidative stress injury [20, 21]. The present study was designed to evaluate the possible protective effect of molsidomine *via* preventing oxidative stress injury on cisplatin-induced nephrotoxicity in a rat model. Histopathological findings, including tubular degeneration and apoptotic changes, and biochemical analyses; such as tissue malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), myeloperoxidase (MPO), blood urea nitrogen (BUN) and serum creatinine (Cr) levels, were evaluated.

Material and Methods

Animals and Experimental Procedure

This animal experimental study was designed according to ARRIVE guidelines [22]. The protocol of this experimental study was approved by the Ethical Committee on Animal Research of Inonu University (Reference No.: 2012/A-38). All experimental procedures in our study were conducted in accordance with the Guidelines for Animal Research from the National Institutes of Health publication. Thirty-two female, post-pubertal (10–12 weeks) Wistar Albino rats, weighing 180–220 g, were obtained from Inonu University Laboratory Animals Research Center. The animals were housed in a temperature – (21 ± 2°C), humidity – (60 ± 5%) and light – (12:12-h light and dark cycle) controlled room on a standard commercial pellet diet and water *ad libitum*. The

rats were randomly assigned to 4 groups (n = 8) as follows: (1) control group rats applied only intraperitoneal (*i.p.*) vehicle; (2) cisplatin group rats received a single *i.p.* dose injection of 5 mg/kg cisplatin (cisplatin DBL, 50 mg, Orna Corp., Istanbul, Turkey); (3) molsidomine plus cisplatin group rats (molsidomine + cisplatin) received single *i.p.* dose of molsidomine (4 mg/kg/day) (molsidomine, Sigma Chemical Co., St Louis, MO, USA) for 3 consecutive days following a single-dose *i.p.* injection of cisplatin (5 mg/kg); (4) molsidomine group rats were treated for 3 consecutive days by *i.p.* with 4 mg/kg/day molsidomine.

Since Bokemeyer et al. [23] reported that cisplatin (5 mg/kg) can cause an elevation in BUN levels at least 3 days after cisplatin has been administered. Therefore, the rats in group 1 and 4 were sacrificed after 3 days of the injections whereas the rats in group 2 and 3 were sacrificed after 3 days of the cisplatin administrations. The dosage of cisplatin and molsidomine, and the duration of drug-treatment were decided according to the previous related studies [24, 25]. After sacrifice by an overdose of the ketamine and xylazine mixture, the renal tissue specimens were quickly and meticulously harvested for biochemical and histopathological analysis. After being divided into 2 equal longitudinal sections, one of them was placed in formaldehyde solution for routine histopathological examination using light microscopy. The other half was placed in liquid nitrogen and stored at –70 °C until assayed for MDA, CAT, SOD, GPX and MPO. Trunk blood was extracted to evaluate serum levels of BUN and Cr using an Olympus Autoanalyzer (Olympus Instruments, Tokyo, Japan).

Biochemical Analyses

Measurement of Malondialdehyde

The MDA contents of the homogenates were measured spectrophotometrically by determining the entity of thiobarbituric acid reactive substances [26]. Three milliliters of 1% phosphoric acid and 1 mL 0.6% thiobarbituric acid solution were added to 0.5 mL of homogenate pipetted into a tube. The mixture was heated in boiling water for 45 min. After the mixture had cooled, the colored part was extracted into 4 mL of *n*-butanol. The absorbance was determined by a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 and 520 nm. The amount of lipid peroxides was calculated as thiobarbituric acid reactive substances of lipid peroxidation. The results were given in nanomole per gram tissue (nmol/g tissue) according to a prepared standard graph.

Measurement of Superoxide Dismutase (SOD) Activity

Total SOD activity was measured according to the method of [27]. The basis of this method is the prevention of nitroblue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of SOD was determined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activities were given as unit per gram protein (U/g protein).

Measurement of Catalase Activity

CAT activity was measured according to Aebi's method [28]. The basis of the analysis is based on the determination of the rate constant (k , s^{-1}) or the H_2O_2 decomposition rate at 240 nm. The results were given as k per gram protein (k/g protein).

Measurement of Glutathione Peroxidase Activity

GPX activity was measured by the method of Paglia and Valentine [29]. An enzymatic reaction in a tube containing NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase, was initiated by addition of H_2O_2 , and the change in absorbance at 340 nm was observed by a spectrophotometer. The activities were given as unit per milligram protein (U/mg protein).

Measurement of Myeloperoxidase

MPO activity was determined using a 4-aminopyrine/phenol solution as the substrate for MPO-mediated oxidation by H_2O_2 and changes in absorbance at 510 nm were recorded [30]. One unit of MPO activity is determined as that degrades 1 μ mol H_2O_2 /min at 25°C. The results were given as unit per gram protein (U/g protein).

Histological Analysis

The kidney tissues were fixed in a 10% neutral formalin solution, and then embedded in paraffin after a routine follow-up of the tissue. The 5 μ cut sections were stained with haematoxylin and eosin (H & E) and evaluated by a Leica DFC 280 light microscope. The glomerular, tubular, interstitial and pelvicalyceal tissue sections were examined by an experienced observer unaware of the identity of the animal treatment groups. The interstitial inflammation and tubular changes, including necrosis and vacuolization, were semi-quantitatively evaluated and graded as follows: 0, normal

histological appearance; I (mild), inflammation, tubular epithelial cell swelling, brush border loss, nuclear condensation and apoptosis involving 1/3 of renal tissue; II (moderate), tubular epithelial changes and focal inflammation (lymphocyte infiltration) involving 2/3 of renal tissue; III (severe), tubular epithelial changes and diffused interstitial inflammation involving more than 2/3 of renal tissue [30]. The results were given as arithmetic median for interstitial inflammation and necrosis and vacuolization of tubules, and presence and absence for glomerular congestion.

Statistical Analysis

In order to determine even minor effects, the required sample sizes used in this experiment were identified through statistical power analysis. The sample sizes required for a power of 0.80 were estimated using NCSS software. Data was analyzed using the SPSS software program for Windows, v. 21.0 (SPSS Inc., Chicago, IL). The normality of the distribution was confirmed using the Kolmogorov-Smirnov test. According to the results obtained from the normality test, one-way analysis of variance (ANOVA) and the Kruskal-Wallis H test were used for the statistical analysis, as appropriate. Multiple comparisons were realized by Tamhane's test (for non-homogeneous variances) after the ANOVA test. The results were expressed as mean $\pm$ standard deviation (S.D.) for MDA, SOD, CAT, MPO, BUN and Cr. After a significant Kruskal-Wallis H test, a Conover test was also conducted for GPX. The values were given as median (min-max). P-values less than 0.05 were regarded as statistically significant.

Results

Body and Kidney Weight

No animals died during or after the injections. When we compared the body and kidney weights of the animals at the beginning and the end of the experiment, there was no significant difference among the groups (data not shown).

Effect of Molsidomine on Serum Parameters

As shown in Table 1, serum levels of BUN and Cr were significantly higher in the cisplatin alone group when compared to the control group. In the molsidomine + cisplatin group, BUN and Cr levels were lower than those in the cisplatin alone group and this difference was significant in the levels of BUN.

Table 1. The serum levels of BUN and Cr

Groups	BUN (mg/dL) mean $\pm$ SD	Cr (mg/dL) mean $\pm$ SD
Group 1 (control)	50.7 $\pm$ 9.14	0.33 $\pm$ 0.04
Group 2 (cisplatin)	98.1 $\pm$ 19.11*	0.50 $\pm$ 0.07*
Group 3 (molsidomine + cisplatin)	53.4 $\pm$ 8.95**	0.44 $\pm$ 0.09
Group 4 (molsidomine)	45.2 $\pm$ 4.14**	0.27 $\pm$ 0.04**,***

* p < 0.05 vs. Group 1; **p < 0.05 vs. Group 2; ***p < 0.05 vs. Group 3.

Table 2. The levels of MDA, SOD, CAT, GPX and MPO in renal tissue

Groups	MDA (nmol/g tissue) mean $\pm$ SD	SOD (U/g prot) mean $\pm$ SD	CAT (k/g prot) mean $\pm$ SD	GPX (U/mg prot) median (min–max)	MPO (U/g prot) mean $\pm$ SD
Group 1 (control)	167.2 $\pm$ 63.5	0.63 $\pm$ 0.07	60.5 $\pm$ 8.3	0.102(0.09–0.15)	3.5 $\pm$ 0.93
Group 2 (cisplatin)	241.8 $\pm$ 53.5*	0.57 $\pm$ 0.02	56.7 $\pm$ 8.4	0.080(0.04–0.09)*	5.6 $\pm$ 2.28*
Group 3 (molsidomine + cisplatin)	224.9 $\pm$ 39.6	0.58 $\pm$ 0.04	67.2 $\pm$ 5	0.081(0.07–0.13)	3.6 $\pm$ 0.62**
Group 4 (molsidomine)	190.2 $\pm$ 45.5	0.55 $\pm$ 0.05*	61.6 $\pm$ 9	0.082(0.04–0.12)	3.3 $\pm$ 1.33**

* p < 0.05 vs. Group 1; **p < 0.05 vs. Group 2.

Effect of Molsidomine on Cisplatin-Induced Changes in Kidney Tissue Enzymes and Lipid Peroxides

The levels of MDA and MPO significantly increased in the cisplatin alone group, whereas GPX activities decreased when compared to the control group (Table 2). The levels of SOD and CAT were decreased in the cisplatin alone group when compared to the control group; however, this reduction was not significant (Table 2).

On the other hand, as shown in Table 2, molsidomine given before cisplatin caused a decrease in the levels of MDA and MPO when compared to the cisplatin alone group; however, this reduction was significant only in MPO levels. Also, this ameliorating effect was found in the levels of SOD, CAT and GPX. Although CAT levels were even higher than the control levels, these elevations were not significant when compared to the control group.

Histological Results

There were no renal histological changes in the control group (Fig. 1). However, there was glomerular congestion and severe tubular degeneration in the cisplatin group. Also, nuclear condensation, apoptosis and scattered patchy inflammation were quite apparent in the cisplatin-treated rats

(Fig. 2A-B). These tubular changes were decreased with molsidomine given before cisplatin in group 3 when compared to group 2 and glomerular congestion was not found (Fig. 3). The histological appearance of molsidomine alone in the group was similar to the control group except for mild vacuolar degeneration in the tubular epithelia (Fig. 4). The histological findings are shown in Table 3.

Discussion

Kidneys, which are very critical organs, have crucial activities, such as providing acid-base balance, the stabilization of the volume of total body fluid and its compound. Several anti-neoplastic

Fig. 1. Control group – normal kidney histology, H & E $\times$ 200

Fig. 2A. Cisplatin group – glomerular congestion and foci of inflammation, H & E $\times 200$

Fig. 2B. Cisplatin group – severe apoptosis in the tubular epithelia and nuclear condensation (shown as white arrows), H & E $\times 200$

Fig. 3. Cisplatin + molsidomin group – reduced apoptosis in the tubular epithelia (shown as white arrows), H & E $\times 200$

Fig. 4. Molsidomin group – extensive vacuolar degeneration in the tubular epithelia and glomerular congestion, H & E $\times 200$

drugs, including cisplatin, used in the treatment of various diseases could lead to deleterious effects, especially on the kidney and nervous system [2–4, 31–33]. Cisplatin accumulates in the proximal tubular cells and this accumulation leads to toxicity in the kidney [34]. Although the exact mechanism of cisplatin-induced nephrotoxicity has not been yet fully understood, many hints indicate that ROS, such as superoxide anion radicals, hydrogen peroxide, hydroxyl radical etc., and reactive nitrogen species (RNS), such as peroxynitrite, peroxynitrous acid, nitrogen dioxide etc., play pivotal roles in this injury [35]. It has been shown that oxidative stress, which occurs due to the overproduction of ROS and a decrease in the antioxidant level, is the most important element underlying renal injury in various pathological conditions [36, 37]. ROS cause the peroxidation of membrane lipid, the oxidation of cell proteins, mitochondrial dysfunction, the damage to DNA helix and apoptosis of renal tubule cells in cisplatin nephrotoxicity [6, 12].

The agents, which have protective effects on oxidative stress decreasing the activity of ROS, seem to be promising in the treatment of many diseases [20, 38, 39]. On the other hand, in the previous studies, massive production of NO, which causes the formation of peroxynitrite in large amounts, is believed to be involved in cisplatin nephrotoxicity [40, 41]. Although the role of NO in cisplatin-induced nephrotoxicity is not clear, there is an alteration in the production and metabolism of NO in cisplatin treatment [35].

In the literature, it has been well established that molsidomine relaxes vascular smooth muscle by stimulating guanylate cyclase. Besides its increasing effect on cyclic GMP, Chander et al. [42] recently reported that molsidomine treatment prevents renal ischemia and reperfusion (I/R)-induced lipid peroxidation and the severe depletion of the antioxidant enzyme pool in rats. Also, the same investigators showed in the different experimental models that molsidomine is able to prevent

renal dysfunction along with the renal oxidative stress induced by cyclosporine [20]. They found that molsidomine decreased the level of MDA and increased the levels of SOD, CAT and GPX. Based on this relationship, Ozturk et al. [25] indicated that molsidomine could exert a protective effect against skeletal muscle injury caused by I/R in rats. They suggested that, at least, the beneficial effects of molsidomine might be related to the reduction of neutrophil infiltration. In accordance with this result, Rodriguez-Pena et al. [43] demonstrated that intra-renal infusion of molsidomine decreases plasma levels of pro-inflammatory cytokines such as TNF- α , IFN- γ and IL-1 β , whereas it increases the levels of anti-inflammatory cytokines IL-10 and IL-6 in renal I/R applied rats. On the other hand, as a donor of NO, molsidomine can cause nitrosative stress *via* forming peroxynitrite, a toxic RNS [44].

The present experimental study demonstrated that cisplatin led to oxidative stress in the renal tissue as evidenced by a significant rise in MDA levels (Table 2), which indicates lipid peroxidation as shown in the previous studies related to cisplatin-induced nephrotoxicity; however, the reduction obtained with the use of molsidomine in addition to cisplatin did not reach a significant level [45, 46]. It has been suggested that ROS production and subsequent lipid peroxidation caused by cisplatin in the tubular cells are responsible for the oxidative renal damage [8, 37, 47–49]. Whereas, in a previous study, it has been revealed that molsidomine decreased the level of MDA in renal tissue in oxidative stress induced by cyclosporine [20]. On the other hand, MPO, which is a pro-oxidant enzyme, is mostly used to demonstrate neutrophil infiltration that causes the release of ROS, cytotoxic proteins and MPO, which generate additional damage in the tissue [50–52]. In the current study, cisplatin increased MPO activities that exacerbate this harmful cascade. On the other hand, we found that molsidomine prevented the elevation in the level of MPO. In accordance with our results, Rodriguez-Pena et al. [43] revealed that MPO elevation could be decreased with molsidomine. In accordance with a previous study [53], in the current study molsidomine decreased neutrophil infiltration, thereby preventing a further increase in oxidative injury in our study.

In organisms, the levels of antioxidants and free radicals, at least, should be in balance to prevent oxidative stress [54]. SOD converts superoxide anion, which is produced during the usual metabolic process in cells, into hydrogen peroxide, thus undesirable radicals can be eliminated [55]. CAT and GPX subsequently inactivate hydrogen peroxide through converting into H₂O and

O₂ [56–58]. In our study, we demonstrated that the level of GPX was significantly decreased by cisplatin administration, which paralleled to the previous studies [39, 59]. We found that the levels of SOD and CAT were reduced by cisplatin; however, this decrease was not significant. One of the possible explanations of this result is that it can be due to the defensive reaction of the organism against cisplatin [60]. Moreover, the previous related experimental studies revealed that the usage of cisplatin might cause an unexpected increase in the levels of SOD and CAT [35, 60]. In the current study, when molsidomine was given before cisplatin, it did not provide a significant elevation in levels of SOD, CAT and GPX. In parallel with our results, Rodriguez-Pena et al. [43] demonstrated that the usage of molsidomine might unexpectedly decrease instead of increase in the levels of SOD, CAT and GPX. Whereas molsidomine has been shown to increase significantly these antioxidant enzyme activities in the renal tissue [20, 42]. On the other hand, it was demonstrated that molsidomine prevented tissue injury reducing plasma levels of pro-inflammatory mediators, which increase oxidative stress and the severity of inflammation process [53]. This pathway could have been utilized against cisplatin nephrotoxicity in addition to its antioxidant properties in our study. The other possible reasons for the insufficient elevation in the levels of these antioxidants can be inadequate dosing and timing for molsidomine administration, and nitrosative stress pathway, which is another pathway used by molsidomine to prevent tissue injury. However, we need further investigations with different dosage and timing scheme, especially about nitrosative stress pathway, to understand the real mechanism(s).

The impairment in glomerular function is accompanied by the increase in BUN and Cr levels in the process of nephrotoxicity. We found that cisplatin caused renal dysfunction as evidenced by a significant elevation in serum BUN and Cr levels. This circumstance was also revealed in the other studies related to cisplatin-induced renal damage [59, 61]. Molsidomine pretreatment of cisplatin administration attenuated the cisplatin-induced nephrotoxicity as shown in decrease in the serum levels of BUN and Cr. Our results are in parallel with the outcomes of the study of Chander et al. [20] in which molsidomine decreased BUN and Cr levels in cyclosporine-induced nephrotoxicity.

The histopathological evidences further confirmed our biochemical findings. Besides significantly decreasing the levels of MPO and BUN, molsidomine pretreatment of cisplatin ameliorated its certain nephrotoxic effects in the histopathological examination, such as tubular degeneration,

nuclear condensation, apoptosis and inflammation. Cisplatin-induced extensive tubular degeneration and the other histological alterations were also revealed in the previous studies [59, 62]. On the other hand, in our study unlike those studies, a single dose of cisplatin administration did not induce significant changes in the body and kidney weights of the rats.

In the current study, the beneficial effects of molsidomine of cisplatin-induced nephrotoxicity were examined for the first time. Although the mechanisms involved in cisplatin-induced nephrotoxicity are complex and may include inflammation, oxidative stress injury and apoptosis. Molsidomine, given at a dose of 4 mg/kg (*i.p.*) before

cisplatin administration, improved the renal damage caused by cisplatin nephrotoxicity. The useful alterations in biochemical analyses, including amelioration in antioxidant status, improvement in BUN and Cr levels were accompanied by beneficial changes in the histopathological aspect of the renal tissue. We propose that molsidomine acts in the kidney as a potent scavenger of free radicals, anti-inflammatory and anti-apoptotic effects to prevent the toxic effects of cisplatin both at the biochemical and histopathological evaluations. Based on our results, after further clinical and experimental trials conducted with a different dosage and timing scheme, molsidomine could be used to protect the kidney against the toxic effect of cisplatin.

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