Turk J. Pharm. Sci. 6 (2), 83-92, 2009

Original article

THE EFFECTS OF L-ARGININE ON URINARY NITRIC OXIDE METABOLITES AND RENAL LIPID PEROXIDATION IN CISPLATIN TREATED RATS

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Abstract

Increase of lipid peroxidation and glutathione (GSH) depletion in kidney tissues have been observed in rats with cisplatin-(CDDP) induced nephrotoxicity. This investigation elucidates the role of L-arginine, the substrate of nitric oxide synthase (NOS), on renal injury, lipid peroxidation and urinary excretion of nitrite (NO_2^-)+ nitrate (NO_3^-) in rats with CDDP induced renal failure. CDDP (3 mg/kg, once a day) was injected intraperitoneally for 5 days. In subgroups, daily L-arginine (0.2g/kg) or N^G-nitro-L-arginine methyl ester (L-NAME) (NOS inhibitor, 20 mg/kg) were administrated intraperitonally 1 hour prior to CDDP treatment. Treatment with CDDP resulted in significant increase plasma creatinine (Cr), urea levels, daily urine volume, urinary gamma glutamyl transferase (GGT) levels and significant decrease creatinine clearance and urinary NO_2^- +NO_3^- excretion. Intraperitoneal administration of L-arginine in the low dose prevented the CDDP induced elevation of plasma Cr and urea levels. When compared with controls, CDDP administration resulted in increased lipid peroxidation and decreased GSH levels in the kidney; L-arginine reversed these effects. In addition, pretreatment of L-arginine was effective in the normalization of daily urine volume and urinary excretion of NO_2^-+NO_3^-. On the other hand, the administration of L-NAME resulted in no protection aganist CDDP-induced renal damage. The findings of this study suggest that intraperitoneal L-arginine administration can prevent the CDDP-induced renal damage by a mechanism which involves the production of NO.

Key words: *Cisplatin, L-arginine, Lipid peroxidation, Urinary nitrate and nitrite*

Sisplatin Uygulanmış Ratlarda İdrar Nitrik Oksit Metabolitleri ve Böbrek Lipid Peroksidasyonu Üzerine L-Arjininin Etkisi

Ratlarda sisplatin uygulanmasına bağlı olarak böbrek lipid peroksidasyon düzeyinin ve indirgenmiş glutatyon (GSH) tüketiminin arttığı gözlenmiştir. Bu çalışmada, nitrik oksit sentazın (NOS) substratı olan L-arjininin sisplatine bağlı böbrek hasarı oluşturulmuş ratlarda böbrek hasarına, böbrek lipid peroksidasyonuna, idrar nitrit (NO_2^-)+nitrat (NO_3^-) düzeylerine etkisi araştırılmıştır. Sisplatin (3 mg/kg) intraperitonal olarak günde bir kez 5 gün uygulandı. L-arjinin (0.2g/kg) ve N^G-nitro-L-arjinin metil ester (L-NAME, NOS inhibitörü, 20 mg/kg) sisplatin uygulanmasından 1 saat önce uygulandı. Sisplatin plazma kreatinin, üre düzeylerinde, günlük idrar hacminde ve idrar gama-glutamil transferaz (GGT) düzeyinde artışa neden olurken kreatinin klirensinde ve idrar NO_2^-+NO_3^- atılımında azalmaya neden oldu. Sisplatin uygulanmasından önce düşük doz intraperitonal L-arjinin verilmesi plazma kreatinin ve üre düzeylerindeki artışı engelledi. Sisplatin böbrek lipid peroksit düzeylerini artırırken GSH içeriğinin de azalmasına neden oldu. L-arjinin bu etkileri tersine çevirdi. L-arjinin günlük idrar atılımının ve idrar nitrik oksit metabolitlerinin normale dönmesini sağladı. Diğer taraftan, L-NAME sisplatine bağlı renal hasarda koruyucu etki göstermedi. Bulgularmıza göre intraperitonal L-arjinin uygulanması NO üretimini içine alan mekanizma ile sisplatine bağlı böbrek hasarında koruyucu etki göstermektedir.

Anahtar kelimeler: *Sisplatin, L-arjinin, Lipid Peroksidasyonu, İdrar nitrat ve nitrit*

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INTRODUCTION

Cisplatin (Cis-diamminedichloro platinum II, CDDP) is an important antineoplastic drug which is widely used in the treatment of several human tumors (1). The major side effect of CDDP is severe nephrotoxicity, that limit its clinical use (2,3). Although the exact mechanisms of CDPP induced nephrotoxicity are not clear, it is associated with increased renal vascular resistance and histologic damage to proximal tubular cells (4). In addition, increase of lipid peroxidation and depletion of glutathione in kidney tissues have been suggested to be responsible for the nephrotoxicity (3,5). Consequently, there is much interest in developing various agents to prevent CDDP-induced renal damage. A combination of these agents such as calcium antagonist nifedipine (6), ebselen (7) and glycine (8) with CDDP have all been shown to reduce CDDP-induced renal failure.

Nitric oxide (NO), previously known as endothelin derived relaxing factor, is a biological mediator and which is produced from L-arginine by nitric oxide synthase (NOS). NO is unstable and oxidizes to nitrites and nitrates. There are three isoforms of NOS: the endothelial type (eNOS), the neuronal type (nNOS), and the inducible type (iNOS). eNOS and nNOS are considered constitutive (cNOS). iNOS is normally inactive until cytokines and liposaccaride stimulation (9). The three NOS isoforms are present in the kidney. NO and has been shown to play important role renal hemodynamics (10). An altered renal production of NO may be involved in the renal disease progression. L-arginine, the substrate of NO, has effective role on regulation of renal functions in several patolojic conditions. It has been observed that oral administration of L-arginine in rats prevents renal failure which is induced drugs (11,12). On the other hand, expression of iNOS results with production of large amounts of NO (13). The overproduction of NO can also caused tissue damage and increase of oxidative modifications. It has been reported that enchanching NO production by providing excess of L-arginine can increase ischemia-reperfusion induced tissue damage in testis (14).

Based on these observations, we have investigated the effects of intraperitoneally L-arginine administration on CDDP-induced renal damage by measuring malondialdehyde (MDA, an end-product of lipid peroxidation), glutathione (GSH) levels, plasma creatinine (Cr), urea, urinary GGT activity. In addition, the present study was designed to verify whether the CDDP and/or L-arginine administration has effect on daily urinary excretion of NO metabolites (NO_2^-+NO_3^-, NO_x).

MATERIALS AND METHODS

Materials

L-arginine, NG-nitro L-arginine methyl ester (L-NAME), N-(1-Naphthyl) ethylenediaminedihydrochloride, and sulphanilamide were purchased from Sigma (St Louis, USA), dithiobis nitrobenzoic acid, thiobarbituric acid, cadmium granules and glycine from Fluka (Steinheim, Switzerland). Other chemicals were of reagent grade obtained from Merck (Darmstadt, Germany).

Animals

A total of 42 male Sprague Dawley weighing 230-300 g were housed controlled in a controlled environment and provided with standart pellet chow and water. The study was approved by the Marmara University School of Medicine Animal Care and Use Committee.

Experimental protocols

Rats were divided to 6 groups of 7 rats each:
Group 1 (Control): daily intraperitoneally injections of isotonic saline for 5 days.

Group 2 (CDDP): daily intraperitoneally injections of CDDP(3 mg/kg) for 5 days (15).

Group 3 (CDDP+L-arginine): daily intraperitoneally of L-arginine (0,2g/kg) 1 hour prior to CDDP treatment for 5 days.

Group 4 (CDDP +L-NAME): daily intraperitoneally of L-NAME (20 mg/kg) 1 hour prior to CDDP treatment.

Group 5 (L-arginine): daily intraperitoneally of L-arginine (0,2g/kg) for 5 days (16).

Group 6 (L-NAME): daily intraperitoneally of L-NAME (20mg/kg) treatment for 5 days (17).

Assays of Serum and Urine samples

Blood samples and urine samples of 24 hours were collected before sacrification. Plasma levels of creatinine and urea were assayed by standard colorimetric procedures, creatinine by reaction with picrate in alkaline solution and urea by reaction with diacetyl monooxime. Creatinine Clearance (C_{Cr}) was calculated on the basis of urinary Cr, serum Cr, urine volume and body weight.

For determination urinary GGT, urine samples were centrifuged at 2500 g for 10 min. GGT activity was determined by the Szasz method (18) using gamma-glutamyl p-nitroanilide as the substrate and glycylglycine as acceptor. The nitroaniline released was measured at 405 nm using spectrophotometer. One unit (U) represents the amount of enzyme catalyzing the release of 1 μmol of nitroaniline/min.

Determination of renal MDA levels and GSH content

Kidneys of rats were homogenized with 0.15 M KCl to make a 10 % homogenate, using a glass teflon homogenizer. Levels of MDA were assayed for products of lipid peroxidation in tissue homogenate (19). The principle of the method is based on measuring the concentration of the pink chromogen compound that forms when MDA couples to thiobarbituric acid. MDA levels were expressed as nmol MDA /g tissue.

GSH was determined by spectrophotometric method, which was based on the use of Ellman's reagent (20). Results were expressed as μmol GSH /g tissue.

Colorimetric assay of nitrate and nitrite

NO_x levels of urine samples were determined with a colorimetric method (21). This method is based on the reduction of nitrate to nitrite by cadmium, involving a shortened incubation period of nitrate with cadmium. Urine samples were diluted glycine (45 g/L) buffer. Cadmium granules (2-2.5 g for each tube) were rinsed three times with deionized distilled water and swirled in a 5 mmol/L $CuSO_4$ solution in glycine-NaOH buffer (15g/L, pH:9.7) for 5 min. The copper-coated granules was used within 10 min. Cadmium granules were added to 1 ml deproteinized urine and stirring for 10 min. Samples were transferred and nitrit levels were determined by Griess reaction with use of reagents sulfanilamide and N-(1-Naphthyl) ethylenediamine. NOx concentration was estimated from a standard curve and the results were given in μmol/ mg creatinine.

Statistics

All data expressed as mean± SD. Data were analyzed with ANOVA followed by a Tukey post-hoc test for multiple comparisons. P-value less than 0.05 was considered significant.

RESULTS

The average body weights of rats before the experiment and at the end of the experiment are shown in Table 1. There was a 10% to 14% decrease in the weight of groups of CDDP and CDDP+L-arginine and CDDP+L-NAME compared with its beginings. This decrease was no statistically significant.

Table 1. Effects of L-arginine and L-NAME on body weights of CDDP-treated rats.

	Start weight(g)	End weight(g)	difference (%)
Control	262.0±11.6	267.5±23.2	2
CDDP	271.5±8.0	235.3±14.0	10
CDDP+ L-arginine	268.8±22.4	243.5±25.1	10
CDDP+L-NAME	259.0±14.3	222.3±18.3	14

Values are mean ±SD

Serum creatinine , urea, urinary excretion of GGT and daily urine volume were significantly increased in the CDDP-treated group compared to control group (Table 2). L-arginine administration before 1 hour from CDDP injection reduced the rise in the level of serum creatinine, urea, urinary GGT activity and urine output as well as a significantly increased creatinine clearance. Treatment of L-NAME+CDDP resulted no significant changes in urine output, urine GGT activity and C_{Cr} when compared with rats given only cisplatin, although L-NAME caused significant falls in creatinine and urea levels relative to the CDDP group.

Table 2. Effects of L-arginine and L-NAME pretreatment on serum creatinine, serum urea, urine volume, urinary GGT levels and C_{cr} of CDDP-treated rats.

	Control	CDDP	CDDP+ L-arginine	CDDP+ L-NAME
Serum creatinine (mg/dl)	0.66±0.10	1.80±0.12*	1.10± 0.14*[a]	1.30±0.20*[a]
Serum urea (mg/dl)	39.18±4.57	110.40±18.0*	73.9± 9.03*[a]	85.60±7.60*[a]
Urine volume (ml/day)	5.01±1.20	14.8±51.70*	9.50±1.60*[a]	11.30±1.10*
Urinary GGT excretion (U/day)	5.6±2.3	13.8±4.7*	7.7±3.6*[b]	10.5±4.5 *
C_{Cr} (ml/min)	0.49±0.16	0.13±0.04**	0.56±0.18[a]	0.27±0.06**

All data represent mean±SD

*p<0.001, **p<0.05 (Compared to controls).

[a]p<0.001, [b]p<0.05 (Compared to CDDP).

The findings for urinary NO_x excretion are given in Table 3. CDDP treated rats had a much lower NO_x excretion compared with the normal rats value ($p<0.01$). Pretreatment of L-arginine raised the excretion of NO_x to normal rat values($p<0.05$). Treatment of CDDP+L-NAME resulted no significant change in urine NO_x levels compared with the CDDP group.

Table 3. Effects of L-arginine and L-NAME on urinary NO_x levels of CDDP-treated rats.

	Control	CDDP	CDDP+ L-arginine	CDDP+ L-NAME
NO_x (nmol/μg creatinine)	0.460±0.21	0.188±0.13*	0.329±0.19[a]	0.210±0.10*

All data represent mean values±SD
*$p<0.01$ (Compared to control group).
[a]$p<0.05$ (Compared to CDDP group).

The mean level of MDA was increased after CDDP administration compared with the control group ($p<0.001$, Table 4). L-arginine administration to the CDDP group caused a marked decrease in mean MDA with CDDP ($p<0.001$). L-arginine alone had no effect compared with the control group in lipid peroxide levels(data not shown). In addition, CDDP-induced renal damage caused significant decrease in GSH levels compared with the control ($p<0.001$). However, the contents of kidney GSH in the CDDP plus L-arginine-treated group increased by 25%, but this was not statistically significant ($p>0.05$). But pretreatment with L-NAME did not caused significant changes in lipid peroxide levels and GSH content relative to the CDDP group.

Table 4. Effects of L-arginine and L-NAME administration on kidney MDA and GSH levels of CDDP-treated rats.

	Control	CDDP	CDDP+ L-arginine	CDDP+L-NAME
MDA(nmol/g tissue)	18.01±1.9	33.85±3.8*	26.3±3.7*[a]	30.23±2.8*
GSH (μmol/g tissue)	4.6±0.3	2.4±0.2*	3.2± 0.2*	2.6±0.2*

All data represent mean values±SD
*$p<0.001$ (Compared to controls).
[a]$p<0.001$ (Compared to CDDP).

DISCUSSION

The major side effect of cisplatin is severe nephrotoxicity (22). The underlying the mechanism of CDDP-induced nephrotoxicity is not clear. However, it has been observed that CDDP primarly causes necrosis in the S3 segment of renal proximal tubule (22,23).

CDDP-induced nephrotoxicity is dose dependent and cumulative (24). Elevation of BUN and creatinine levels which are indicative of renal injury can last for more than 2 years if CDDP is given over 5 days at high doses (25). In addition; after the administration of CDDP, a decrease

in renal blood flow and glomerular filtration rate in rats can be observed (17). In the present study, cisplatin (3mg/kg) was administrated intraperitonally 5 days to induce nephrotoxicity. A significant increase in serum creatinine, urea, urine creatinine clearance and daily urine volume, which indicates renal failure, was observed in the CDDP administrated group compared to the control group. It was shown that a minimal dose of CDDP was sufficient to induce nephrotoxicity in rats. When L-arginine was administrated alone, it did not cause any significant changes in the biological parameters and daily urine volume. On the other hand, pretreatment with L-arginine in CDDP injected rats resulted in normalization of these biochemical parameters. L-NAME is an inhibitor of iNOS and eNOS (26). Although pretreatment with L-NAME induced changes in the serum creatinine and urea, it resulted insignificant changes in urine volume and Ccr compared to the CDDP group. It has been reported that L-NAME decreases renal blood flow and the renal clearance of [^{3}H] inulin when given to normal rats with infusion. In contrast, when L-NAME was given to rats those injected with CDDP, there was no changes in CDDP-induced decrease in renal blood flow and the renal clearance of inulin (27). In a study by Li et al (17); administration of L-NAME (1.0 mg kg^{-1}, i.v.) to rats those received cisplatin and glycine, significantly inhibited the reno-protective effect of glycine. However, L-NAME administration to rats those with cisplatin did not result in any potentiation of cisplatin nephrotoxicity in our study.

Increase in the urinary excretion of renal enzymes is a manifestation of kidney damage (28). GGT also is a membrane bound enzyme which is also present on luminal surface of proximal tubule epithelial cells. Urinary GGT is of great clinical interest as a marker for several renal diseases (29). It has been reported that urinary GGT level is high in rats which has gentamycin-induced renal damage and it may be used as an indicator of renal injury (30). Similar findings were found with CDDP administration. CDDP admimistration (6.5 mg/Kg) increases urinary excretion of GGT (two fold) (31). Fatima et al. (32) also has shown that activity of renal brush border enzymes were decreased by CDDP administration in the brush border membrane as well as in the homogenates of cortex and medulla. In our study, CDDP administration was also found to be increasing urinary GGT levels and L-arginine reversed this effect of CDDP.

CDDP induced nephrotoxicity is associated with initiation of lipid peroxidation and depletion of non protein thiols (15,33). GSH, non protein thiols in the cells, protects cells from toxic effects of free radicals and plays a role of conjugation with electrophilic drug metabolites. GSH depletion increases the sensivity of a cell to oxidative stress and chemical injury (34). NO can reacts with intracellular GSH and forms S-nitrosogluathione (35). The formation of S-nitrosogluathione can also protect the cell against injury (36). Oral L-arginine administration prevents the cells from increased lipid peroxidation and depletion of antioxidant enzymes such as glutathione peroxidase, catalase and glutathione S-transferase(12). We showed that intraperitoneally administration of L-arginine one hour ago from CDDP injection also provided a significant reduction on kidney MDA levels and a insignificant increase on kidney GSH contents. In our study, L-NAME did not show protective or potentive effect against CDDP-induced lipid peroxidation. Inactiveness of L-NAME could be due to nonselective inhibitor of NOS.

NO has organ specific regulatory functions. Several agents which increase production of NO in cell can be effective in protecting the kidney from failure. The effects of these agents with CDDP combinations on kidney function have been explained in various studies (12,37). It has been demonstrated that N-Acetyl cysteine (NAC) administration protects the rats which are CDDP injected from renal failure. NAC, as an antioxidant, increased GSH content in the cell and also regenerated NO production (37). It has been shown that glycine also prevents CDDP–induced decrease of renal blood flow. Glycine acts indirectly as a nitrogen source for the synthesis of L-arginine for NO formation (17). In mammalian cells, L-arginine is the semi-essential amino acid. Therefore, intracellular L-arginine levels and NO production are sensitive

to exogenous L-arginine administration (38). It was observed that oral administration of L-arginine also increased renal blood flow and glomerular filtration rate in normal rats through an NO mediated mechanism. It antagonized cisplatin's renal haemodynamic effects (11,12). NO maintains renal vasodilatation as a renoprotective agent (39).

Urinary excretion of NO_x may depend on various factors such as glomerular filtration, tubular handling, diet and de novo synthesis of NO (40). However, the relative stability of nitrate in plasma and urine have encouraged its use as an index of body NO production (41). NO production can increase within 1 or 2 days after induction of renal disease (42, 43). Kim et al. (44) reported that rats with Cronic Renal Failure were characterized by an almost threefold lower daily urine NO_x excretion compared to normal rats. In another study, it has been showed that feeding with excess cholesterol decreased urinary NO_3^- excretion in rabbits and chronic oral administration of L-arginine reversed this effect (45). Our results confirm these findings. Nitrite excretion in urine was decreased in CDDP and significantly reversed by L-arginine. The marked increase of nitrite in urine with L-arginine may be due to increased renal blood flow and glomerular filtration rate in CDDP group through an NO mediated mechanism.

CONCLUCION

We observed that urinary nitrite+nitrate levels increase in CDDP-induced renal injury. In vivo activation of L-arginine-NO pathway via intraperitonealy L-arginine administration has a beneficial effect on CDDP-induced renal failure, increased lipid peroxidation and normalized urine NO_x levels.

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Received: 28.05.2008
Accepted: 12.02.2009