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ORIGINAL ARTICLE

Effects of mycophenolate mofetil on cisplatin-induced renal dysfunction in rats

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Abstract
Purpose Inflammation and oxidative stress are important events among the plethora of mechanisms involved in cisplatin (CDDP)-induced nephrotoxicity. The aim of this study was to evaluate the effect of mycophenolate mofetil (MMF), an immunosuppressive, in the protection against CDDP-induced renal dysfunction.
Methods Rats were divided into four groups; untreated-control group, CDDP-treated group (7 mg/kg, single intraperitoneal dose), MMF-treated group (40 mg/kg/day orally for 5 successive days) and the fourth group was treated with both drugs and MMF treatment was started 1 day prior to CDDP administration. Nephrotoxicity was assessed 7 days after the CDDP treatment by measuring serum indices of nephrotoxicity, kidney weight as a percentage of total body weight, kidney's tissue peroxidative alterations and total nitrate/nitrite concentration (NOx) and the results were confirmed histopathologically.
Results Rats treated with CDDP showed marked nephrotoxicity as evidenced from the significant increase in serum creatinine and urea levels and decrease in serum calcium and albumin levels. Kidneys of CDDP-treated rats showed significant increases in kidney weight and malondialdehyde (MDA) production level and decreases in total NOx concentration, glutathione peroxidase (GPx) activity and reduced glutathione (GSH) content levels. Histopathological assessment of kidneys of CDDP-treated rats revealed extensive tubular necrosis with "sloughing off" of the renal tubular lining cells, intratubular hyaline casts and mononuclear cell infiltration. Treatment with MMF significantly protected the rats against CDDP-induced nephrotoxicity. The rise in serum creatinine and urea levels, kidney weight and kidney tissue MDA production, depletion of "endogenous antioxidant reserve" including GPx activity and reduced GSH content levels and the deleterious histopathological changes induced by CDDP treatment were significantly mitigated by MMF treatment.
Conclusions MMF treatment dramatically ameliorates CDDP-induced renal dysfunction.

Keywords Cisplatin · Mycophenolate mofetil · Nephrotoxicity · Rats

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Introduction

Nephrotoxicity is a major side effect of cisplatin (CDDP), a widely used cancer therapy drug. Depending on its concentration, CDDP induces necrosis or apoptosis of tubular cells in the kidneys, whereas the underlying injury mechanism is unclear. Recent evidence supports a role for an inflammatory pathogenesis of CDDP nephrotoxicity, but immune cell-mediated mechanisms in this disease are still largely unknown [1]. The renal microenvironmental changes following CDDP treatment are a complex process and could be broadly categorized into three main pathological

events, which at times might overlap: initial cytotoxic events, inflammatory events and fibroproliferative events [2]. Stress responses and heat shock proteins generated following CDDP treatment are actively involved in the initiation and progression of these events. Recently, it is reported that extracellular signal-regulated kinase (ERK1/2) pathway mediates CDDP-induced caspase activation and apoptosis in cultured renal tubular cells [2].

Reactive oxygen species (ROS) have been suggested as important mediators of CDDP-induced acute renal failure in vivo. Hydrogen peroxide is involved in the CDDP-induced necrosis, whereas hydroxyl radical is responsible for the CDDP-induced apoptosis via cytochrome c release, caspase activation [3] and death receptors activation [4]. Nitrosative stress involving the formation of 3-nitrotyrosine and peroxynitrite is considered to be an important mechanism participating in CDDP-induced nephrotoxicity [5, 6]. CDDP nephrotoxicity is characterized by activation of proinflammatory cytokines and chemokines. Tumor necrosis factor-alpha (TNF-α) appears to play a central role in the activation of this cytokine response and also in the pathogenesis of CDDP renal injury [7].

Tumor necrosis factor-alpha and interleukin-1β (IL-1ß) are proinflammatory cytokines and are often elevated in parallel. Indeed, TNF-α and IL-1ß stimulate the production of one another and act synergistically to stimulate the production of other cytokines and chemokines [8]. RANTES, MCP-1 and MIP-2 are chemotactic for a variety of leukocytes, including neutrophils, monocytes, natural killer cells and T lymphocytes [9, 10]. Which of these leukocyte populations may participate in CDDP nephrotoxicity is unknown.

During the last years, the novel immunosuppressive drug mycophenolate mofetil (MMF) has been introduced into the clinical protocol to overcome severe side effects associated with cyclosporine or tacrolimus. Meanwhile, it has become part of the immunosuppressive regimen after organ transplantation [11]. MMF effects are based on the inhibition of inosine monophosphate dehydrogenase (IMPDH) and the prevention of guanosine monophosphate synthesis from inosine monophosphate, a rate-limiting step in the purine biosynthesis in lymphocytes. Consequently, MMF blocks the proliferation and clonal expansion of T and B lymphocytes, and prevents the generation of cytotoxic T cells, as well as other effector T cells [12]. Additional mechanisms may also contribute to the efficacy of MMF in preventing allograft rejection. By depleting guanosine nucleotides, MMF suppresses glycosylation and the expression of some adhesion molecules, thereby decreasing the recruitment of lymphocytes and monocytes into sites of inflammation and graft rejection [12]. MMF possesses antitumoral properties particularly to colon and prostate carcinoma cells. MMF acts on adhesion proteins, which are relevant for tumor recurrence and dissemination [13].

T lymphocytes are direct mediators of experimental CDDP-induced nephrotoxicity [1]. So targeting T lymphocytes could lead to improved ways to administer CDDP safely. The aim of this work was to evaluate the effect of MMF administration on CDDP-induced renal dysfunction.

Materials and methods

Age-matched male Wister rats weighing 200 ± 20 g were used. Throughout the investigations the animals were fed a standardized diet (Purina chow) and had free access to drinking water. The study adhered to the guidelines of the National Institutes of Health for experimental use of animals. The study was approved by our Institutional Review Board. Animals were divided into four groups of ten animals each: control untreated, MMF (Mycophenolate mofetil, CellCept® Roche) treated 40 mg/kg/day for 5 days orally (suspended in 0.5% carboxymethyl-cellulose); CDDP (Cisplatin, David Bull Laboratories 'DBL') treated, 7 mg/kg single intraperitoneal injection and a fourth group was treated with both drugs, where MMF treatment was started 1 day prior to CDDP administration and continued for 5 consecutive days.

Seven days after CDDP administration, animals were anesthetized with ether and blood samples were taken by heart puncture and the kidneys were dissected out. One kidney from each animal was fixed in 10% neutral formaldehyde for histopathological evaluation. Kidneys were processed, embedded in paraffin, sectioned (3-μm thick), and stained with H&E. Each specimen was scored for the histopathological changes according to the following criteria: 0 for no histopathological changes; 1+ for interstitial nephritis and inflammation; 1+ for tubular atrophy and intratubular dilation with or without intralumenal hyaline casts and 1+ for renal tubular necrosis with "sloughing off" of the renal tubular lining cells. These changes were judged as significant if seen in three or more higher-power fields. Another kidney from each animal was washed with ice-cold saline, blotted with a piece of filter paper, weighed, de-capsulated and homogenized (Biohomogenizer) in ice-cold bi-distilled water.

Serum creatinine, urea and albumin levels were measured according to the methods of Bonsnes and

Taussky [14], Hallet and Cook [15] and Wrenn and Feichtmeir [16], respectively. Serum calcium and total protein levels were estimated by Randox Kits (Randox Laboratories Ltd, UK).

Tissue total nitrate/nitrite (NOx), malondialdehyde (MDA) concentration, glutathione peroxidase (GPx) activity and reduced glutathione (GSH) content were determined according to the methods of Miranda et al. [17], Ohkawa et al. [18], Paglia and Valentine [19] and Ellman [20], respectively.

The statistical significance of difference noted in the biochemical parameters was evaluated using one-way analysis of variance (ANOVA) followed by Dunnett T3 multiple comparisons test as a post hoc. A P value of 0.05 or less was taken as criterion for a statistically significant difference. For histopathological scoring, statistical differences were evaluated by Kruskal–Wallis one-way ANOVA.

Results

Administration of MMF to normal rats produced significant increase in serum urea level and decrease in albumin level amounting to 110 and 8.2% of the control group value, respectively. Also, MMF-treated rats showed significant reduction in total NOx concentration in kidney tissue amounting to 21.7% of those of the control untreated rats ($P < 0.01$, Table 1).

Table 1 shows that CDDP-treated rats showed significant ($P < 0.01$) increase in serum creatinine and urea levels and decrease in both calcium and albumin levels by 324, 712, 53 and 10.6% of the control group values, respectively. Moreover, kidney tissue of CDDP-treated rats resulted in significant increases in kidney weight as a percentage of total body weight (TBW) and MDA production level and decreases in total NOx concentration and "endogenous antioxidant reserve", including GPx activity and reduced GSH content levels amounting to 57.2, 28.7, 74, 31.6 and 53.6% of those results of the control untreated rats, respectively ($P < 0.01$).

Oral administration of MMF to CDDP-treated rats significantly ($P < 0.01$) mitigated CDDP-induced nephrotoxicity and its indices. MMF significantly reduced serum creatinine and urea and increased serum calcium levels by 64.5, 68.5 and 32% of those results obtained with CDDP-treated group, respectively. Moreover, the administration of MMF to CDDP-treated animals showed significant reduction in kidney weight as a percentage of TBW, NOx concentration and MDA production levels in addition to restoration of GPx activity and GSH content levels amounting to 23, 13.5, 32, 24.2 and 94.6% compared to CDDP-treated control rats, respectively (Table 1).

Microscopical examination of kidney tissue revealed that CDDP treatment produced extensive tubular necrosis with "sloughing off" of the renal tubular lining cells and mononuclear cell infiltration plus intratubular hyaline casts. However, CDDP/MMF-treated rats showed marked mitigation of CDDP-induced histopathological changes exhibiting nearly no more than intratubular dilation (Fig. 1, Table 2).

Discussion

Dose-limiting toxicity secondary to antineoplastic chemotherapy is due to the inability of cytotoxic drugs to differentiate between normal and malignant cells. Chemoprotectants have been developed as a means of ameliorating the toxicity associated with cytotoxic agents by providing site-specific protection for normal tissues without compromising antitumor efficacy.

Table 1 Effect of mycophenolate mofetil (MMF) on serum nephrotoxicity indices and renal peroxidative alterations induced by cisplatin (CDDP) treatment in normal rats

Group parameter	Control	MMF	CDDP	CDDP/MMF
Creatinine (mg/dl)	0.501 ± 0.152^{a}	0.545 ± 0.154^{a}	2.126 ± 0.295	0.754 ± 0.237^{a}
Urea (mg/dl)	28.86 ± 4.59	60.57 ± 13.51^{b}	234.29 ± 52.44	73.86 ± 26.74^{b}
Calcium (μmol/l)	2.12 ± 0.128^{c}	$2.0 \pm 0.19^{c,d}$	1.33 ± 0.09	1.76 ± 0.08^{d}
Albumin (g/l)	4.14 ± 0.12	3.80 ± 0.09^{e}	3.70 ± 0.166^{e}	3.56 ± 0.08^{e}
Kidney's weight and peroxidative alterations				
Kidney weight % of TBW	0.388 ± 0.02^{a}	$0.423 \pm 0.046^{a,b}$	0.610 ± 0.019	0.469 ± 0.028^{b}
Total NOx (μmol/g wet tissue)	456 ± 23.16	357 ± 40.86^{c}	$326 \pm 22.62^{c,d}$	281 ± 37.80^{d}
MDA (nmol/g wet tissue)	380.7 ± 43.83^{e}	$400 \pm 55.94^{e,f}$	662.6 ± 33.32	450.7 ± 33.39^{f}
GPx (IU/mg protein)	0.0567 ± 0.0045^{g}	0.0632 ± 0.0041^{g}	0.0388 ± 0.0026	0.0482 ± 0.0024
GSH (μmol/g wet tissue)	5.95 ± 0.352^{h}	5.68 ± 0.433^{h}	2.76 ± 0.355	5.37 ± 0.404^{h}

For details of treatment, refer to the text. Data are the mean ± SD of six to eight rats. Means marked by the same superscript letters are not significantly different ($P > 0.05$, ANOVA followed by Dunnett T3 test as a post hoc)

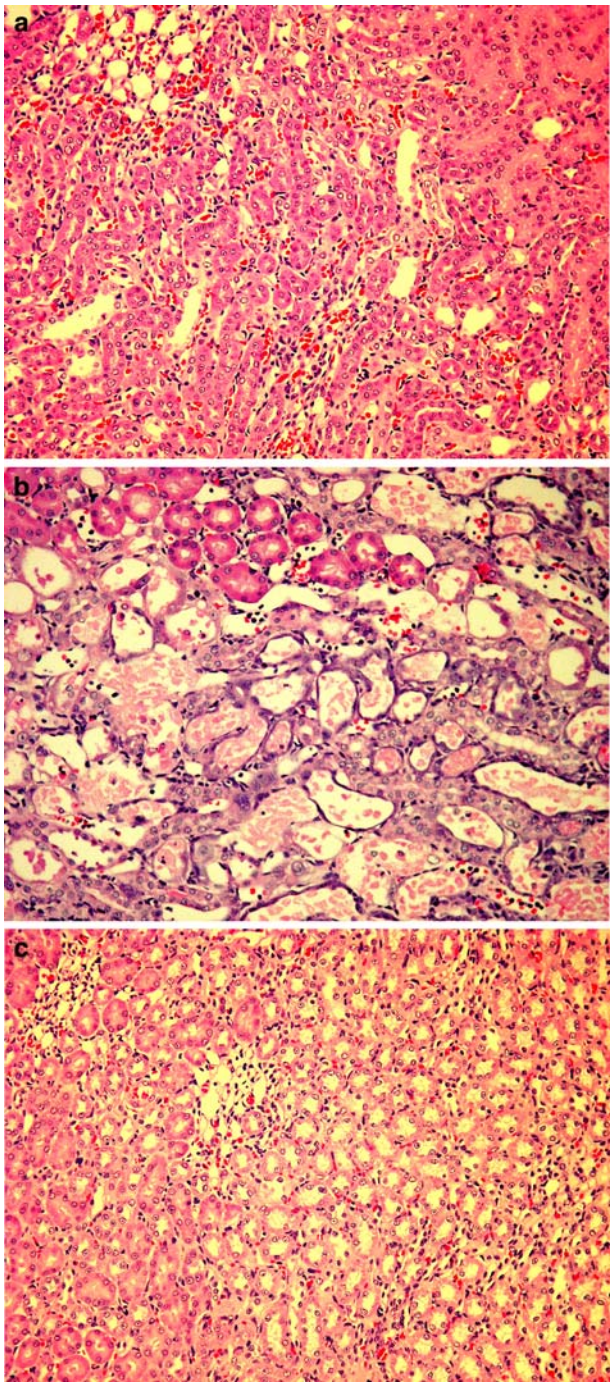

Fig. 1 **a** Photomicrograph of a kidney section taken from a rat treated with MMF depicting no pathological changes H&E X400. **b** Photomicrograph of a kidney section taken from a rat treated with CDDP showing extensive tubular necrosis, hyaline casts and inflammatory cell infiltration H&E X400. **c** Photomicrograph of a kidney section taken from a rat treated with CDDP/MMF concomitantly revealing minor tubular dilation H&E X400

Nephrotoxicity is an inherent adverse effect of certain anticancer drugs. Mechanisms of chemotherapy-induced renal dysfunction generally include damage to vascular or structures of the kidneys, hemolytic uremic syndrome and pre-renal perfusion deficits [21]. CDDP causes dose-related renal dysfunction through preferential accumulation in cells of the S3 segment of the renal proximal tubule and toxification intracellularly by hydration [22]. Several agents have been tested to see whether they could ameliorate or augment the nephrotoxicity of CDDP. Pharmaceutical antidotes to CDDP-induced nephrotoxicity include amifostine, sodium thiosulfate and diethyldithiocarbamate [23].

Table 2 Reno-protective effects of mycophenolate mofetil (MMF) against cisplatin (CDDP)-induced deleterious histopathological changes in the rat's kidney

Group	Median histopathological score (Score, +)
Control	0[a]
MMF-treated	0[a]
CDDP-treated	3 (2–3)
CDDP/MMF-treated	0.5 (0–1)[a]

For details of treatment and criteria of histopathological scoring, refer to the text. Values represent the median with the range in parenthesis ($n = 6$–7). Medians marked by the same superscript letters are not significantly different ($P > 0.05$, Kruskal–Wallis ANOVA)

This report documents the effect of MMF on indices of nephrotoxicity induced by CDDP treatment in vivo. We found that CDDP treatment produced significant increases in serum creatinine and urea as well as decreases in serum calcium and albumin levels. Also, kidneys of CDDP-injected rats showed significant increases in kidney weight as a percentage of TBW and MDA production level, in addition to reductions in total NOx concentration, GPx activity and reduced GSH content levels. These results were confirmed histopathologically, revealing extensive tubular necrosis, inflammatory cell infiltration and tubular hyaline casts. The obtained results are in accordance with those of our previous report [24].

The cytotoxic effects of CDDP are found to occur via several mechanisms, including inhibition of protein synthesis, DNA damage and mitochondrial injury which lead ultimately to the activation of apoptotic cell death in both tumor cells and renal tubular cells [25, 26]. CDDP-induced renal dysfunction occurs via nitric oxide (NO) depletion leading to stimulation of MCP-1 expression, upregulation of connective tissue growth factor and TNF-α which appears to play a central role in CDDP-induced proinflammatory cytokines and chemokines [8–10]. Moreover, CDDP-induced hydroxyl radicals, either directly or indirectly, activate p38 MAPK that plays an important role in mediating CDDP-induced acute renal injury and inflammation [27, 28].

The dose of MMF is an important factor in animal studies. In this study, 40 mg/kg/day of MMF was chosen. The review of animal studies using MMF shows the range of dose between 10 and 80 mg/kg [12]. The administration of MMF to CDDP-treated rats produced significant improvement in serum indices of nephrotoxicity and peroxidative alterations in the kidney due to CDDP treatment; however, MMF produced non-additive decrease in the total NOx concentration in the kidney tissue. The reduction in serum albumin in both MMF-treated controls and CDDP/MMF-treated rats might be due to MMF-related gastrointestinal side effects. Only the CDDP/MMF-treated animals showed signs of moderate diarrhea; however, this explanation could not be ruled out because we did not measure the animals food consumption.

Inflammation is associated with afferent arteriolopathy contributing to glomerular hemodynamic disturbances that result in the progression of renal disease [29]. MMF treatment might ameliorate CDDP-induced oxidative stress by reducing the infiltration and activation of superoxide-producing cells [30]. Also, MMF inhibits NO generation, preventing nitrotyrosine formation via depression of inducible nitric oxide synthase (iNOS) gene expression [31]. MMF-induced reduction in the total NOx of kidney tissue of normal control plus the inability of MMF to restore the kidney total NOx concentration in CDDP-treated rats might be due to a reduction in NO production via the inhibition of the biosynthesis of tetrahydrobiopterin, an essential co-factor of NOS [32]. Interestingly, it might be argued that the protective effects of MMF against CDDP-induced nephrotoxicity may result from a variety of actions including, antiproliferative effect on T and B lymphocytes, inhibition of glycosylation of cell surface adhesion proteins involved in a cell–cell contact, recruitment of circulating leukocytes to the sites of renal damage and inflammation and reduction of the proinflammatory cytokine TNF-α [33]. Iguchi et al. [34] reported that treatment with CDDP induces renal osteopontin (OPN) at both mRNA and protein expression levels contributing to renal cortical necrosis [34]. Thus, the reno-protective effect of MMF against CDDP-induced nephrotoxicity might be in part due to an inhibition of OPN expression in the kidney tissue [35].

In addition, increased renal angiotensin II (Ang II) production contributes to CDDP-induced nephropathy. The MMF administration to CDDP-treated rats might provide its protective effect via mitigation of infiltration and activation of immune cells producing Ang II, lowering the interstitial Ang II expression that elicits inflammatory signaling pathways and thickening of afferent arterioles [36].

Moreover, damage involving tubular interstitial compartment due to CDDP treatment might affect the functionality of nephron segments and renal afferent arterioles. Co-administration of MMF with CDDP might improve kallikrein expression, since enzymes and substrates of kallikrein-kinin system are synthesized by the renal connecting and collecting tubules [37]. Also, the protective effect of MMF against CDDP-induced nephrotoxicity could be due to interstitial fibroblast infiltration and interstitial type II collagen deposition reduction [38]. Progressive glomerular disease is usually associated with exacerbation of expression and activity of platelet-derived growth factor (PDGF) that has been consistently implicated in cell proliferation and extracellular accumulation [39]. Thus, the administration of MMF to CDDP-treated rats might inhibit PDGF-induced cellular ROS and ERK 1/2 and p38 MAPK activation [40]. In addition, MMF treatment-induced PDGF inhibition could result in a diminution of interstitial fibrosis due to CDDP treatment [41].

In conclusion, MMF has a marked protective effect against CDDP-induced renal dysfunction. Antitumor activity as well as the possible pharmacokinetic interaction of the two drugs concomitantly might warrant further investigations.

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