Cisplatin nephrotoxicity and protection by silibinin

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Abstract

Background. The anticancer drug cisplatin is known to have toxic side-effects on different segments of the nephron. The flavonoid silibinin has previously been shown to be protective in models of hepatotoxicity. The aim of the present study was to evaluate, whether silibinin can also ameliorate alterations in renal glomerular and tubular function and tubular morphology induced by cisplatin.

Methods. In a rat model renal damage was induced by a single injection of cisplatin (5 mg/kg body weight). The protective effects of silibinin were studied in rats that received the flavonoid (200 mg/kg body weight, i.v.) 1 h prior to the administration of cisplatin. Kidney function was monitored by analysing urinary markers of glomerular and tubular function over a period of 11 days. Animals of a second group, with identical treatment, were sacrificed 4 days after drug application for an evaluation of tubular morphology at the light-microscopical level.

Results. Administration of cisplatin caused a decline in kidney function within a day following treatment. Symptoms observed were for example decreases in creatinine clearance and increases in proteinuria, in the urinary activity of the proximal tubular enzymes alanine aminopeptidase and N-acetyl-β-D-glucosaminidase and in renal magnesium wasting. The effects of cisplatin on creatinine clearance and proteinuria were totally prevented by a pretreatment of the animals with silibinin. Impairment of proximal tubular function was ameliorated, that is enzymuria and magnesium wasting was less pronounced. Silibinin alone had no effect on kidney function. Treatment with silibinin distinctly diminished morphological alterations observed in the S₃-segment of the proximal tubule 4 days after cisplatin administration.

Conclusions. The effects of cisplatin on glomerular and proximal tubular function as well as proximal tubular morphology could totally or partly be ameliorated by silibinin. It is concluded that silibinin can act as a nephroprotectant and it is suggested that it could have beneficial effects on the kidney in clinical settings.

Key words: nephrotoxicity; nephroprotection; cisplatin; silibinin; glomerulus; tubular apparatus

Introduction

The anticancer drug cis-diamminodichloroplatinum (cisplatin) is a very effective compound in the treatment of a variety of cancers. Its clinical use, however, is associated with severe side-effects. To reduce the nephrotoxicity of this compound, patients are usually hydrated. Despite these precautions, cisplatin has been found to leave permanent damage in some patients [1]. The effects of cisplatin on renal function have been extensively studied in animal models. In the rat, like in humans, nephrotoxicity affects different segments of the nephron such as the tubular apparatus and the glomerulus. Functional and morphological impairment of the proximal and distal tubules is well demonstrated and can result in a reversible polyuric renal failure [2]. Impaired transport processes occur at the luminal and to a lesser degree at the contraluminal side of the proximal tubular membrane [3,4]. Morphological examination showed reversible damage up to necrosis in the proximal tubule, especially in the S₃ segment, 3–4 days after treatment [5]. Glomerular toxicity is both acute and chronic and becomes manifest in a reduction of the glomerular filtration rate due to reduced glomerular blood flow [6]. The mechanisms of this toxicity are not yet fully understood. In the tubular cells, the generation of free oxygen radicals has been proposed as a mechanism of toxicity [7–9]. Other evidence points to the inhibition of protein, RNA and DNA synthesis caused by the intrinsic property of cisplatin to bind to DNA and to interfere with protein synthesis [10,11].

Cytoprotective agents can be applied in therapy to ameliorate functional disorders. Cytoprotection is also regarded as a suitable tool to elucidate the pathogenesis of chemically induced injury [12]. Silibinin is a flavonoid extracted from Silybum marianum that has...
already successfully been applied as a protective agent in various clinical and both in-vivo and in-vitro experimental models of hepatotoxicity [13,14]. Silibinin possesses both antioxidant, anti-inflammatory and RNA and protein synthesis stimulating properties [13–17].

The study presented here attempted to evaluate the nephroprotective effects of the flavonoid silibinin on acute cisplatin toxicity. Parameters in serum and urine that allow assessment of the function or integrity of the different nephron segment such as the glomerulus or the proximal tubular apparatus were studied in a rat model following treatment with cisplatin and/or silibinin. These functional data were supplemented by an evaluation of kidney morphology at the light-microscopical level on day 4 after treatment.

Subjects and methods

Animals

Female Wistar rats (170–230 g) were obtained from the Laboratory of Experimental Animals, Medical School Hannover. Animals were housed at 3–4 animals per cage under standardized laboratory conditions with controlled light–dark cycle, room temperature (21°C), and moisture (50%). Animals had free access to tap water and to pellet diet (Altromin R, Altrogge, Lage, Germany). All animals were adapted to handling and the metabolic cages repeatedly during a 5-day period prior to the experiments.

Groups studied

For the functional studies 45 animals were randomly divided into four groups and treated as outlined below:

Group 1 (n = 12): NaCl
Group 2 (n = 10): silibinin
Group 3 (n = 12): cisplatin
Group 4 (n = 11): silibinin and cisplatin

For the morphological studies another 15 animals were used. They received the same treatment as the animals for the functional studies. The group sizes were n = 3 for NaCl and silibinin respectively, n = 5 for cisplatin, and n = 4 for silibinin plus cisplatin.

Treatment of animals

Following a control period of 1 day, treatment with the compounds or the vehicle was performed on day 0 of the observation period.

Animals treated with the vehicle alone were given 500 μl of 0.9% saline and served as controls (group 1). Silibinin was given as silibinin-C-2,3-dihydrogen succinate, disodium salt (Madaus AG, Cologne, Germany). The compound was dissolved in saline, the animals received 200 mg/kg body weight in a volume of 500 μl (200 mg/kg silibinin = 300 mg/kg silibinin-C-2,3-dihydrogen succinate, disodium salt) (group 2). Cisplatin (Medac, Hamburg, Germany) dissolved in saline was administered at a concentration of 5 mg/kg body weight to induce nephrotoxicity (group 3). Animals of group 4 received one injection of silibinin like group 2 and 1 h later the same dose of cisplatin as group 3. All injections were given i.v. into the tail vein. Cisplatin injections were applied between 9 and 12 a.m. to prevent any circadian variations described for cisplatin toxicity [18].

Sample collection

Urine and plasma samples were collected during a control phase (day–1), treatment began the following day (day 0 of the observation period). Further samples were collected on days 1, 3, 5, 7, 9 and 11 after treatment. For sample collection, animals were housed in individual metabolic cages, which allowed collection of urine samples without food or faeces contamination. Urine was collected overnight under paraffin oil to avoid evaporation. After each collection period a venous blood sample was drawn from the orbital plexus under light ether anaesthesia (Hoechst AG, Frankfurt, Germany). After collection, urine was cooled to 4°C and spun at 100 g for 5 min to remove any debris. Urines were supplemented with 0.01% NaN₃, 1 mM PMSF (phenylmethylsulphonyl fluoride) in DMSO was additionally added to samples to be used for protein analysis. Serum and urine samples were frozen in aliquots at −20°C.

Body weight and general parameters of renal function

Body weights of all rats were recorded every 2 days. Twenty-four-hour urinary volumes were measured gravimetrically. Total urinary protein was measured with the Coomassie brilliant blue binding method [19]. Electrophoretic separation of proteins on an SDS-PAGE polyacrylamide gradient gel followed standard procedures [20]. Subsequent staining with Coomassie brilliant blue allowed the assessment of the proportions of high- and low-molecular-weight proteins in the urine.

Tubular parameters

Urinary osmolality was measured using a Wescor vapor osmometer (Wescor, Logan, USA). Activity of the lysosomal enzyme β-N-acetyl-β-d-glucosaminidase (NAG, E.C. 3.2.1.30) was measured according to Price [21] by end-point determination after 1 h of incubation at 30°C, pH 4.3, using p-nitrophenyl β-N-acetyl-β-d-glucosaminide (Sigma, Deisenhofen, Germany) as a substrate at a concentration of 5.72 nM. L-alanine-aminopeptidase (AAP, E.C. 3.4.11.2) was measured with a kinetic determination at 25°C, pH 7.6, using L-alanine-nitro-anilinohydrochloride as a substrate. Urine for enzyme activity measurements was dialysed in Spectra Por 2 membranes (MWCO 14.000) against phosphate-buffered saline (0.01 M, pH 6.5) to remove chromogenic substances and enzyme inhibitors. Serum and urinary magnesium was analysed with the xylidil blue method (Magnesium test kit, Merck, Darmstadt, Germany). Fractional magnesium clearance was calculated as (urine [Mg²⁺]/plasma [Mg²⁺]) × 100, assuming that in the experimental set-up, the fraction of non-filtrable protein-bound Mg²⁺ is constant after cisplatin treatment, as has been demonstrated [22,23].

Glomerular parameters

Serum and urinary creatinine were measured using a Beckman creatinine analyser and reagents supplied by the manufacturer (Creatinine analyser 2 Reagents, Beckman, Munich, Germany). Urinary fibronectin levels were measured using a sandwich enzyme-linked immunosorbsorbentassay
Cisplatin nephrotoxicity and protection by silibinin (ELISA) as previously described [24]. Blood urea nitrogen levels were measured with a test kit (Harnstoff Test-Kit, Boehringer Mannheim, Germany).

**Histology**

Histological evaluation of the kidneys was performed on separate groups of rats. The animals were sacrificed on day 4 after treatment. Creatinine clearance and proteinuria was recorded on day–1, 0, 1 and 3 to verify that these animals showed the same alterations in kidney function as the animals that were followed for the functional studies for a longer period of time. The kidneys were first perfused with ice-cold saline to remove blood. This was followed by a perfusion fixation. Both perfusions were pressure controlled. Fixative was a solution of 2% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer. Embedding in paraffin as well as staining with haematoxylin and eosin was carried out according to standard procedures. A scoring system for the light-microscopical ranking was used: 0, normal renal architecture; +1, minimal necrosis of tubular cells; +2, moderate to severe tubular necrosis, no proteinic material in the tubules; +4, severe tubular necrosis, proteinic material in tubules.

**Calculations and statistics**

Data are presented as means ± SD. Ratios for the creatinine clearance ($C_{\text{creatinine}}$) and osmotic clearance ($C_{\text{oO}}$) were calculated using the general clearance formula for a substance $y$: $C_y = (\frac{\text{[urine]}}{\text{[plasma]}}) \times \text{urine volume}$. Statistical analysis was performed with SPSS 4.1 (SPSS, Chicago, IL, USA). Statistical evaluation of changes in the excretion rate of urinary analytes was performed with the $t$ test. ANOVA procedures were applied in the follow-up of groups to evaluate whether the variance of the excretion rates of the analytes was influenced by the time point of collection. Significance was defined as $P \leq 0.01$.

**Results**

Cisplatin affected the excretion of several analytes studied. Silibinin administered alone did not affect any of the investigated parameters of renal function. The excretion rates of the various analytes were normalized for body weight. Animals of the cisplatin-treated group had the most pronounced weight loss. However, differences between the groups described below could also be seen when values were compared in a non-normalized way.

**Body weight and total urinary protein excretion**

Cisplatin induced a significant but reversible loss of body weight (Figure 1a). This loss was most pronounced on days 5 and 7. Pretreatment with silibinin ameliorated this reduction in body weight and led to a quicker recovery. Total urinary protein excretion was reversibly elevated on days 3 and 5 after injection of cisplatin. Following treatment with cisplatin and silibinin, proteinuria was not changed in comparison to the control period (day–1) (Figure 1b). Further analysis of total urinary proteins of cisplatin-treated animals in SDS-PAGE revealed increases in both high- and low-molecular-weight protein fraction (data not shown). This indicates combined tubular and glomerular functional alterations.

**Tubular function**

The function of the more distal segments of the tubular apparatus was examined with a determination of the osmotic clearance. Following cisplatin administration a reversible polyuria, associated with a disability to concentrate the urine was observed. On day 1 after treatment, the mean urinary volume in the cisplatin treated animals significantly rose to $22.9 ± 9.8 \text{ ml} \times 24 \text{ h}^{-1} \times 100 \text{ g body weight (bw)}^{-1}$
Urinary osmolality (mosmol • kg H2O⁻¹) dropped significantly in both groups treated with cisplatin. The recovery was slightly faster following pretreatment with silibinin (day 7 versus day 9 in animals given cisplatin alone) (Figure 2a). To assess proximal tubular damage, urinary enzyme excretion was measured. Activity of the brush border enzyme AAP was significantly elevated after cisplatin treatment. Peak values were reached on day 3 (Figure 2b). Rats treated with silibinin and cisplatin also showed an increase. However, this increase was significantly (P<0.01) lower than in the cisplatin treated animals. Three- to fivefold increases in NAG excretion in comparison to the control phase were observed in rats with cisplatin treatment. The increases appeared to be less pronounced following a combined treatment with cisplatin and silibinin. However, statistical significance could only be assigned to the differences on day 7 (Table 1).

Mean fractional magnesium excretion ranged from 10 to 15% of the filtered Mg²⁺ load in the animal groups studied during the control phase (day 1). Following cisplatin administration there was an approximately 2.5-fold increase in magnesium excretion in cisplatin treated animals (Figure 2c). None of the other groups studied showed significant alterations during the follow-up (ANOVA, P>0.01). By day 7 after treatment serum magnesium levels were significantly reduced in animals that only received cisplatin (0.82 ± 0.05 mmol/l on day−1, 0.62 ± 0.13 mmol/l on day 7, P<0.01). No such decline was observed following pretreatment with silibinin.

Glomerular function

Glomerular function was measured by determination of the clearance of endogenous creatinine, BUN levels, and excretion of the extracellular matrix protein fibronectin. Animals treated with cisplatin alone showed a rapid and significant decline of the creatinine clearance which eventually dropped to 0.15 ml x 24 h⁻¹ x 100 g bw⁻¹ on day 3. The group pretreated with silibinin had a lower creatinine clearance during the control phase, but remained stable from day 1 through to the end of the observation period (ANOVA, P>0.01) (Figure 3a).

The alterations in creatinine clearances were concomitant with rises in blood urea levels. Low creatinine
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Table 1. Urinary NAG excretion rates and urea plasma levels

<table>
<thead>
<tr>
<th>Group</th>
<th>NAG (mU/24 h x 100 g bw⁻¹)</th>
<th>Urea (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day -1 Day 3 Day 7</td>
<td>Day -1 Day 3 Day 7</td>
</tr>
<tr>
<td>NaCl</td>
<td>32±11 37±12 52±13</td>
<td>25±2 26±3 25±4</td>
</tr>
<tr>
<td>Silibinin</td>
<td>61±11 55±12 47±11</td>
<td>24±3 25±4 25±3</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>42±15 130±20* 138±41*</td>
<td>26±5 56±18* 38±9*</td>
</tr>
<tr>
<td>Silibinin + cisplatin</td>
<td>49±18 102±41* 89±17*</td>
<td>31±3 31±8 34±6</td>
</tr>
</tbody>
</table>

*P<0.001, bP<0.01 versus day -1 (ANOVA); cP<0.01 versus group treated with cisplatin alone (Student t test).

clearances were reflected by increases in BUN. Following cisplatin treatment urea levels were significantly elevated on days 1–9. Combined treatment with cisplatin and silibinin prevented an increase (Table 1).

In states of unaltered glomerular protein permeability urinary fibronectin reflects the turnover of this protein by glomerular cells. In states of increased protein permeability it is filtered from the serum [25]. The excretion of the extracellular matrix protein fibronectin was significantly elevated on days 5 and 7 after cisplatin treatment (Figure 3b). Because of the mixed glomerular–tubular type of proteinuria in the cisplatin treated animals, it is assumed that fibronectin in these experiments reflects glomerular leakage of plasma fibronectin rather than the turnover of cellular fibronectin.

Histology

The focus of the study was on functional alterations. Morphological examinations at the light-microscopical level were performed to supplement the data derived from functional studies. Structural changes were documented to show that there is a histological correlate to the tubular changes. Figure 4 shows representative examples of changes observed. Following a single i.v. injection of 5 mg/kg cisplatin, histological alterations of the kidney were restricted to the S3 segments of the proximal tubule situated in the outer stripe of the outer medulla. A spectrum of changes was found from condensation of nuclear chromatin to widespread tubular necrosis (histological score +3, Figure 4a). Pretreatment with silibinin protected the kidneys from tubular necrosis. Only slight degenerative changes of the S3 segments occurred and single-cell necrosis was a rare event (histological score +1, Figure 4b). Silibinin alone had no effect on renal morphology. The kidneys did not differ from the ones of rats treated with NaCl alone (Figure 4c).

Discussion

The present study examined the protective effects of the flavonoid silibinin on function and morphology of different nephron segments after treatment with the nephrotoxin cisplatin. The integrity of the glomerulus,
Fig. 4a–c. Histological examination. (a) Areas of severe necrosis of the proximal tubules, 4 days after injection of 5 mg/kg cisplatin. (b) Renal tubular epithelium showing only single cell degeneration (arrows), 4 days after treatment with 200 mg/kg silibinin and 5 mg/kg cisplatin. (c) Saline-treated animal as control. Haematoxylin and eosin, ×300.

the proximal tubule and the thick ascending loop of Henle with the distal tubule was investigated. Cisplatin affected the function of all three nephron segments. It could be shown that pretreatment with a single dose of silibinin significantly ameliorated both proximal tubular and glomerular damage induced by cisplatin.

Within 1 day after cisplatin application, animals showed signs of the well-described acute polyuric renal failure, i.e. polyuria, decreased urinary osmolality and a decrease in glomerular filtration (Figures 2a, 3) [6,26,27]. Polyuria must be attributed to dysfunction at the level of the distal tubule and has previously been linked to an altered release of the antidiuretic hormone [28]. In the experiments described here polyuria was not statistically different between the cisplatin and silibinin plus cisplatin treated groups. Polyuria can therefore not account for the different excretion rates of analytes such as AAP.

Silibinin partly or totally ameliorated cisplatin induced alterations in parameters associated with proximal tubular function. Tubular enzymuria (AAP, NAG) and fractional magnesium excretion were significantly less impaired following pretreatment with silibinin (Figure 2b, Table 1). Hypomagnesaemia, comparable to that observed by Mavichak and co-workers [23], was observed following cisplatin treatment but not after treatment with cisplatin and silibinin. In addition to changes in excretion rates of urinary markers related to the function of the proximal tubule morphological alterations of this nephron segment were observed following cisplatin. These changes, being less pronounced after silibinin treatment, show that the functional changes may have a morphological correlate.

Creatinine clearance was found to be decreased following cisplatin administration. Silibinin prevented this drop (Figure 3a). This parameter was included in the protocol of the study as a marker of glomerular filtration rate (GFR) and glomerular haemodynamics. But from the data obtained it can not be excluded that this decrease and the increases in BUN values (Table 1) are the result of tubular backleak. Acute renal failure, indicated by a reduced creatinine clearance, occurred prior to the development of tubular necrosis in previous studies on cisplatin nephrotoxicity. This finding has been interpreted as a support of the hypothesis that tubular obstruction and/or tubular fluid backleak are not involved in the initiation of acute renal failure in this model of nephrotoxicity [33].

A functional study such as the one presented here can only give limited evidence as to the mechanisms of damage or protection. Tubular defects resulting from cisplatin treatment have been ascribed largely to the generation of free radicals [8]. Cisplatin-induced damage could be increased by depleting cells of protective radical scavengers like glutathione or superoxide dismutase [7,29]. Silibinin possesses anti-oxidant and membrane-stabilizing properties that have already been elucidated in hepatocytes challenged with a variety of radical-generating drugs [13,14,30]. Silibinin showed a dose-dependent protection of hepatocytes in both in-vivo and in-vitro models of phenylhydrazine and ethanol application. In these models, both MDA content and chemically induced chemoluminescence were reduced by silibinin treatment, suggesting a strong antioxidant potential of this drug. In contrast, in ischaemic renal failure induced in dogs, silibinin—but also other compounds such as allopurinol or superox-
ide dismutase—failed to show any protection [31]. Experiments with cisplatin-treated rats given two injections of superoxide dismutase (100 mg) or vitamin E (50 mg), did not show protective effects of these free radical scavengers (data not shown). These findings further support the notion that free radical mediated damage does not play a major role in the model of cisplatin-induced nephrotoxicity used here.

Another possible mechanism of tubular toxicity may result from the intrinsic property of cisplatin to depress DNA, RNA and protein synthesis demonstrated in in-vitro studies [10,11]. Silibinin, in contrast, is known to upregulate the function of DNA-dependent RNA polymerase I in liver cells [32]. This leads to an increased number of ribosomes and it might counteract the decrease in macromolecule synthesis in the kidney.

Flavonoids like silibinin that have anti-inflammatory, antioxidant, and also antiallergic properties, display a remarkable array of biochemical and pharmacological actions [17]. Flavonoids have already been successfully applied in clinical studies on hepatic disorders like alcohol-induced liver diseases [34,35]. Silibinin improved liver function and haematological values in workers chronically exposed to organic solvents [36]. The finding that silibinin is also a protective agent against cisplatin nephrotoxicity suggests that the application of the flavonoid may be clinically advantageous. Other substances like L-methionine or glutathione ester have already been found to be effective in protecting against cisplatin toxicity [37,38]. However, protective agents should not lead to increases in the effective dosage needed for the antitumour activity of cisplatin [39]. In in-vitro experiments with testicular cancer cell lines of human origin we could show that silibinin does not compromise the cytotoxicity of cisplatin [40]. It is therefore suggested that silibinin could be tested in clinical studies on cytostatic-induced nephrotoxic side-effects.

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