NEPHROPROTECTION BY OLEANOLIC AND URSOLIC ACID AGAINST CISPLATIN IS COMPARABLE TO AMIFOSTINE

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ABSTRACT

OBJECTIVES: To compare the protective effects low-dose intravenously administered Oleanolic acid (OA) and Ursolic acid (UA) with intravenous amifostine (AMF) against cisplatin (CP)-induced nephrotoxicity in rats. METHODS: Nephrotoxicity was induced by intraperitoneal injection of CP (5 mg/kg). OA and UA were administered at 0.5, 1.0 and 1.5 mg/kg doses, twice a day, for 5 days post CP injection. AMF was administered intravenously at 90 mg/kg dose 30 minutes prior to CP injection. Serum and urine samples were collected for each group and the rats were sacrificed to collect kidneys for biochemical and histopathological examinations. RESULTS: In CP-treated rats there was significant increase in the serum biomarkers of nephrotoxicity and reduction in serum alkaline phosphatase (ALPase) levels. Kidney homogenates of CP-treated rats revealed increased oxidative stress. Intravenous administration of OA and UA to CP-treated rats reduced oxidative stress by inhibiting depletion of oxidative stress markers from the kidneys. OA and UA treatments dose-dependently inhibited alternations in the serum and urine biomarkers of nephrotoxicity and oxidative stress. The kidney histology of OA and UA treated rats revealed a protection from CP-induced damage. It is noteworthy that OA and UA administered at a considerably lower dose of 0.5, 1 and 1.5 mg/kg twice a day for five days exert protection against CP-induced nephrotoxicity comparable to intravenous AMF. CONCLUSIONS: OA and UA protected the kidney from CP-induced nephrotoxicity by inhibiting oxidative stress and have the potential to use as an adjuvants to main therapy.

KEYWORDS: Cisplatin-induced nephrotoxicity; Oleanolic acid; Ursolic acid; Amifostine;
Nephroprotection.

INTRODUCTION
Orally administered oleanolic acid (OA) and ursolic acid (UA) exert protective effects against nephrotoxicity induced by gentamicin, paracetamol and streptozotocin in experimental animals. However, a recent study on pharmacokinetics of OA reports that oral bioavailability of OA is 0.7%\(^1\). Poor aqueous solubility of OA and UA has till date restricted the evaluation of their biological activity through intravenous administration. Different approaches including formulations of liposome to improve bioavailability of OA and UA are under investigation\(^2,3\). Recently, N, N-dimethylacetamide (NND) is reported a as a suitable solvent for the intravenous administration of OA. This provides an opportunity to re-evaluate the efficacy of OA and UA as protectants against drug induced nephrotoxicity.

Clinical use of higher doses of cisplatin (CP) in treating cancers is limited due to its dose dependent nephrotoxicity\(^4,5\). The mechanisms of CP-induced nephrotoxicity include formation of reactive oxygen species (ROS) and depletion of renal antioxidant defense system\(^6,7\). Numerous pharmacological strategies like diuresis\(^8\), pre-treatment with antioxidants and organ protective drugs like AMF improve therapeutic index of CP\(^9\). In present study, protective effects of intravenously administered OA and UA were evaluated against CP-induced nephrotoxicity in rats.

MATERIALS AND METHODS
OA and UA were purchased from 3W Botanical Extract Inc., Changsa, Hunan, China. Purity of both the compounds was confirmed by HPLC analysis. CP, AMF and 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Neprod Life Sciences Pvt. Ltd. Mumbai, Dabur India Ltd. Mumbai, and Himedia Laboratories, Mumbai, India respectively. Phosphoric acid and \(N\)-\(N\)-dimethyl acetamide (NND) were purchased from Sigma-Aldrich. Tween 80 and PEG 400 were purchased from Merck Ltd., Bangalore, India. Biochemical estimation kits were purchased from Span Diagnostic Ltd, Surat, India and Agappe Diagnostic Pvt. Ltd., Mumbai, India. Other chemicals used in the study were of analytical grade.

Experimental animals
Healthy male Wistar rats (n = 63) of 200-250 g body weight were used in this study. The rats were housed in polypropylene cages under standard conditions (12 hrs light / dark cycles, at
22 ± 2°C and 35 - 60% humidity). Standard palletized feed (Amrut Pellets for rat, Pune, India) and tap water were provided ad libitum. The study was approved by Institutional Animal Ethical Committee.

**Intravenous administration of oleanolic acid and ursolic acid to rats**

For intravenous administration of OA and UA, a mixture of NNDA: PEG 400: Tween 80: Normal saline (1:4:2:3) and then this whole solution was diluted to 3 ml with normal saline. OA and UA were administered 30 minutes prior to cisplatin through tail vein at interval of 12 hours to maintain constant plasma concentration.

**Experimental design**

The rats were randomized and allocated to 9 groups, each containing 7 rats. The labels and treatments for the groups were as follows

- **Vehicle treated**: vehicle administered at 2.5 ml / kg dose intravenously
- **CP-treated**: intraperitoneal CP (5 mg/kg)
- **AMF-treated**: single dose of AMF (90 mg/kg, i.v.) administered thirty minutes prior to CP (5 mg/ kg, i.p.) injection
- **Low dose-OA**: OA (0.5 mg/kg, i.v.) twice a day for 5 days starting from 30 minutes prior to CP (5 mg/kg, i.p.) injection
- **Medium dose-OA**: OA (1.0 mg/kg, i.v.) twice a day for 5 days starting from 30 minutes prior to CP (5 mg/kg, i.p.) injection
- **High dose-OA**: OA (1.5 mg/kg, i.v.) twice a day for 5 days starting from 30 minutes prior to CP (5 mg/kg, i.p.) injection
  
  (OA was administered between 8.00 to 9.00 am and between 8.00 to 9.00 pm every day.)

- Low dose, medium dose and high dose-UA treated groups received doses of UA and CP similar to OA treated groups. On the fifth day, blood samples were collected from all the rats through tail vein and processed for separation for serum and determination creatinine, urea, blood urea nitrogen, total protein, albumin and alkaline phosphatase (ALPase) levels and then individual rats were placed in the metabolic cages for collection of urine samples. During urine collection, rats had access to water but not food. Initial 5 hours urine samples were used for determination of creatinine, urea, total protein, albumin and ALPase levels. Urine collection was continued for next 19 hours for determination of total urine output in 24 hours. At the end of the treatments, blood samples were collected from all the rats through tail vein and processed for separation of serum and determination of biochemical parameters. Rats
were sacrificed by cervical dislocation and kidneys were quickly separated, right kidney was kept in ice cold normal saline at -20ºC until analysis and left kidney was preserved in 10% neutral formaldehyde solution for histopathological examination by light microscopy.

**Homogenization of the kidney tissues**

Homogenization of kidney tissue was carried out in a Teflon - glass homogenizer with a buffer containing 1.5% KCl to obtain 1:10% (w/v) using a homogenizer (Remi motors, Mumbai, India). Estimation of GSH was performed by Ellman’s reagent method whereas the LPO and catalase were performed.

**Histopathological examination**

The kidneys were excised after sacrifice and fixed in 10% formalin, embedded in paraffin and sectioned using microtome. The sections of 5 μm thickness were stained according to haematoxyline eosin staining protocol. Histological alteration in the kidney sections were graded by a blinded observer.

**Statistical analysis**

The parameter values are expressed as mean ± S.E.M and compared for difference in the central tendencies by Analysis of variance (ANOVA) followed by Bonferroni’s post-test. Each study group contained 6 animals (n=6). Statistical analysis was performed using Graphpad prism 5.0.

**RESULTS**

**OA and UA inhibit alterations in serum indicators of CP-induced nephrotoxicity**

Serum creatinine and Blood Urea Nitrogen (BUN) levels were significantly increased in CP-treated group as compared to vehicle treated group (p < 0.05). Treatment of rats with high dose of OA and UA prevented the CP-induced increase in BUN and serum creatinine (p < 0.001; p < 0.01). Low and medium doses of UA and OA had no significant effect on the CP-induced rise in these parameters (Table 1). CP treatment induced significant decrease in the serum albumin, serum protein and ALPase activity. Treatment of CP-treated rats with high dose OA and UA inhibited such alterations in these parameters. The medium doses of OA and UA significantly inhibited the change in serum ALPase activity. The effect of medium dose on serum albumin and serum protein levels was not statistically significant (Table 1). The inhibitory effects of low dose OA and UA on CP-induced alterations in serum albumin, serum protein and ALPase activity were not statistically significant.
Table 1: OA and UA inhibit alterations in serum indicators of CP-induced nephrotoxicity

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Serum creatinine (mg dl(^{-1}))</th>
<th>BUN (mg dl(^{-1}))</th>
<th>Serum ALPase (U/l)</th>
<th>Serum albumin (gm dl(^{-1}))</th>
<th>Serum protein (gm dl(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>1.2 ± 0.2</td>
<td>35.0 ± 2.5</td>
<td>321.2 ± 48.3</td>
<td>5.4 ± 0.6</td>
<td>5.4 ± 0.5</td>
</tr>
<tr>
<td>AMF-treated</td>
<td>1.7 ± 0.2</td>
<td>59.4 ± 6.6</td>
<td>550.9 ± 81.2</td>
<td>3.8 ± 0.4</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>CP-treated</td>
<td>3.1 ± 0.3</td>
<td>139.5 ± 26.7</td>
<td>150.3 ± 26.0</td>
<td>1.7 ± 0.3</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>Low dose OA</td>
<td>2.6 ± 0.3</td>
<td>98.4 ± 17.6</td>
<td>646.2 ± 46.6(*)</td>
<td>2.2 ± 0.2</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>Medium dose OA</td>
<td>2.1 ± 0.3</td>
<td>75.6 ± 14.7</td>
<td>528.4 ± 25.0(**)</td>
<td>3.0 ± 0.2</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>High dose OA</td>
<td>1.6 ± 0.3(**)</td>
<td>48.9 ± 8.1(**)</td>
<td>411.3 ± 54.8(**)</td>
<td>4.0 ± 0.3(**)</td>
<td>5.0 ± 0.5(**)</td>
</tr>
<tr>
<td>Low dose UA</td>
<td>2.5 ± 0.2</td>
<td>93.1 ± 21.8</td>
<td>695.8 ± 60.1(**)</td>
<td>1.9 ± 0.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Medium dose UA</td>
<td>1.9 ± 0.2(*)</td>
<td>58.3 ± 10.6(*)</td>
<td>560.7 ± 66.3(**)</td>
<td>2.5 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>High dose UA</td>
<td>1.5 ± 0.2(**)</td>
<td>35.8 ± 4.1(**)</td>
<td>403.6 ± 42.1(**)</td>
<td>3.2 ± 0.3</td>
<td>3.7 ± 0.5</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M. of six rats for each group. Statistical analysis: One way - ANOVA followed by Bonferroni’s Multiple Comparison test. *** p < 0.001, ** p < 0.01 as compared to the CP-treated group.

Table 2: Intravenous OA, UA and AMF normalize kidney weight and urine biochemistry in CP-treated rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Relative kidney weight</th>
<th>Urine output (ml/24 hrs)</th>
<th>Urea (mg dl(^{-1}))</th>
<th>Creatinine (mg dl(^{-1}))</th>
<th>Albumin (gm dl(^{-1}))</th>
<th>Urine protein (gm dl(^{-1}))</th>
<th>ALPase (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>0.34±0.01</td>
<td>7.8±0.5</td>
<td>14.5±0.5</td>
<td>11.6±0.3</td>
<td>5.1±0.9</td>
<td>79.1±13.9</td>
<td>274.0±25.2</td>
</tr>
<tr>
<td>AMF-treated</td>
<td>0.29±0.05</td>
<td>8.3±0.4</td>
<td>35.1±4.2</td>
<td>14.8±0.6</td>
<td>6.5±1.6</td>
<td>47.2±9.9</td>
<td>206.4±20.5</td>
</tr>
<tr>
<td>CP-treated</td>
<td>0.41±0.02</td>
<td>10.7±0.6</td>
<td>42.7±3.5(**)</td>
<td>25.3±1.1</td>
<td>1.7±0.3</td>
<td>17.7±4.6</td>
<td>97.57±11.5</td>
</tr>
<tr>
<td>Low dose OA</td>
<td>0.35±0.02</td>
<td>9.2±1.1</td>
<td>16.5±0.9(**)</td>
<td>22.2±0.5</td>
<td>2.8±0.7</td>
<td>34.1±10.5</td>
<td>130.6±9.3</td>
</tr>
<tr>
<td>Medium dose OA</td>
<td>0.41±0.02</td>
<td>8.8±0.4</td>
<td>18.8±0.4(**)</td>
<td>18.1±0.6(**)</td>
<td>4.6±0.7</td>
<td>37.6±10.5</td>
<td>168.9±17.3</td>
</tr>
<tr>
<td>High dose OA</td>
<td>0.40±0.04</td>
<td>8.5±0.6</td>
<td>18.1±0.7</td>
<td>16.6±1.0(**)</td>
<td>4.7±0.8</td>
<td>34.8±9.6</td>
<td>203.4±22.1</td>
</tr>
<tr>
<td>Low dose UA</td>
<td>0.50±0.03</td>
<td>9.0±1.4</td>
<td>41.7±2.7</td>
<td>23.0±0.8</td>
<td>2.9±0.8</td>
<td>39.0±8.2</td>
<td>120.8±12.5</td>
</tr>
<tr>
<td>Medium dose UA</td>
<td>0.38±0.02</td>
<td>8.5±0.3</td>
<td>18.9±0.6(**)</td>
<td>19.6±0.9 (**)</td>
<td>5.4±0.7</td>
<td>38.4±9.5</td>
<td>150.9±14.1</td>
</tr>
<tr>
<td>High dose UA</td>
<td>0.39±0.04</td>
<td>8.3±0.8</td>
<td>19.2±0.4(**)</td>
<td>16.6±1.1(**)</td>
<td>4.4±0.5</td>
<td>42.3±8.5</td>
<td>177.9±19.4</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M (n = 6) Statistical analysis was performed using one way - ANOVA followed by Bonferroni’s Multiple Comparison test. Values represent percentage change to control. ***p < 0.001, **p < 0.01 as compared to CP-treated group.
Intravenous OA, UA and AMF normalize kidney weight and urine biochemistry in CP-treated rats

Urine creatinine and BUN were significantly increased in CP-treated group as compared to vehicle treated group (p < 0.001). The study groups that received low, medium and high dose of OA and UA were protected from such rise in the urine creatinine and BUN in a dose dependant manner (Table 2). Urine levels of albumin, protein and ALPase in urine were significantly decreased in the CP-treated group. Both OA and UA treatments reversed these changes however the effects of only high dose OA was statistically significant (Table 2).

CP-induced oxidative stress is reduced by intravenous OA and UA treatment

Alteration in the GSH, SOD and CAT levels are expressed in terms of percentage of vehicle treated group readings (Table 3). Treatment with CP induced a significant decrease in the levels of SOD, CAT and GSH in the kidney tissues (p < 0.05). Treatment with high dose of OA and UA causes significant changes in the activities of GSH, SOD and CAT compared to vehicle treated group. A significant recovery relating to SOD, CAT and GSH was observed in response to high dose of OA and UA along with cisplatin. The lipid peroxidation- estimated in terms of TBARs was significantly increased in the CP-treated group. High dose OA and UA inhibited such rise in the TBARs in the kidney tissues (Table 3).

Table 3: CP-induced oxidative stress is reduced by intravenous OA and UA treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD</th>
<th>CAT</th>
<th>GSH</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle treated</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>AMF-treated</td>
<td>83.7 ± 1.6 ***</td>
<td>80.1 ± 4.1 **</td>
<td>80.7 ± 5.4</td>
<td>126.5 ± 4.5</td>
</tr>
<tr>
<td>CP-treated</td>
<td>41.9 ± 1.2 ***</td>
<td>40.5 ± 3.2 ***</td>
<td>41.6 ± 10.0 ***</td>
<td>207.4 ± 13.0 ***</td>
</tr>
<tr>
<td>Low dose OA</td>
<td>52.9 ± 2.6 ***</td>
<td>47.4 ± 4.3 ***</td>
<td>79.8 ± 3.8</td>
<td>158.9 ± 12.3 *</td>
</tr>
<tr>
<td>Medium dose OA</td>
<td>69.5 ± 2.2 ***</td>
<td>70.4 ± 4.2 ***</td>
<td>86.4 ± 5.4</td>
<td>135.6 ± 8.8</td>
</tr>
<tr>
<td>High dose OA</td>
<td>77.8 ± 1.9 ***</td>
<td>84.5 ± 3.4</td>
<td>85.2 ± 3.7 ***</td>
<td>112.3 ± 10.3</td>
</tr>
<tr>
<td>Low dose UA</td>
<td>45.2 ± 2.3</td>
<td>43.9 ± 1.5 ***</td>
<td>75.3 ± 4.5 **</td>
<td>162.5 ± 13.0 **</td>
</tr>
<tr>
<td>Medium dose UA</td>
<td>59.0 ± 3.6 ***</td>
<td>58.5 ± 3.5 ***</td>
<td>78.4 ± 3.3</td>
<td>143.3 ± 12.7</td>
</tr>
<tr>
<td>High dose UA</td>
<td>67.6 ± 1.6 ***</td>
<td>71.6 ± 4.4 ***</td>
<td>81.8 ± 4.0</td>
<td>139.4 ± 15.2</td>
</tr>
</tbody>
</table>

Data represent means ± S.E.M.  (n = 6). Statistical analysis was performed using one way ANOVA followed by Bonferroni’s Multiple Comparison test; ***p < 0.001, **p < 0.01 as compared to CP-treated group.
Histopathology

All the CP-treated animals developed kidney lesions that could be identified by microscopic evaluation as shown in figure (Fig. 1). The characteristics of these lesions include tubular necrosis, glomerulosclerosis, glomerular congestion, hemorrhages and edema formation. The data on these lesions are summarized in Fig. 1. The data of lesions were obtained from one animal from each group.

DISCUSSION

OA and UA are extensively investigated through preclinical and clinical studies for their anti-inflammatory, anti-diabetic and anticancer potentials\textsuperscript{10, 11, 12}. The rise in interest in the activities of these compounds is due to their low toxicity and abundant availability in the plant kingdom\textsuperscript{13,14}. Both these triterpenoids possess potent antioxidant activities and exert organ-protective effects against oxidative toxicants\textsuperscript{15, 16, 17}. Orally administered OA (40, 60
and 80 mg/kg/day for 7 days) protects rats against gentamicin-induced nephrotoxicity. However, recently developed data on the pharmacokinetics of OA has revealed that orally administered OA has very low bioavailability (0.7%). In present study, protective effects of intravenously administered OA and UA solutions against CP-induced nephrotoxicity were evaluated. The solvent used for intravenous administration was constituted using NNDA, PEG-400 and saline. The amount of NNDA that was injected per rat per day was even less than 6 mg/day. Intravenous administration of NNDA at this dose level is reported to be safe and within limits of 10.9 mg/day (CDER Feb., 2012). For solubilization of other drugs like rapamycin, paclitaxel, betulinic acid, NNDA and PEG-400 have been used.

The 0.5, 1.0 and 1.5 mg/kg doses of OA and UA were selected considering that the plasma levels of OA after intravenous administration of 2.0 mg/kg dose are about seven times higher than the levels achieved after oral administration of 50 mg/kg of OA.

CP is a potent anticancer drug that is administered by intravenous route and use of its higher doses is restricted because of its nephrotoxicity. As the cancer patients are accessible by intravenous route, there is need of protective agents that can be administered by intravenous route. Presently available therapy against CP-induced nephrotoxicity is prior intravenous administration of AMF. It is expected that the organ protectors administered with a cancer chemotherapeutical agent should not interfere with its anti-cancer activity. Both UA and OA are known to possess anticancer potentials. Hence, in present study, protective effects of intravenously administered OA and UA against CP-induced nephrotoxicity are evaluated.

CONCLUSION

The results of this study indicate that intravenously administered OA and UA provide significant protection against the CP-induced nephrotoxicity. The effects of 1mg/kg and 1.5 mg/kg of OA and UA administered twice a day for five days were comparable with the single dose of AMF administered 30 minutes prior to the CP injection. The dose level at which OA and UA have shown the protective effects indicates that these drugs may be further evaluated for their protective activity against CP-induced nephrotoxicity.

REFERENCE


