Oxytocin alleviates cisplatin-induced renal damage in rats

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ARTICLE INFO	ABSTRACT
	Objective(s): The purpose of the present study was to investigate the protective effect of oxytocin on cisplatin (CP)-induced renal damage in rats. *Materials and Methods:* Fourteen adult Sprague Dawley rats, weighing 200 to 210, were administered by cisplatin (CP, 2 mg/kg/day) twice a week for five weeks. Then, they were randomly divided into two groups and treated with either saline (1 ml/kg/day) or OT (200 µg/kg/day) for five weeks. Seven rats served as control group. At the end of the treatment period, animals were sacrificed and their kidneys were assessed histologically. In addition, C-reactive protein (CRP), TGF-β and Akt expression were evaluated immunohistochemically. *Results:* Both tubules and glomeruli were found to be severely damaged with marked medullary tubulo-interstitial inflammation due to chronic cisplatin exposure, particularly in the saline-treated group (Group 1) compared to control group. Oxytocin treatment spared renal tissue significantly by suppressing CRP and TGF-β, and enhancing Akt expression. *Conclusion:* We conclude that renal damage due to cisplatin toxicity was prevented to a great extent by the anti-inflammatory effect of oxytocin.

Introduction

Cisplatin (CP) is an alkylating agent widely used in cancer treatment. It inhibits transcription-replication by covalently binding to DNA, RNA and proteins in the cell. This agent is one the major chemotherapeutics, selectively preferred in treating testis, ovary, prostate, head-neck tumors and osteosarcomas (1, 2). Being also ototoxic and neurotoxic, cisplatin is known to be primarily nephrotoxic, limiting its high dose applications (3, 4). Its tissue destructive feature operates via apoptosis and necrosis following DNA and protein damage (5). Cisplatin induces additive oxidative stress mostly owing to over-expression of the reactive oxygen species (ROS), such as superoxide anion (6-8). It also disturbs and degrades renal capillaries leading to glomerular and tubular damage (9). This agent is the major cause of inflammation triggering nuclear factor-kappa B (NF-κB) activity in the kidney, thus leading to over-expression of tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), monocyte chemo-attractant protein-1 (MCP-1), intercellular adhesion molecule (ICAM), hemoxygenase-1, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (10). Cisplatin causes lymphocyte, TGF-β and TNF-α releasing macrophage infiltration, and resultant fibrosis in the renal medullary peritubular interstitial zone. These cytokines participate in matrix formation from myofibroblastic cells (11-13).

Consisting of nine amino acids and secreted by the hypothalamic supraoptic and paraventricular nuclei, oxytocin (OT) is a peptide hormone exerting its physiological and biological actions via its G-protein coupled receptor. OT receptors are widespread in the nervous system, vascular smooth muscle, myocard, thymus, pancreas, and adipocytes (14-16). It has anti-oxidative, anti-inflammatory, and anti-apoptotic effects (17-20). Recently, we have shown its beneficial effects against rotenone-induced Parkinson's disease and sepsis-induced polyneuropathy in rats (19, 20).

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Hoping to contribute to the attempts for ameliorating the highly adverse and dose-limiting features of the most widely prescribed chemotherapeutic, cisplatin and optimizing the relevant cancer treatment protocols, we hypothesized that OT maybe of benefit in protection of the rat renal tissue during chronic exposures to this agent. We designed this preliminary experimental study to "translate" its outcomes for clinical implications in the future.

Materials and Methods
Animals

Twenty-one adult Sprague Dawley rats, weighing 200–220 g were used in this study. Animals were housed in groups of four in cages and maintained under standard conditions with 12/12 hr light/dark cycle at room temperature ($22 \pm 2°C$). They were fed by standard pellet diet and tap water *ad libitum* along the study. The protocol employed in this study was approved by the Institutional Animal Care and Ethical Committee of the University of Ege.

Experimental protocol

Seven rats were grouped as naive control (NC), and randomly chosen 14 rats were injected IP. by 2 mg/kg/day cisplatin (Sisplatin Flakon, Kocak Farma), twice a week for 5 weeks. Total cisplatin dose was 20 mg/kg/rat. Seven randomly chosen cisplatin (CP) animals were also injected IP every day by 200 µg/kg/day OT (oxytocin, Pituisan®, Ege Vet, Alfasan International B.V., Holland) for five weeks (CP+OT) while the other seven cisplatin (CP) animals received 1 ml/kg/day, IP saline (%0.9 NaCl) in the same regime (CP+S). During the treatment period, all animals monitored daily for health conditions. Then, they were sacrificed and their kidneys were resected for histological and immunohistochemical evaluations.

Histopathological examination of kidney

For histological and immunohistochemical studies, all animals were anesthetized by ketamin (40 mg/kg, Alfamine®, Alfasan International BV, Holland) and xylazine (4 mg/kg, Alfazyne®, Alfasan International B.V., Holland) IP and perfused with 200 ml of 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS). Formalin-fixed kidney sections (4 µm) were stained with hematoxylene and eosine. All sections were photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope.

Morphological evaluation was performed by computerized image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA) on 10 microscopic fields per section examined at a magnification of ×20, by the observer blind to the study group.

Kidney sections from every rat in all groups were evaluated semi-quantitatively in terms of the extent of tubular necrosis/atrophy, glomerular damage, and interstitial inflammation by being rated as follows: 0-5% = score 0; 6-20% = score 1; 21-40% = score 2; 41-60% = score 3; 61-80% = score 4; and 81-100% = score 5 (21, 22).

Akt, CRP and TGF-β immunohistochemistry

For immunohistochemistry, sections were incubated with H_2O_2 (10%) for 30 min to eliminate endogenous peroxidase activity and blocked with 10% normal goat serum (Invitrogen) for 1 hr at room temperature. Subsequently, sections were incubated in primary antibodies against Akt, CRP and TGF-β (Bioss, Inc.; 1/100) for 24 hr at 4°C. Antibody detection was performed with the Histostain-Plus Bulk kit (Invitrogen, Inc.) against rabbit IgG, and 3,3' diaminobenzidine (DAB) was used to visualise the final product. All sections were washed in PBS and photographed with an Olympus C-5050 digital camera mounted on Olympus BX51 microscope. Brown cytoplasmic staining was scored positive for immune-expression. The number of immune-expression positive cells was assessed systematically, scoring at least 50 glomeruli and tubuler cells per field in 10 fields of tissue sections at a magnification of 100x.

Statistical analysis

All data were analyzed by non-parametric (Mann-Whitney U) test. Data are presented as mean values ± standard error of the mean (SEM). *P*-values of 0.05 or less were regarded as statistically significant.

Results
Histopathological evaluation

Figure 1 demonstrates the histopathological alterations in renal sections. Glomerular structure shrinkage was prominent in all CP rats, with the CP+S group showing the highest damage possibly owing to apoptosis and necrosis. Moreover, the CP+S group's slides displayed intensive and extensive tubular and peritubular mononuclear cell infiltration. Peritubular fibrotic areas and tubular atrophy could also be traced in the same animals (Figure 1). CP+OT group rats' sections displayed significantly much less glomerular and tubular damage compared to the saline group (*P*<0.005 and *P*<0.05, respectively; Table 1). The most striking difference was observed in the tubulo-interstitial areas that expressed significantly much less inflammation than those of the saline treated animals (*P*<0.005, Table 1).
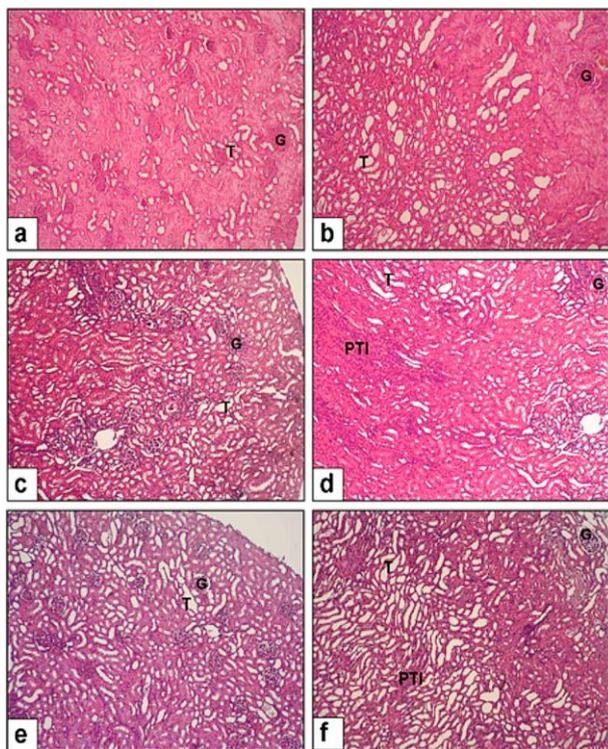
Figure 1. Evaluation of renal damage in cisplatin-induced toxicity. All images 10X magnification. (a-b) NC group displayed normal glomerular (G) and tubular (T) structure,(c-d) CP+S group developed severe glomerular (G) damage and peritubular infiltration (PTI), (e-f) CP+OT group displayed much less peritubular mononuclear cell infiltration with relatively spared tubular structure and much less apparent peritubular fibrosis
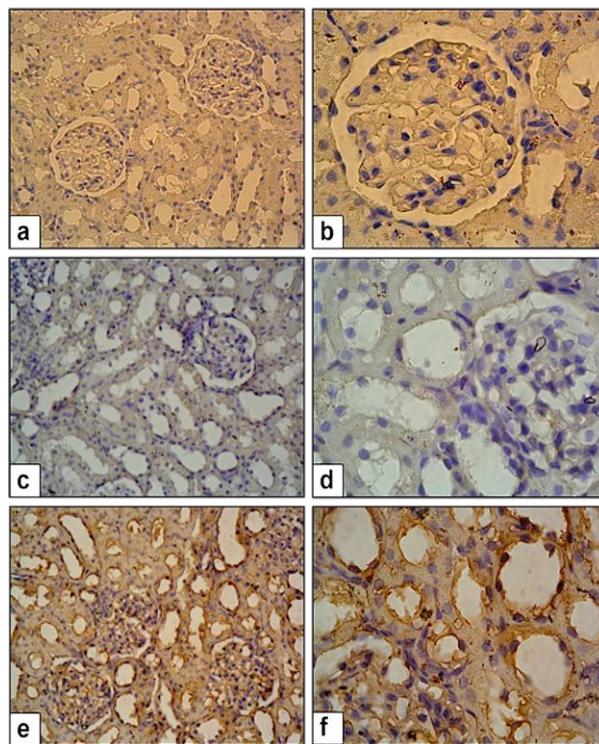

Figure 2. Akt expression in control and study groups. (a-b) NC group. (c-d) CP+S group, (e-f) CP+OT group enhanced Akt expression. 40X and 100X magnification
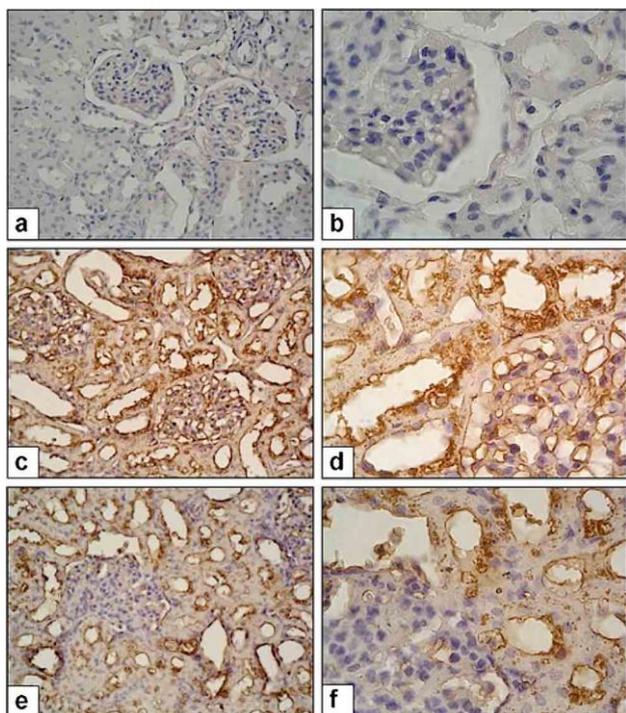

Figure 3. CRP expression in control and study groups. (a-b) NC Group, (c-d) CP+S group, CRP expression is significantly higher than NC group, (e-f) CP+OT group expression is significantly lower than CP+S group. 40X and 100X magnification
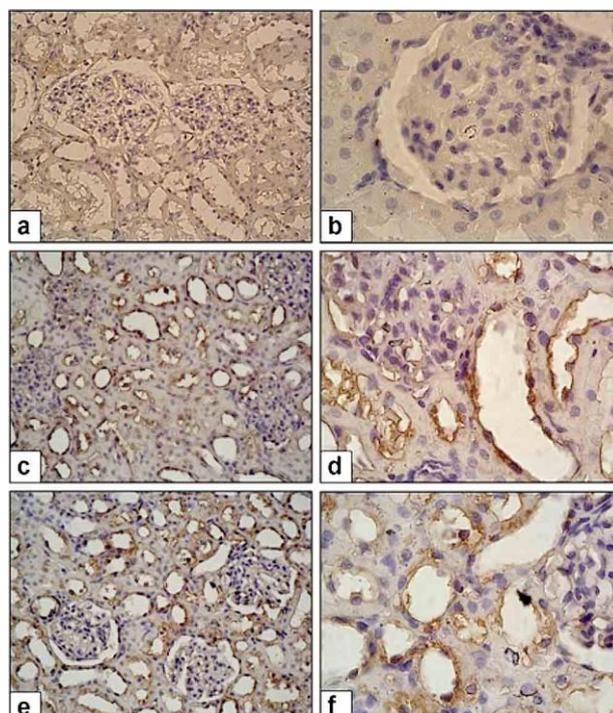

Figure 4. TGF-β expression in control and study groups. (a-b) NC group, (c-d) CP+S group, TGF-β expression is higher than NC group, (e-f) P+OT group, TGF-β expression is lower than CP+S Group. 40X and 100X magnification

Table 1. Comparison of the group average scores in tubular necrosis /atrophy, glomerular damage, and interstitial inflammation

	Tubular necrosis /atrophy	Glomerular damage	Interstitial inflammation
NC	ND	ND	ND
CP+S	4.16 ± 0.30	4.5 ± 0.22	4.33 ± 0.21
CP+OT	2.33 ± 0.21 **	3.16 ± 0.30 *	1.66 ± 0.42 **

Data were expressed as mean±SEM. NC: naive control, ND: not detected, CP: Cisplatin, S: Saline, OT: Oxytocin
** *P*<0.005, CP+OT vs. CP+S group
*   *P*<0.05, CP+OT vs. CP+S group

Immunoexpression of Akt, TGF- β and CRP

Figure 2 shows the Akt immunostaining in renal sections. Also, the ratios of Akt (+) cells in renal sections for all groups are given in Table 2. CP+OT rats had significantly higher Akt (+) cells than CP+S group (*P*<0.01, Table 2). Figure 3 and 4 illustrate the immunoexpression of CRP and TGF-β in renal sections of all groups. As summarized in Table 2, CP+S rats had significantly higher TGF-β (+) and CRP (+) cells than in the naïve control group (*P*<0.01, Table 2). Treatment of CP with OT (CP+OT group) significantly diminished TGF- β and CRP immunexpression when compared with saline treated (CP+S) group. (*P*<0.01 and *P*<0.05, repectively; Table 2).

Discussion

The present study demonstrated that OT attenuated the renal tubulo-interstitial fibrosis-induced by chronic cisplatin treatment in the rat. Additionally, OT also ameliorated tubular injury by decreasing the cisplatin associated renal over-expression of TGF-β1 and CRP, meantime, activating Akt signaling. OT treatment significantly reduced interstitial macrophage infiltration thus leading to suppression of inflammation and cytotoxicity induced by cisplatin. These findings suggest that the nephro-protective effects of OT are likely to be achieved, at least partly, via suppression of TGF-β1/Smad2 signaling pathways and the resultant relative anti-apoptotic activity in these rats.

Cisplatin, a major anti-neoplastic drug of solid tumors, has multiple intracellular effects, such as direct toxicity with reactive oxygen species, enhancement of mitogen activated protein kinases (MAPK), triggering apoptosis, and stimulating inflammation and fibrogenesis (23, 18). Cisplatin-induced renal injury is characterized by a series of ultra-structural changes, including tubular necrosis, loss of microvilli, alterations in number and size of lysosomes, and mitochondrial vacuolization (23).

Many potential therapeutic approaches targeting these steps for the prevention of cisplatin-induced renal injury has been extensively studied (23, 18). In particular, recent evidence indicates that inflammation has an important role in the pathogenesis of cisplatin induced renal injury. In the present study, we observed that oxytocin significantly decreased intra-renal CRP and TGF-β1 expression in accordance with the dosage administered, which was paralleled by a significant attenuation of tubulo-interstitial fibrosis at 4 wk. Our results suggest that oxytocin suppresses the progression of tubulo-interstitial inflammation and tubulo-interstitial fibrosis through a mechanism involving decreased CRP and TGF-β1 expression in cisplatin-induced nephropathy.

Excessive deposition of extracellular matrix (ECM) in the tubulo-interstitium causes decline in renal function in progressive renal disease (24). Epithelial-mesenchymal transition (EMT) of tubular epithelial cells, characterized by loss of epithelial cell characteristics and gain of ECM-producing myofibroblast characteristics, is associated with tubulointerstitial fibrosis. Tubular EMT can be stimulated by TGF-β1 (25-27) advanced glycation end products (28, 29), and angiotensin II (30). However, TGF-β1 is probably the key inducer of EMT because TGF-β1 signaling is sufficient to induce EMT in cultured epithelial cells (26, 27). In a normal rat tubular epithelial cell line, NRK-52E; Li *et al* (31) found that TGF-β1-induced Smad 2 phosphorylation resulted in stimulation of EMT with the loss of E-cadherin, and *de novo* expression of α-SMA and collagens I, III, and IV. Moreover, they observed that over-expression of Smad 7 resulted in inhibi-tion of TGF-β1-induced Smad 2 activation with the

Table 2. Quantitative comparison of Akt, C-reactive protein expression and Transforming growth factor-beta immunoexspressions

	Akt expression (%)	CRP expression (%)	TGF-β expression (%)
NC	10.4 ± 2.3	6.2 ± 3.5	5.8 ± 3.2
CP+S	12.6 ± 3.4	38.6 ± 5.6 *	43.2 ± 5.4 *
CP+OT	56.8 ± 7.2 ††	20.8 ± 4.8 †	22.6 ± 2.1 ††

Data were expressed as mean ± SEM. NC: naive control, ND: not detected, CP: Cisplatin, S: Saline, OT: Oxytocin, TGF: Transforming growth factor, CRP: C-reactive protein

* *P*<0.001, CP+S vs. NC group
† *P*<0.05, CP+OT vs. CP+S group
†† *P*<0.01, CP+OT vs. CP+S group

prevention of EMT and collagen synthesis. TGF-β1 induced EMT in renal tubular epithelial cells, directly through activation of MAPK and indirectly through extra-cellular signal regulated kinases (ERKs), directed Smad 2 phosphorylation (31, 32).

Stuveling *et al* demonstrated that CRP is an inflammatory biomarker and a strong predictor of renal function abnormalities in humans (33). The production of CRP is presumably restricted to the liver, but a recent study has suggested that the kidney may be a second site of CRP formation (34).

Padilla *et al* showed that rat CRP, similarly to human CRP, can activate autologous complement (35). This finding suggests that biological functions of CRP are conserved among species. In this study, we evaluated intrarenal CRP expression to test anti-inflammatory effects of OT on cisplatin-induced renal injury. The results of this study revealed that OT treatment decreased cisplatin-induced over-expression of CRP expression. On the basis of present and previous studies, we propose that the attenuation of tubulo-interstitial inflammation by OT treatment in cisplatin treated rat kidneys may be related, in part, to the decrease in intrarenal CRP expression.

In this study, we showed that OT has cytoprotective effects against cisplatin-induced tubular toxicity and these effects are mediated by the anti-apoptotic effects of OT and involve Akt activation. Cell fate also depends on the activation of anti-apoptotic pathways these results from loss of equilibrium between apoptotic and anti-apoptotic mediators. Of note, among the signaling pathways that participate in the inhibition of apoptosis, the phosphatidylinositol 3 phosphate kinase (PI3K)/Akt axis is considered a master regulator of cell survival and activating mutations of this pathway are widely implicated in numerous cancer types (36). PI3K activation causes Akt phosphorylation, and then anti-apoptotic mediators such as Bcl-xl and X-linked inhibitor of apoptosis protein (XIAP) are activated (37, 38).

Our study neatly showed that OT has been protective during chronic cisplatin-induced nephro-toxicity and interstitial fibrosis. For the first time in literature, we thereby identified this peptide's anti-apoptotic activity specifically attributing its operating mechanism via Akt signaling. We believe that this is of importance because of being strongly suggestive that OT could directly activate anti-apoptotic signaling at the cellular level. We reckon that these present findings referring to a receptor mediated activity could be an important additive actor involved in the other previously proposed/defined potent tissue protection mechanisms of OT.

Conclusion

In summary, OT treatment decreased renal TGF-β1 expression and reduced tubulo-interstitial fibrosis in the rats. CRP and macrophage infiltration was strongly attenuated by OT in this animal model of cisplatin induced renal damage. Moreover, the anti-inflammatory and anti-fibrotic effects of OT were accompanied by suppression of TGF-β1/Smad2 in the cisplatin-injured kidneys. Our data suggest that OT protects rat kidneys against cisplatin nephro-toxicity through anti-apoptotic mechanisms. Therefore, we conclude that our results further imply that administration of OT may slow the progression of tubulo-interstitial nephritis by inhibiting the inflammatory and fibrotic processes.

Declaration of interest

The authors report no declaration of interest.

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