

# Enhancement of Cisplatin Nephrotoxicity by Morphine and Its Attenuation by the Opioid Antagonist Naltrexone

Atefeh Aminian<sup>1,4</sup>, Shiva Javadi<sup>1</sup>, Reza Rahimian<sup>1,2</sup>, Ahmad Reza Dehpour<sup>1,2</sup>, Fahimeh Asadi Amoli<sup>3</sup>, Payman Moghaddas<sup>1</sup>, and Shahram Ejtemaei Mehr<sup>1</sup>

<sup>1</sup> Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

<sup>2</sup> Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran

<sup>3</sup> Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

<sup>4</sup> Department of Pharmacology, School of Medicine, Arak University of Medical Sciences, Arak, Iran

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**Abstract-** Nephrotoxicity is a major side effect of cisplatin, a widely used chemotherapy agent. Morphine and other opioids are also used extensively in different types of cancer for the clinical management of pain associated with local or metastatic neoplastic lesions. In addition to its analgesic effects, morphine has also been reported to possess potential immunomodulatory and antioxidant properties. Herein, we investigated the effects of morphine in a rat model of cisplatin-induced nephrotoxicity. Following administration of a single dose of cisplatin (5 mg/kg), animals received intraperitoneal injections of morphine (5 mg/kg/day) and/or naltrexone (20 mg/kg/day), an opioid antagonist, for 5 days. Cisplatin-induced nephrotoxicity was detected by a significant increase in plasma urea and creatinine levels in addition to alterations in kidney tissue morphology. Levels of TNF- $\alpha$  and IL-1 $\beta$  were significantly increased in the renal tissue in cisplatin group. Moreover, glutathione (GSH) concentration and superoxide dismutase activity were significantly reduced in renal tissue in cisplatin group compared with control animals. Treatment with morphine aggravated the deleterious effects of cisplatin at clinical, biochemical and histopathological levels; whereas naltrexone diminished the detrimental effects of morphine in animals receiving morphine and cisplatin. Morphine or naltrexone alone had no effect on the mentioned parameters. Our findings indicate that concomitant treatment with morphine might intensify cisplatin-induced renal damage in rats. These findings suggest that morphine and other opioids should be administered cautiously in patients receiving cisplatin chemotherapy.

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**Keywords:** Cisplatin; Morphine; Naltrexone; Antioxidant; Nephrotoxicity; Immunomodulatory

## Introduction

Cisplatin (CP) as an efficient chemotherapeutic agent, is widely used in diverse types of cancers (1,2). Cisplatin's mechanism of action involves DNA cross-linking followed by inhibition of DNA replication and cell death (2,3). However, administration of cisplatin is associated with a number of side-effects including nephrotoxicity, neurotoxicity, and electrolyte imbalance. Among these, cisplatin-induced nephrotoxicity is the major restrictive factor which limits the dose of the drug (4,5). Although the molecular mechanisms underlying cisplatin nephrotoxicity are not fully known, several lines of evidence suggest that oxidative stress and inflammation might be involved in the process (6-10).

Cisplatin has been shown to reduce renal glutathione (GSH) concentration and superoxide dismutase (SOD) activity, leading to the susceptibility of renal tissue to oxygen radicals with subsequent tubular epithelial cell damage (6). Moreover, expression of a number of proinflammatory cytokines including IL-1 $\beta$ , IL-18, IFN- $\gamma$ , IL-6 and TNF- $\alpha$  has been reported to increase in the kidney after cisplatin administration (11-13). Perturbed kidney function will manifest and could be monitored by enhanced blood urea nitrogen, serum creatinine and alteration in urine volume (14).

Morphine and other opioids have been widely used clinically for the management of severe pain in patients suffering from different types of cancer (15,16). In addition to morphine's analgesic effects, there have been

**Corresponding Author:** Sh. E. Mehr

Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran  
Tel: +98 21 88973652, Fax: +98 21 66402569, E-mail address: ejtemam@gmail.com

controversial findings regarding its immunomodulatory and antioxidant properties (17-19) as well as its toxic effects caused by oxidative-induced cell injury in various organs (20,21). The chronic use of clinically relevant doses of morphine has been reported to cause renal dysfunction associated with structural kidney abnormalities in a murine model of cancer (22). Likewise, opioid antagonists have been reported to diminish the severity of renal damage and reduce markers of nephrotoxicity in an experimental model of cholestasis which is associated high levels of endogenous opioids. Similar protective effects have been reported for opioid antagonists in the context of renal allograft survival and ischemic kidney damage (23,24).

Given the common use of morphine in cancer pain, herein we investigated the effects of this compound in cisplatin-induced nephrotoxicity. Experiments were performed to analyze the morphological as well as biochemical and inflammation-related aspects of cisplatin nephrotoxicity in the absence or presence of

morphine and the opioid antagonist, naltrexone.

## Materials and Methods

### Animals

Forty-two male Sprague–Dawley rats weighing 250–300 g were obtained from Department of Pharmacology in Tehran University of Medical Sciences. Animals were housed in a temperature-controlled room (24±1°C, 50±5% humidity) on a 12h light/dark cycle and were given free access to food and water. All procedures were approved by a group from the Ethics Committee of Tehran University of Medical Sciences (TUMS), and experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

### Experimental design

Rats were randomly divided into seven groups (Table 1).

**Table 1. Experimental groups and treatments**

Groups	Treatment(s) *	CP single injection (5 mg/kg)	n
1	Saline	--	6
2	MOR (5mg/kg)	--	6
3	--	+	6
4	MOR (5mg/kg)	+	6
5	NTX (20mg/kg)	--	6
6	NTX (20mg/kg)	+	6
7	MOR (5mg/kg) plus NTX (20mg/kg)	+	6
<b>total</b>	--	--	42

CP: cisplatin; MOR: morphine; NTX: naltrexone

\* Once daily for 5days

Animals received a single intraperitoneal injection of cisplatin (5 mg/kg) which is known to cause nephrotoxicity without lethality after a 5-day period (25). Morphine sulphate (5 mg/kg, Iran Darou Co. Ltd, Tehran, Iran) or naltrexone hydrochloride (20 mg/kg, Iran Darou, Tehran, Iran) were also injected intraperitoneally half an hour before administration of cisplatin (EBWE Pharma A-4866 Unterach, Austria) or an equivalent volume of saline. Daily intraperitoneal injections of morphine and/or naltrexone were continued for 5 days to provide steady plasma levels (26).

### Renal function assessment

On day 6 after CP injections, animals were sacrificed

using ketamine/xylazine anesthesia. Blood samples were collected by heart puncture and centrifuged at 200×g for 5 min. Serum levels of urea (BUN) and creatinine (CR) were measured using autoanalyzer (Olympus® AU 600, Japan).

### Determination of oxidative stress

The right kidneys of rats were removed immediately after sacrifice, frozen in liquid nitrogen and stored at -80°C. The frozen kidney tissues were weighed and homogenized in ice cold 0.15M KCl. The supernatant was divided into three parts and put in separate tubes. Tissue glutathione (GSH) concentration was measured using a Glutathione Assay Kit (BioVision Co., St. Linda

## Cisplatin nephrotoxicity

Vista, CA, USA) according to manufacturer's instructions. Total Superoxide Dismutase (SOD) activity (cytosolic and mitochondrial) was also determined using SOD activity assay kit (BioVision Co., St. Linda Vista, CA, USA) according to manufacturer's instructions.

### Quantification of inflammatory cytokines

Levels of IL-1 $\beta$  and TNF- $\alpha$  in supernatants were assessed using sandwich ELISA kits (eBiosciences Co., Vienna, Austria) according to the manufacturers' instructions. All measurements were performed in triplicates.

### Histological analysis

The left kidneys were placed in 10% neutral buffered formalin after removal. Following primary fixation, the tissues were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Five micrometer sections were stained with hematoxylin and eosin (H&E) and photographed using Light microscopy (Axioskop2; Carl Zeiss Microimaging, Inc.). Tubular necrosis was scored by a blinded investigator according to the following scale: - no cell necrosis; + mild (only single cell necrosis or slight degenerative changes); ++ moderate (tubular necrosis at different foci throughout the cortex); +++ severe (extensive and marked tubular necrosis throughout the cortex) (27).

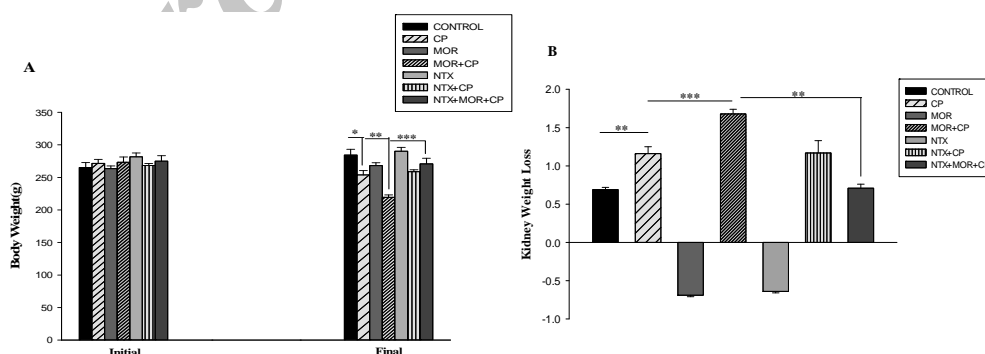
### Statistical analysis

Results are reported as mean $\pm$ S.E.M. Statistical significance between all measurements was analyzed using One-way ANOVA followed by the Tukey post-test. In all experiments, a probability level of  $P < 0.05$  was considered significant.

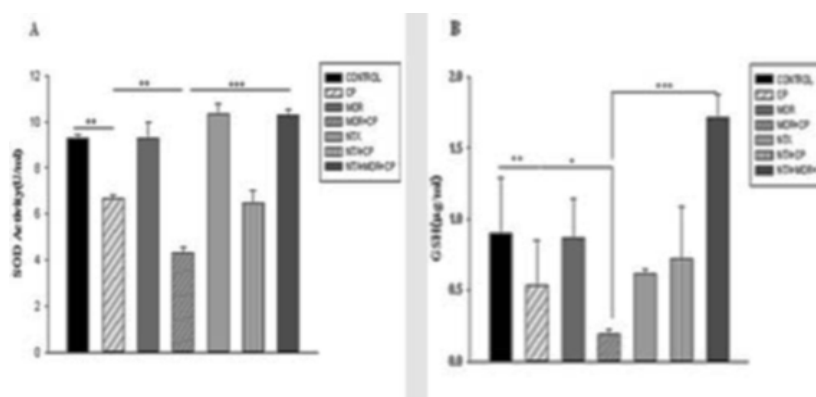
## Results

### Effects of morphine treatment on animals' body and kidney weight, serum creatinine and urea

As shown in Figure 1, a single dose of cisplatin (5 mg/kg, i.p.) caused a decrease in body weight ( $P < 0.05$ ) and an increase in kidney weight ( $P < 0.01$ ) in animals on day 6 compared with vehicle-treated controls. Interestingly, morphine (5 mg/kg/day, i.p.) enhanced the effects of cisplatin on body weight loss ( $P < 0.01$ ) and kidney weight gain ( $P < 0.001$ ) in MOR+CP group, whereas naltrexone (20 mg/kg/day, i.p.) dramatically attenuated the effect of morphine-induced reduction in body weight ( $P < 0.001$ ) and increase in kidney weight ( $P < 0.01$ ) in NTX+MOR+CP group. As expected, the levels of blood urea nitrogen (BUN,  $P < 0.01$ , Figure 2A) and creatinine ( $P < 0.05$ , Figure 2B) were increased in cisplatin-treated animals compared with controls, which indicated the occurrence of nephrotoxicity. Interestingly, serum BUN and creatinine levels in MOR+CP group were higher than CP group ( $P < 0.001$ , Figure 2), while these levels were markedly lower in NTX+MOR+CP group compared with MOR+CP group ( $P < 0.001$ , Figure 2). Animals receiving MOR or NTX alone displayed no differences in the body or kidney weight, plasma BUN or creatinine compared with control group ( $P > 0.05$ , Figure 1 and Figure 2). Naltrexone substantially reduced the effect of CP on BUN and creatinine ( $P < 0.05$ , Figure 2), but it did not alter the weight loss in NTX+CP group ( $P > 0.05$ , Figure 1).



**Figure 1.** Effect of cisplatin (CP) or its combination with morphine (MOR) and Naltrexone (NTX) on initial and final body weight (Figure 1A) and kidney weight loss (Figure 2A). Final body weight (BW) and kidney weight were measured at the fifth day after CP injection. Each column represents the mean  $\pm$  S.E.M of six animals.

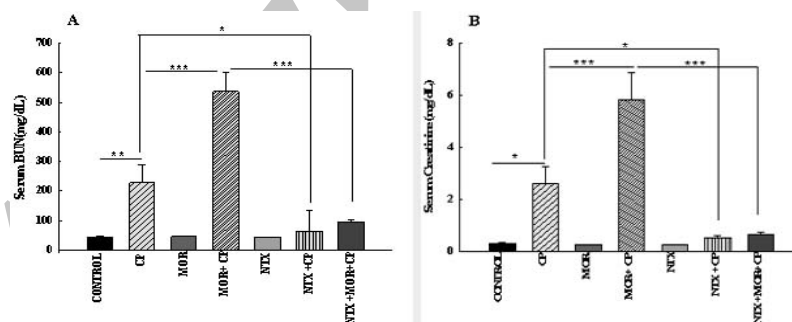


**Figure 2.** Effect of cisplatin (CP) or its combination with morphine (MOR) and Naltrexone (NTX) on kidney SOD activity (Figure 3A) and GSH content (Figure 3B). SOD activity and GSH content were measured at the fifth day after CP injection. Each column represents the mean  $\pm$  S.E.M of six animals.

### Effects of morphine treatment on kidney GSH and SOD activity

To investigate the effect of cisplatin and morphine/naltrexone treatment on markers of oxidative stress in kidney, GSH concentration as well as SOD activity was measured in kidneys of different groups of animals as mentioned in materials and methods (Figure 3). Cisplatin treatment significantly reduced GSH levels and SOD activity compared with control animals ( $P < 0.01$ ), findings that indicated the occurrence of oxidative stress in kidneys. Of interest, the levels of GSH concentration SOD activity in the group receiving MOR+CP were significantly lower compared with the

group treated with CP alone ( $P < 0.05$  and  $P < 0.01$ , respectively, Figure 3). In contrast, treatment with NTX+ MOR+CP markedly increased these levels in comparison with MOR+CP treatment ( $P < 0.001$ , Figure 3). Values obtained from rats treated with either morphine or naltrexone alone was not significantly different from those treated with control ( $P > 0.05$ ). Nonetheless, NTX had no effect on the reduced levels of GSH concentration or SOD activity in NTX+CP group compared with CP group ( $P > 0.05$ ). Overall, these results indicated that morphine treatment aggravated the oxidative stress induced by cisplatin, an effect that was inhibited by morphine antagonist.



**Figure 3.** Effect of cisplatin (CP) or its combination with morphine (MOR) and Naltrexone (NTX) on plasma creatinine (Figure 1A) and urea (Figure 1B) levels ( $n = 6$ ). Blood urea nitrogen and creatinine were measured at the fifth day after CP injection. Each column represents the mean  $\pm$  S.E.M of six animals.

### Effects of morphine treatment on levels of pro-inflammatory cytokines in renal tissue

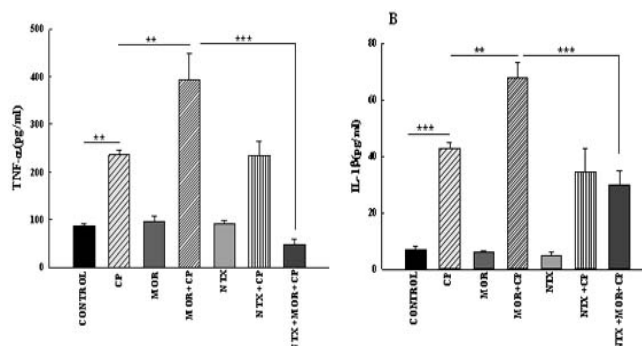
In order to examine the effect of cisplatin and morphine on the production of proinflammatory cytokines in renal tissue, the levels of two major innate immune cytokines, TNF- $\alpha$  and IL-1 $\beta$  were measured in the supernatants of homogenized kidney tissues. The

measurements showed significantly higher levels of TNF- $\alpha$  and IL-1 $\beta$  in kidney tissues derived from the cisplatin group in comparison with control group ( $P < 0.01$  and  $P < 0.001$ , figure 4). The levels of these inflammatory cytokines were significantly higher in the group receiving MOR+CP ( $P < 0.01$ , Figure 4) compared with the group treated with cisplatin alone. Concomitant

## Cisplatin nephrotoxicity

treatment with naltrexone decreased the TNF- $\alpha$  and IL1 $\beta$  levels in animals receiving morphine and cisplatin ( $P < 0.001$ , Figure 4). Morphine or naltrexone alone did not influence TNF- $\alpha$  or IL1 $\beta$  levels ( $P > 0.05$ , Figure 4). Moreover, naltrexone treatment had no effect on renal

tissue cytokine levels in NTX+CP ( $P > 0.05$ , figure 4) in comparison with CP. Altogether, these results show that morphine treatment could enhance cisplatin-induced inflammatory effects in kidney, an effect that is blocked by an opioid antagonist.

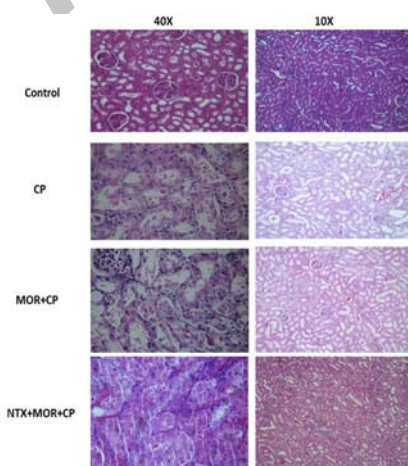


**Figure 4.** Effect of cisplatin (CP) or its combination with morphine (MOR) and Naltrexone (NTX) on kidney TNF- $\alpha$  (Figure 1A) and IL-1 $\beta$  (Figure 1B) levels in rats ( $n = 6$ ). Kidney TNF- $\alpha$  and IL-1 $\beta$  were measured at the fifth day after CP injection. Each column represents the mean  $\pm$  S.E.M of six animals.

### Effects of morphine treatment on kidney histology

We next evaluated potential histological alterations caused by different treatments (Figure 5). Sections from control animals showed normal morphology with well-preserved brush border membranes and no loss of tubular epithelial cells. Cisplatin-treated kidneys exhibited tubular necrosis (++) , tubular dilation, and cast formation throughout the cortical region (Figure 5B). Kidneys from MOR+CP rats displayed even more severe histological changes which included severe tubular necrosis (+++) (Figure 5C). Treatment with naltrexone reduced tubular necrosis in NTX+MOR+CP

group compared with CP+MOR group (+) (Figure 5D). Kidney sections from animals treated with MOR or NTX alone showed no abnormality (Figures not shown). Moreover, histopathological analysis showed no difference between NTX+CP and CP groups (Figures not shown). Taken together, the results of histological analyses were consistent with clinical findings of altered BUN and creatinine levels in different groups of animals and indicated the morphine could accentuate cisplatin-induced nephrotoxicity while naltrexone treatment could reverse the effect.



**Figure 5.** Light microscopy of renal tissue from rats receiving different treatments. (A) control, (B) cisplatin (CP) alone, (C) pre-treatment with morphine and cisplatin (MOR+CP), (D) pre-treatment with Naltrexone and morphine and cisplatin (NTX+MOR+CP) (H and E  $\times 66$ ). (A) Renal tubules are normal. (B) Tubules show severe (++) necrosis. (C) Tubules show severe (+++) necrosis. (D) Tubules show only single cell necrosis and slight degenerative changes (+).

## Discussion

Simultaneous administration of opioids and chemotherapeutic agents is a recurring theme in the treatment of cancer. In the current study, we sought to determine the impact of morphine administration on cisplatin-mediated nephrotoxicity using an animal model. Our results showed marked augmentation of cisplatin-induced kidney damage in rats at clinical and morphological levels. Results of biochemical and molecular analyses were indicative of oxidative stress and enhanced levels of proinflammatory cytokines in animals receiving morphine together with cisplatin.

Nephrotoxicity is the chief adverse effect that limits the use of cisplatin and its derivatives in cancer therapy. Of note, over one quarter of acute renal failure cases among hospitalized patients are due to cisplatin-mediated nephrotoxicity (5). Cisplatin nephrotoxicity is primarily induced by accumulation of cisplatin and its analogues in kidney cells, followed by necrosis and apoptosis of renal epithelial cell (6). This can, in turn, lead to reduced glomerular filtration rate and subsequent increase in serum urea and creatinine levels (14). Morphologically, cisplatin induces severe degeneration in glomeruli, loop of Henle and proximal and distal tubules (5). Pain management represents another dimension of cancer treatment and morphine, and its derivatives are the most widely used agent in this context. However, hepatotoxicity and nephrotoxicity are among the toxic effects of morphine at the cellular level (28-30). Studies have shown that chronic administration of morphine might induce an increase in the serum urea, uric acid and creatinine levels (28). This has been associated with profound degeneration of tubular epithelial cells and formation of cellular casts within the lumen of renal tubules (28). That said, opioids have also been reported to exert protective effects against different injurious stimuli. Nevertheless, there is limited information about side-effects of simultaneous cisplatin and morphine administration in *in vivo* settings.

Our data indicate that continued administration of morphine for 5 days increases the intensity of cisplatin nephrotoxicity as evaluated by elevated levels of creatinine and urea in serum, and increased tubular necrosis in kidneys.

The exact mechanisms of cisplatin nephrotoxicity remain unclear. Some studies have pointed to the accumulation of free radicals and the subsequent oxidative stress in renal tissue after cisplatin treatment (8-10). Induction of oxidative stress by opioids has also

been shown in various organs including the immune system, kidneys, liver, CNS and epithelial cells (20,21,31-33). In mice liver, morphine is known to suppress superoxide dismutase, catalase and GSH peroxidase activity and the GSH/oxidized GSH ratio followed by oxidative damage to DNA, proteins, and lipids (34). Morphine – induced nephrotoxicity has also been shown to be associated with lipid peroxidation which is a marker of oxidant-induced cell injury (30). Likewise, in the current study, we observed a marked decrease in renal levels of GSH concentration and SOD activity of rats treated with MOR+CP compared with CP-treated rats, indicating that morphine potentiated the detrimental effects of cisplatin on antioxidant enzymes in the kidney. In addition to free radicals and oxidative injury, production of proinflammatory cytokines including TNF- $\alpha$  and IL1 $\beta$  is another aspect of cisplatin-induced nephrotoxicity (8-10,35). TNF- $\alpha$  and IL-1 $\beta$  are proinflammatory cytokines and act synergistically to stimulate the production of other cytokines and chemokines (9). Treatment with TNF $\alpha$  inhibitors has been reported to reduce cisplatin-induced nephrotoxicity. Moreover, TNF knockout mice show less renal injury than wild type mice and have markedly higher survival rates following cisplatin administration (10). Morphine can also stimulate the production of proinflammatory cytokines including TNF $\alpha$ , likely through activation of NF- $\kappa$ B pathway (ADD REFS 35,36). However, morphine can also decrease the levels of TNF $\alpha$  in cells or animals after repeated administration which is associated with adaptive changes in gene expression in the brain (36). Our findings have revealed an obvious increase in kidney TNF- $\alpha$  and IL-1 $\beta$  levels in rats receiving MOR+CP in comparison with CP-treated rats which are in corroboration with previous findings. Moreover, an opioid antagonist, naltrexone reversed the morphine's effects in NTX+MOR+CP group which confirmed that these effects of morphine were opioid-receptor mediated. Of interest, naltrexone also reduced the effect of cisplatin on BUN and creatinine, in the absence of exogenous morphine. This raises the possibility that endogenous opioids might be involved in some aspects on cisplatin nephrotoxicity. Deroee *et.al.*, have shown that naltrexone (20 mg/kg) could decrease markers of nephrotoxicity such as N-acetyl- $\beta$ -D-glucosaminidase (NAG) in an experimental model of cholestasis, a condition which is associated with enhanced levels of endogenous opioids (37). In our study, naltrexone (20 mg/kg) modified the plasma markers of the kidney in NTX+CP group, but it had no

## Cisplatin nephrotoxicity

effect on pathological changes. It remains to be investigated whether endogenous opioids are a significant contributor to cisplatin nephrotoxicity.

Overall, the present study describes detrimental effects of continuous treatment with low dose of morphine (5 days) on cisplatin-induced nephrotoxicity in rats at clinical, morphological and biochemical levels. These data suggest that morphine and other opioid analgesics might need to be used more cautiously in cancer patients receiving cisplatin chemotherapy.

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