

Original Paper

Impact of Pulmonary Exposure to Cerium Oxide Nanoparticles on Experimental Acute Kidney Injury

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Key Words

Cerium oxide nanoparticles • Lung inflammation • Acute renal failure

Abstract

Background/Aims: Cerium oxide nanoparticles (CeO₂ NPs) are released from diesel engines that use cerium compounds as a catalytic agent to decrease the diesel exhaust particles, leading to human exposure by inhalation to CeO₂ NPs. We have recently demonstrated that pulmonary exposure to CeO₂ NPs induces lung inflammation, thrombosis, and oxidative stress in various organs including kidneys. It is well known that particulate air pollution effects are greater in patients with renal diseases. The aim of this study is to investigate the effects of pulmonary exposure to CeO₂ NPs in a rat model of acute kidney injury (AKI). **Methods:** AKI was induced in rats by a single intraperitoneal injection of cisplatin (CP, 6 mg/kg). Six days later, the rats were intratracheally (i.t.) instilled with either CeO₂ NPs (1 mg/kg) or saline (control), and various renal and pulmonary endpoints were assessed 24 h afterward using histological, colorimetric assay, enzyme-linked immunosorbent assay and Comet assay techniques. **Results:** CP alone decreased body weight, and increased water intake, urine volume and relative kidney weight. CP also increased the plasma concentrations urea and creatinine, and decreased creatinine clearance. In the kidneys, CP significantly increased renal injury molecule-1, interleukin-6 (IL-6), tumor necrosis factor α (TNF α) and glutathione concentrations, and caused renal tubular necrosis, and DNA injury assessed by Comet assay. All these actions were significantly aggravated in rats given both CP and CeO₂ NPs. Histopathological changes in lungs of CeO₂ NPs-treated rats included marked interstitial cell infiltration and congestion.

These were aggravated by the combination of CP + CeO₂ NPs. Moreover, this combination exacerbated the increase in the concentrations of TNF α and IL-6, and the decrease in the activity of pulmonary catalase and total nitric oxide concentration, and lung DNA damage.

Conclusion: We conclude that the presence of CeO₂ NPs in the lung exacerbated the renal and lung effects of CP-induced AKI.

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Introduction

There has been an accelerated increase in the use of different types of nanomaterials as a result of their unique physicochemical and bioreactive characteristics. Nanomaterials possess different physicochemical and biological properties than larger materials of identical chemical composition. They are used in industries including sunscreens, food, paints, textile, electronics, sports, and biomedical application and imaging [1]. Nonetheless, extensive use of nanomaterials may lead to exposure of humans by various routes including inhalation, dermal and oral routes, raising concerns about their potential toxicity [1, 2].

Cerium oxide nanoparticles (CeO₂ NPs) have been used in Europe, Asia and North America as fuel additive, which serves as a combustion catalyst leading to enhanced fuel efficiency [3-6]. Although this addition enhances the ability of diesel engines and reduces the emission of diesel exhaust particles (DEP), it leads, however, to direct emission of CeO₂ NPs in the environment leading to human exposure by inhalation [7]. The latter could have adverse health effects [7].

It has been shown that exposure to CeO₂ NPs causes inflammation and oxidative stress leading to apoptosis and autophagy *in vitro* [8] and lung inflammation and fibrosis after their pulmonary exposure *in vivo* [9, 10]. Furthermore, it has been demonstrated that following their pulmonary deposition, CeO₂ NPs are able to pass through the air-blood barrier and reach the systemic circulation, and from there distribute to various sites in the body [11]. We have recently demonstrated that pulmonary exposure to CeO₂ NPs induces lung inflammation and thrombotic events, and cause inflammation and oxidative stress in various extrapulmonary sites, including the kidneys [12, 13].

It is well-established that there is a crosstalk between the lung and the kidney, and that their functions are tightly associated under physiological and pathophysiological situations [14]. In fact, it is well-known that different diseases that may affect one organ can have direct consequences on the other one [14]. It has been shown that lung injury can worsen kidney failure [14]. Moreover, it has been reported that patients with pre-existing diseases such as kidney failure are more susceptible to the adverse health effect of particulate air pollution [15]. Actually, it has been demonstrated that living near a major roadway contributes to the decline in renal function [16]. We have also recently provided experimental evidence that pulmonary exposure to diesel exhaust particles (DEPs) aggravate both acute and chronic renal failure [17, 18]. However, as far as we are aware, little is known about the impact of lung exposure to CeO₂ NPs in animal model of acute renal failure. As far as we know, only one study has reported that CeO₂ NPs attenuate acute kidney injury induced by intra-abdominal infection in Sprague-Dawley rats [19]. The latter findings are in complete disagreement with various publications reporting that CeO₂ NPs induce inflammation, oxidative stress and DNA damage in various organs including the kidneys [8-10, 12, 13].

Consequently, the purpose of this study was to evaluate whether and to what extent pulmonary exposure to CeO₂ NPs may have an exacerbating effects in an animal model of acute kidney injury (AKI) induced by cisplatin (CP, [cis-diaminedichloroplatinum II]), an anticancer drug, which induces nephrotoxicity through inflammation, apoptosis and generation of reactive oxygen species [20], by assessing various renal and pulmonary variables.

Materials and Methods

Particles

CeO₂ NPs, 10 wt % in water with average diameter at ~20 nm, were obtained from Sigma-Aldrich (St Louis, MO, USA). CeO₂ NPs samples diluted in saline were used for rat exposures. To minimize aggregation, particle suspensions were always sonicated for 5 min (Clifton Ultrasonic Bath, Clifton, New Jersey, USA). Particle suspensions were prepared promptly before use and were vortexed to offer well mixed suspension prior each instillation. The same particles from the same source were characterized and used recently by Ma and co-workers [10, 21].

The endotoxin concentration in the CeO₂ NPs and saline used was quantified, as described by the manufacturer, by chromogenic Limulus Amebocyte Lysate (Pierce, Rockford, IL) test. The concentrations were lower than the detection limit (0.1 EU/ml) in the saline, and CeO₂ NPs solutions.

Animals and i.t. instillation

This project was reviewed and approved (ERA_2016_4408) by the Institutional Review Board of the United Arab Emirates University, College of Medicine and Health Sciences, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Male Wistar rats (Taconic Farms Inc., Germantown, New York, USA), aged 10–12 weeks and initially weighing 223 ± 13 g, were given a standard laboratory chow and water *ad libitum*. They were randomly divided into four groups and individually housed in metabolic cages, to facilitate urine collection, at a temperature of 23 ± 2°C, relative humidity of 50–60% and a 12-h dark–light cycle. An acclimatization period of four days was allowed for the rats before any experimentation. The rats were weighed at the beginning of the experiment and just before sacrifice. Rats were cared for under a protocol approved by the Animal Research Ethics Committee of our college, and according to the NIH Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985.

Treatments

The AKI in rats was induced by a single intraperitoneal (i.p.) injection of CP (David Bull Laboratories, PTY Ltd, Victoria, Australia) at a dose of 6 mg/kg [22, 23]. Control animals received similar volume of normal saline i.p. On day 6 of treatment, the animals were anesthetized by isoflurane inhalation, and placed supine with extended neck on an angled board. A Becton Dickinson 18 Gauge cannula was inserted via the mouth into the trachea. CeO₂ NPs suspension (150 µl) or saline-only were instilled (150 µl) via a sterile syringe and followed by an air bolus of 100 µl.

The four groups were treated as follows:

Group 1: single normal saline (control, 500 µl /rat) given i.p., and on day 6 of the treatment, a single i.t. administration of saline (150 µl per rat);

Group 2: single normal saline (control, 500 µl /rat) given i.p., and on day 6 of the treatment, a single i.t. administration of CeO₂ NPs (1 mg/kg);

Group 3: single CP (6 mg/kg) given i.p., and on day 6 of the treatment, a single i.t. administration of saline (150 µl per rat);

Group 4: single CP (6 mg/kg) given i.p., and on day 6 of the treatment, a single i.t. administration of CeO₂ NPs (1mg/kg);

On day 6, immediately after i.t. administration of saline or CeO₂ NPs, rats were placed in metabolic cages, and urine of each rat was collected over a 24-h period and the volume measured.

Blood collection, histology and biochemical analysis

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and blood was drawn from the inferior vena cava in ethylenediaminetetraacetic acid (4%). The collected blood was centrifuged at 4°C for 15 min at 900× *g*, and the plasma samples were stored at –80°C pending analysis.

The animals were sacrificed with an overdose of anesthesia. Right kidneys were excised, washed with ice-cold saline, blotted with filter paper and weighed. Each kidney was cassetted and fixed directly in 10% neutral formalin for 24 hours, which was followed by dehydration in increasing concentrations of ethanol, clearing with xylene and embedding with paraffin. Four-µm sections were prepared from paraffin blocks and stained with hematoxylin and eosin. The stained sections were evaluated by the histopathologist (S.A.),

using light microscopy. The microscopic scoring of the kidney sections was carried out in a blinded fashion by our histopathologist who was unaware of the treatment groups and assigned a score which represents measurement of extent of acute tubular injury area in the cortical and medullary tubules on a scale of 0–4 (0, no acute tubular injury; 1, a few focal acute tubular injury areas of $\leq 25\%$ of the kidney; 2, acute tubular injury area was about 26–50% of kidney; 3, acute tubular injury area was 51–75% of kidney; 4, showing acute tubular injury in 76–100% of kidney) [24]. Image J software (NIH, USA) was used to measure the extent of necrosis.

Right lungs were excised, washed with ice-cold saline, blotted with filter paper and weighed. Then they were fixed by with 10% buffered formalin. Each lung was sectioned, casseted and dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded with paraffin. Three- μm sections were prepared from paraffin blocks and stained with haematoxylin and eosin. The stained sections were evaluated using light microscopy.

The concentrations of urea in plasma, and creatinine in both plasma and urine were spectrophotometrically measured using commercial kits (Roche Diagnostics, Indianapolis, IN, USA).

Left kidneys and left lungs of rats from the different groups were quickly collected and rinsed with ice-cold PBS (pH 7.4) before homogenization, as described before [13, 25]. The homogenates were centrifuged for 10 min at 3000 $\times g$ to remove cellular debris, and the supernatants were used for further analysis [25]. Protein content was measured by Bradford's method. The concentrations of tumor necrosis factor α (TNF α) (Duo Set, R & D systems, Minneapolis, MN, USA), interleukin 6 (IL-6) (Duo Set, R & D systems, Minneapolis, MN, USA), kidney injury molecule 1 (KIM) (Duo Set, R & D systems, Minneapolis, MN, USA) and glutathione (GSH) (Sigma-Aldrich Fine Chemicals, St Louis, MO, USA) were determined in kidney homogenates using commercial Kits.

In lung homogenates, IL-6 and TNF α , as well as catalase activity were measured using a kit from Cayman Chemicals Company (Ann Arbor, MI, USA) and the determination of NO was performed with a total NO assay kit from R & D systems (Minneapolis, MN, USA) which measures the more stable NO metabolites NO $_2^-$ and NO $_3^-$ [26, 27].

Assessment of DNA damage by COMET assay

Immediately after sacrifice, the lung and kidneys were removed from each animal. Single-cell suspensions of the different lungs and kidneys were obtained and analyzed according to the method described in our previous publications [28–31]. Each collected organ was washed in a chilled medium (RPMI 1640, 15% DMSO, 1.8% (w/v) NaCl). The lung and kidney tissues were put in 1.5 ml medium and cut finely into pieces in a Petri dish. The slices were allowed to deposit and the supernatant was collected in a 15 ml tube. The collected cell suspension was centrifuged at 1000 rpm for 5 min at 4°C. The supernatant was removed and the pellets were suspended in 0.5 ml of the medium. The cell suspensions were mixed with low melting point agarose solution (0.65%) and spread onto agarose (1.5%)–precoated microscope slides. For each group, five slides were prepared and incubated in ice cold lysis buffer (2.5M NaCl, 10mM Tris, 100mM EDTA, 1% Triton X-100 and 10% DMSO) at 4°C for at least one hour to remove the cell membranes. Following incubation, slides were placed in a horizontal electrophoresis unit and incubated in electrophoresis buffer (0.2M EDTA, 5M NaCl, pH 10) for 20 min for DNA unwinding and the expression of alkali labile sites. Then, electrophoresis was conducted for 20 min at 25V and 300mA. After that, the slides were neutralized with Tris buffer (0.4M Trizma base, pH 7.5) for 5 min and washed with methanol. Then the slides were stained with propidium iodide, as previously described [29, 32]. All these steps were performed in darkness to prevent additional DNA damage. The slides were mounted on a fluorescent microscope and cell scoring was performed. The measurement of length of the DNA migration (i.e. diameter of the nucleus plus migrated DNA) was calculated using the image analysis Axiovision 3.1 software (Carl Zeiss, Canada) [29, 33].

Statistics

All data were analyzed with GraphPad Prism Version 4.01 for Windows software (Graphpad Software Inc., San Diego, USA). Data were analyzed for normal distribution using the D'Agostino and Pearson omnibus normality test. Data are expressed as means \pm SEM. Comparisons between groups were performed by one way analysis of variance (ANOVA), followed by Newman Keuls test for comparing groups treated with control data. P values ≤ 0.05 are considered significant.

Results

Body weight, relative kidney weight, water intake and urine volume

Fig. 1A displays no difference in body weight between saline and CeO₂ NPs groups. Nevertheless, rats treated concomitantly with CP and saline lost about 17.2 % (P<0.001 versus saline group), while those administered with CP and CeO₂ NPs lost about 20.5 % (P<0.001 versus DEP group), respectively. Fig. 1B shows that the relative kidney weight was significantly augmented in rats given CP + saline compared with those administered with saline only (P<0.01). Moreover, the relative kidney weight was significantly increased in rats administered with CP + CeO₂ NPs compared with those given either CeO₂ NPs only (P<0.001) or those given saline + CP (P<0.001). Fig. 1C shows that water intake was significantly increased in the CP + saline group compared with the saline alone (P<0.05), and the CP + CeO₂ NPs group compared with either CP + saline group (P<0.05) or CeO₂ NPs alone (P<0.001). Similarly, Fig. 1D shows that urine volume was significantly augmented in the CP + saline group compared with the saline group (P<0.001), and the CP + CeO₂ NPs group compared with either CP + saline group (P<0.01) or CeO₂ NPs group (P<0.001).

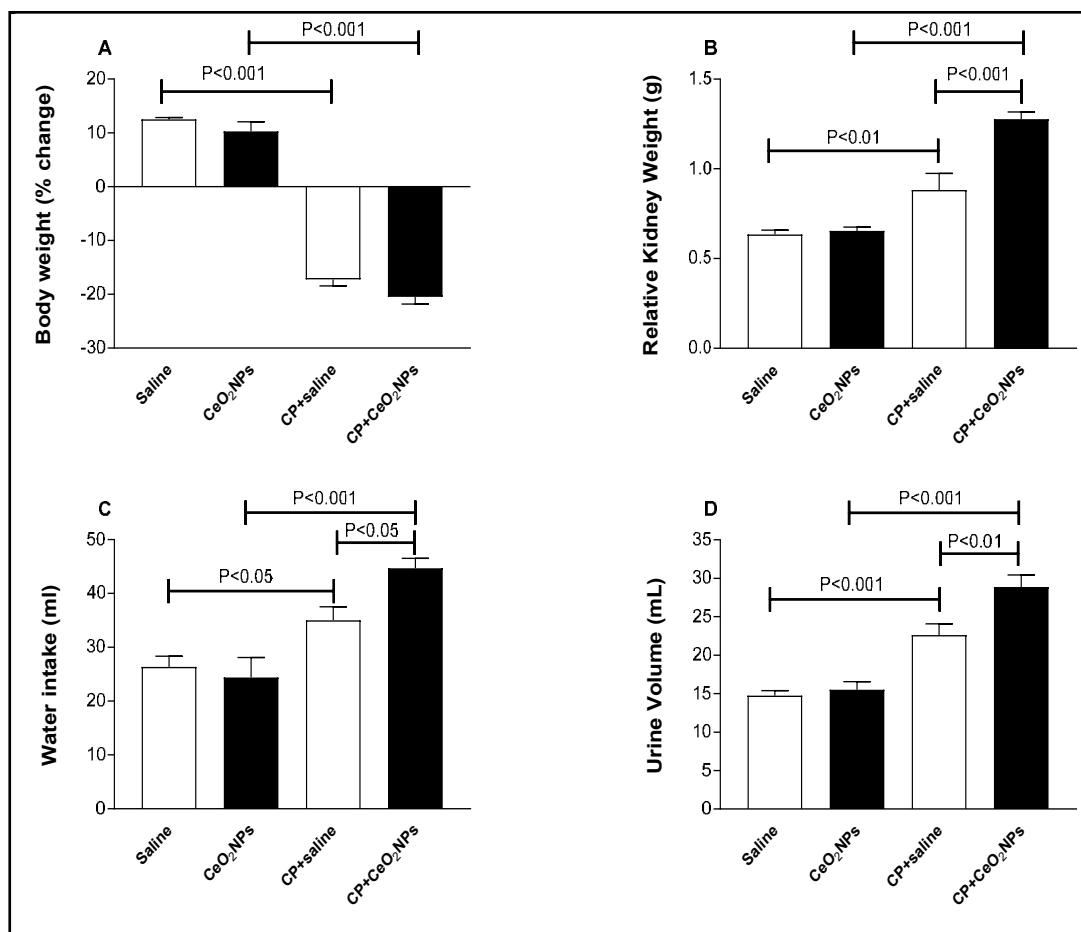


Fig. 1. Body weight (A), relative kidney weight (B), water intake (C) and urine volume (D) in Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs) with or without cisplatin (CP) administration. Data are mean ± SEM. Body and kidney weights: saline (n=8), CeO₂ NPs (n=8), CP+saline (n=8) and CP+ CeO₂ NPs (n=8). Water intake: saline (n=6), CeO₂ NPs (n=8), CP+saline (n=8) and CP+ CeO₂ NPs (n=6). Urine volume: saline (n=8), CeO₂ NPs (n=8), CP+saline (n=8) and CP+ CeO₂ NPs (n=7).

Plasma creatinine and urea concentration and creatinine clearance

Fig. 2A shows no difference in urea concentration has been observed between saline and CeO₂ NPs groups. However, rats treated with CP + saline showed a significant increase in the concentration of urea compared with the saline group (P<0.001). Remarkably, CP + CeO₂ NPs treatment significantly augmented the concentration of urea more than in rats given either CP + saline (P<0.05) or CeO₂ NPs alone (P<0.001). Likewise, as shown in Fig. 2B, the concentration of creatinine in plasma significantly increased in the CP + saline group compared with the saline one (P<0.01), and in the CP + CeO₂ NPs group compared with the CP + saline (P<0.05) or CeO₂ NPs group (P<0.01). Fig. 2C displays that creatinine clearance was significantly reduced in rats treated with CP + saline (P<0.001), and in rats treated with CP + CeO₂ NPs compared with those treated with either CP + saline (P<0.05) or those treated CeO₂ NPs alone (P<0.001).

KIM-1, IL-6, TNFα and GSH concentrations in kidney homogenates

Fig. 3A illustrates that treatment with CP + saline significantly increased the concentration of KIM-1 compared with that of the saline-treated group (P<0.01). Simultaneous treatment with CP and CeO₂ NPs significantly augmented the concentration of KIM-1 in kidney homogenates compared with either CP + saline group (P<0.05) or CeO₂ NPs group (P<0.001). Fig. 3B and 3C show that the concentrations of IL-6 and TNFα in kidney homogenates were significantly increased in CeO₂ NPs group compared with saline group. Moreover, the concentrations of IL-6 and TNFα were significantly increased in CP + saline group compared with saline group, and in CP + CeO₂ NPs compared with either CP + saline or CeO₂ NPs group. Fig. 3D shows that compared with saline group, treatment with CeO₂ NPs induced a significant increase in GSH levels. Moreover, the levels of GSH were also significantly augmented in CP + saline group versus saline group and, in CP + CeO₂ NPs group versus either CP + saline group or CeO₂ NPs group.

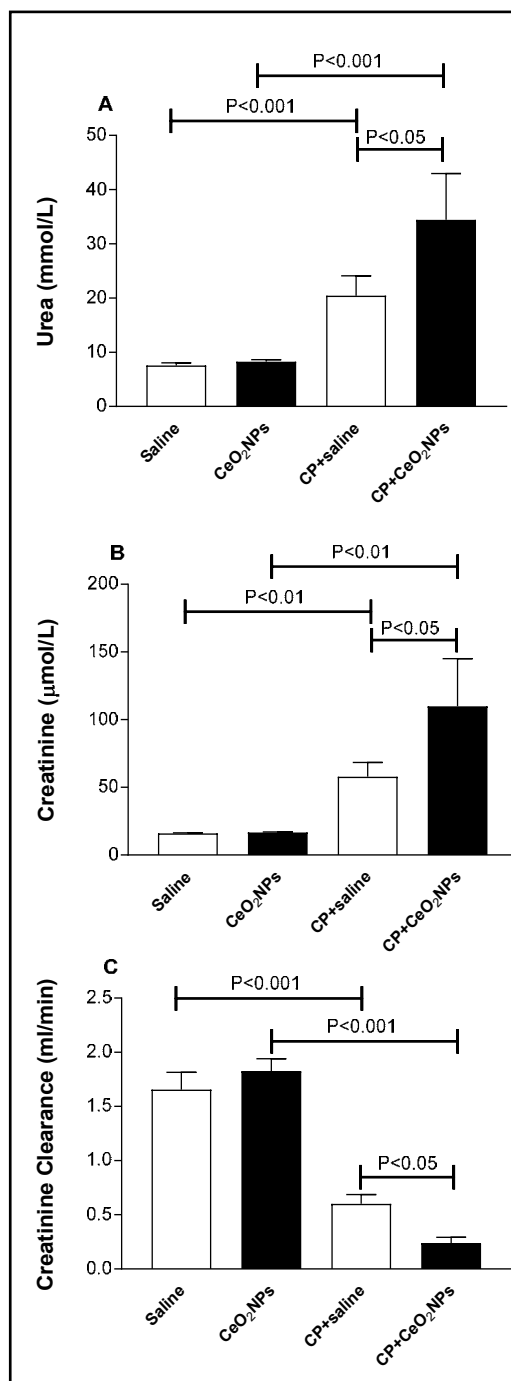


Fig. 2. Plasma concentrations of urea (A) and creatinine (B) and creatinine clearance (C) in Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs) with or without cisplatin (CP) administration. Data are mean ± SEM. Urea: saline (n=8), CeO₂ NPs (n=8), CP+saline (n=6) and CP+ CeO₂ NPs (n=6). Creatinine and creatinine clearance: saline (n=8), CeO₂ NPs (n=8), CP+saline (n=8) and CP+ CeO₂ NPs (n=7).

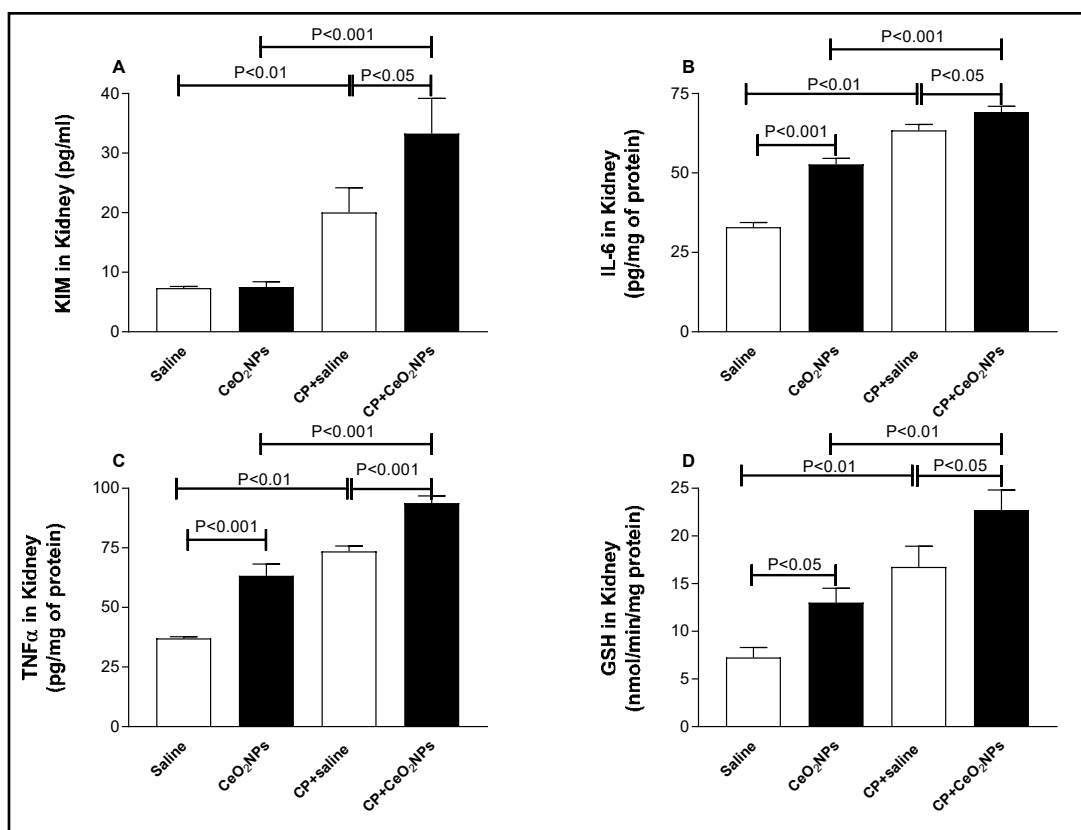


Fig. 3. Kidney injury molecule 1 (KIM-1; A), interleukin 6 (IL-6, B), tumor necrosis factor α (TNF α , C) and glutathione (D) in Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs) with or without cisplatin (CP) administration. Data are mean \pm SEM. KIM: saline (n=8), CeO₂ NPs (n=8), CP+saline (n=7) and CP+ CeO₂ NPs (n=6). IL-6: saline (n=8), CeO₂ NPs (n=8), CP+saline (n=7) and CP+ CeO₂ NPs (n=8). TNF α : saline (n=8), CeO₂ NPs (n=8), CP+saline (n=8) and CP+ CeO₂ NPs (n=8). GSH: saline (n=6), CeO₂ NPs (n=6), CP+saline (n=6) and CP+ CeO₂ NPs (n=6).

DNA damage in the kidney

Fig. 4 depicts the evaluation of DNA injury in the kidney by the Comet assay. Compared with saline group, pulmonary exposure to CeO₂ NPs induced a significant increase in DNA damage (P<0.05). DNA damage was significantly augmented in CP + saline group compared with saline group (P<0.05). Furthermore, the DNA damage observed in CeO₂ NPs + CP group was significantly higher compared with either CP + saline (P<0.01) or CeO₂ NPs alone (P<0.01).

Kidney histopathology

Fig. 5 and Table 1 illustrate kidney histopathology and the percentage and scores for tubular injury in the four studied groups. Saline-treated group showed normal kidney architecture and histology and was given a score of 0 (Fig. 5A-B). In CP + saline group (Fig. 5C-D), there was an acute tubular injury in 37.50 ± 2.49 % of examined tissue areas (score 2) which showed the presence of tubular distention with necrotic material involving loss of brush border; tubular dilatation, tubular cells necrosis, tubular nuclear pyknosis, tubular nuclear enlargement with hyperchromasia, tubular cells flattening, macrophages within the lumen, epithelial cells within the lumen and intra-luminal eosinophilic material. In CeO₂ NPs group (Fig. 5E-F), there were focal areas of acute tubular injury accounting 7.37 ± 0.94 of total examined areas involving proximal convoluted tubules characterized by mild tubular dilatation, intracytoplasmic aggregates of giant lysosomes within tubular cells, epithelial cells falling within the lumen of tubular cells and intratubular secretion. In CP + CeO₂ NPs group

(Fig. 5G-H), there was an increased frequency of acute tubular injury when compared with either CP + saline group or CeO₂ NPs group. The acute tubular injury observed CP + CeO₂ NPs involved 58.63 ± 6.27% of examined areas (score 3). The latter showed the presence of tubular distention with necrotic material involving, loss of brush border, tubular dilatation, tubular cells necrosis, tubular nuclear pyknosis, tubular nuclear enlargement with hyperchromasia, tubular cells flattening, macrophages within the lumen, epithelial cells within the lumen and intra-luminal eosinophilic material.

Lung histopathology

Saline-treated group showed normal lung architecture with unremarkable changes (Fig. 6A).

In CP + saline group, there was a moderate widening of interalveolar interstitial spaces with mixed inflammatory cells consisting of neutrophil polymorphs, lymphocytes and macrophages (Fig. 6B). In CeO₂ NPs (Fig. 6C), there was focal mild widening of interalveolar interstitial spaces with mixed inflammatory cells consisting of neutrophil polymorphs, lymphocytes and macrophages. In CP + CeO₂ NPs (Fig. 6D), there was a severe widening of interalveolar interstitial spaces with mixed inflammatory cells consisting of neutrophil polymorphs, lymphocytes and macrophages.

IL-6, TNF α , catalase and total NO levels in lung homogenates

Fig. 7A-B illustrates that treatment with CP + CeO₂ NPs significantly increased the concentration of IL-6 and TNF α compared with CP + saline group or CeO₂ NPs group. On the other hand, the activity of catalase was significantly reduced in CP + CeO₂ NPs compared with either CP + saline group or CeO₂ NPs group (Fig. 7C). Moreover, the catalase activity decreased significantly in rats treated with CeO₂ NPs compared with those given saline, and in rats treated with saline + CP compared with those treated with saline (Fig. 7C). The total NO activity was significantly reduced in CP + CeO₂ NPs compared with either CP + saline group or CeO₂ NPs group (Fig. 7C-D).

DNA damage in the lung

As shown in Fig. 8, compared with saline group, treatment with CeO₂ NPs induced a significant increase in DNA damage (P<0.001). DNA damage was significantly increased in CP + saline group compared with saline group (P<0.001). Additionally, the DNA damage seen in CeO₂ NPs + CP group was significantly higher compared with either CP + saline (P<0.05) or CeO₂ NPs alone (P<0.001).

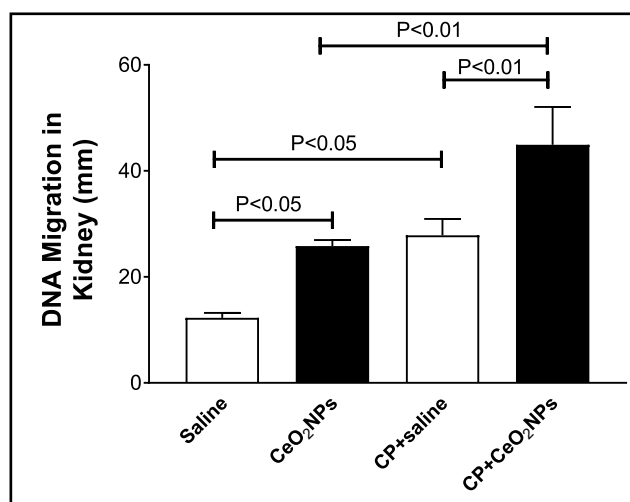


Fig. 4. DNA migration (mm) in the kidney tissues evaluated by Comet assay, in Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs) with or without cisplatin (CP) administration. Data are mean ± SEM (n=5).

Fig. 5. A-H: representative light microscopy sections of H and E stained kidney tissues of Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs) with or without cisplatin (CP) administration. A and B: representative kidney sections obtained from saline-treated rats showing normal kidney architecture and histology. There was no tubular injury (0%, n=6) in the examined section. C and D: representative kidney sections obtained from CP + saline-treated rats showing the presence of acute tubular injury in 37.50 ± 2.49 % (n=6) of examined tissue areas (thin arrows) (score 2). E and F: representative kidney sections obtained from CeO₂ NPs-treated rats showing the presence of focal acute tubular injury in 7.37 ± 0.94% (n=6) of examined tissue areas with intracytoplasmic aggregates of giant lysosomes within proximal tubular cells (arrowhead), and epithelial cells falling within the lumen of tubular cells (thin arrow) (score 1). G and H: representative kidney sections obtained from CP + CeO₂ NPs-treated rats showing the presence higher percentage of acute tubular injury when compared with either CP + saline group or CeO₂ NPs group. There is acute tubular injury (thin arrow) involving 58.63 ± 6.27% (n=6) of examined areas (score 3).

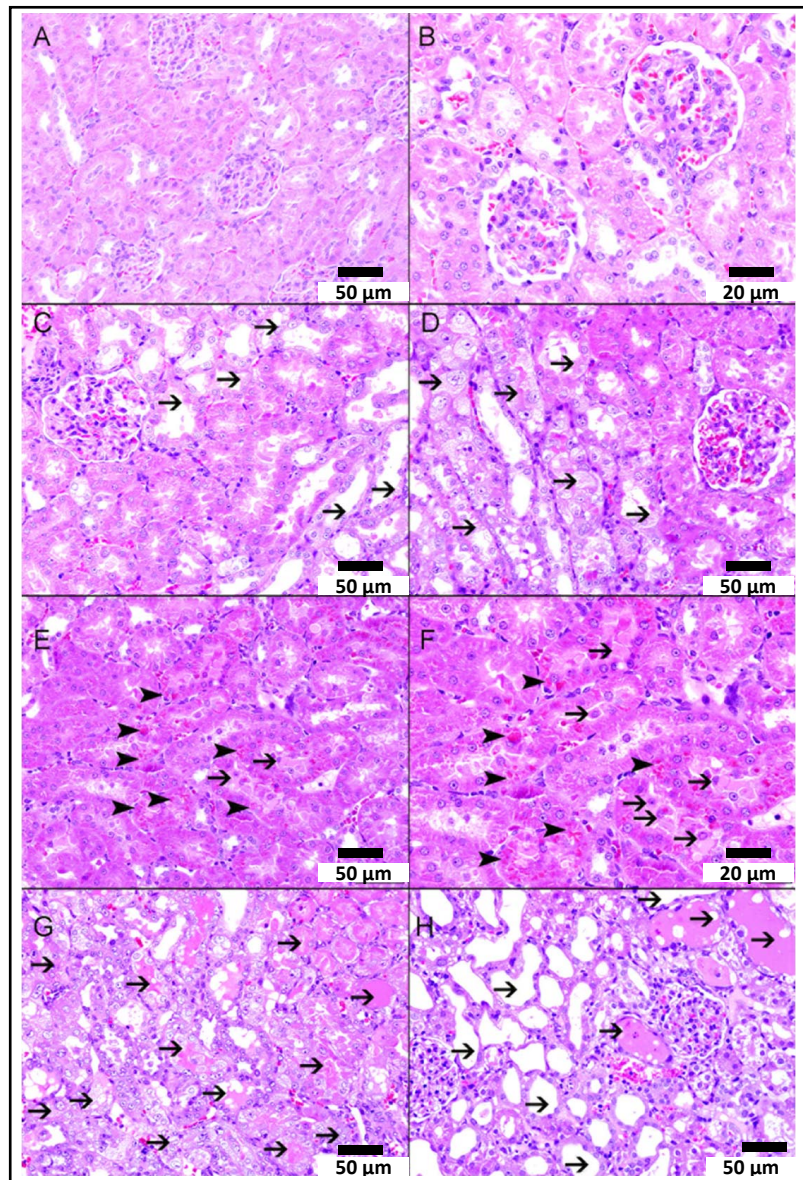


Table 1. Semi quantitative assessment of acute tubular injury in rats treated with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs, 1 mg/kg) with or without cisplatin (CP, 6mg/kg) administration. ^aP<0.0001 compared with the saline-treated group. ^bP<0.0001 compared with the CeO₂ NPs-treated group. ^cP<0.001 compared with the CP + saline-treated group. Data are presented as mean ± SEM (n=6 for each group). Statistical analysis was performed by Newman-Keuls test

Groups	Mean % of acute tubular injury ± SEM	Score of acute tubular injury
Saline	0	0
CP + saline	37.50 ± 2.49 ^a	2
CeO ₂ NPs	7.37 ± 0.94	1
CP + CeO ₂ NPs	58.63 ± 6.27 ^{a,b,c}	3

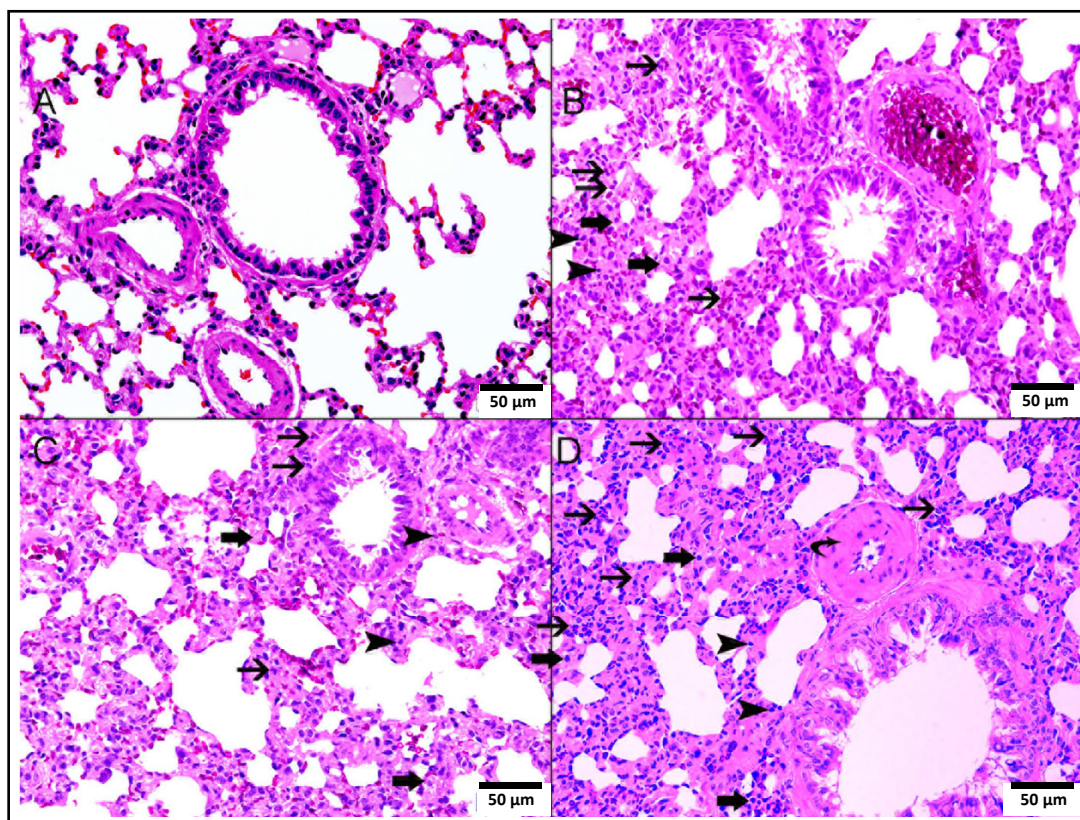


Fig. 6. A-D: representative light microscopy sections of H and E stained lung tissues of Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO_2 NPs) with or without cisplatin (CP) administration. A: representative lung sections obtained from saline-treated rats showing normal lung architecture and histology. B: representative lung sections obtained from CP + saline-treated rats showing the presence of moderate widening of interalveolar interstitial spaces with mixed inflammatory cells consisting of neutrophil polymorphs (thin arrow), lymphocytes (arrowhead) and macrophages (thick arrow). C: representative lung sections obtained from CeO_2 NPs-treated rats showing the presence of foci of mild widening of interalveolar interstitial spaces with mixed inflammatory cells consisting of neutrophil polymorphs (thin arrow), lymphocytes (arrowhead) and macrophages (thick arrow). D: representative lung sections obtained from CP + CeO_2 NPs-treated rats showing the presence severe widening of interalveolar interstitial spaces with mixed inflammatory cells consisting of neutrophil polymorphs (thin arrow), lymphocytes (arrowhead) and macrophages (thick arrow).

Discussion

CP is one of the most effective anticancer drugs used in the therapy of a variety of solid tumours [34], mostly owing to its high efficacy and low cost. CP efficacy increases with dose, but high doses are associated with many severe adverse effects, particularly nephrotoxicity, including apoptosis, inflammation, necrosis and death in proximal tubules and collecting ducts [34]. The mechanism underlying the side effects induced by CP is not fully understood but it has been suggested to be related to oxidative stress and inflammation [35]. Several studies have established the occurrence of renal failure with a single i.p. dose of 6 mg/kg in rats [17, 22, 36]. The dose of CeO_2 NPs tested here has been chosen from earlier studies using animal models of i.t. or oropharyngeal instillation of CeO_2 NPs and evaluating their impact on lung inflammation, oxidative stress, thrombosis and ischemia-reperfusion injury in mice and rats [10, 12, 13, 37]. Here, the exposure of rats to CeO_2 NPs was performed by i.t. instillation technique which offers a more accurate way of administration, since rats are obligate nose breathers that filter most inhaled particles [38].

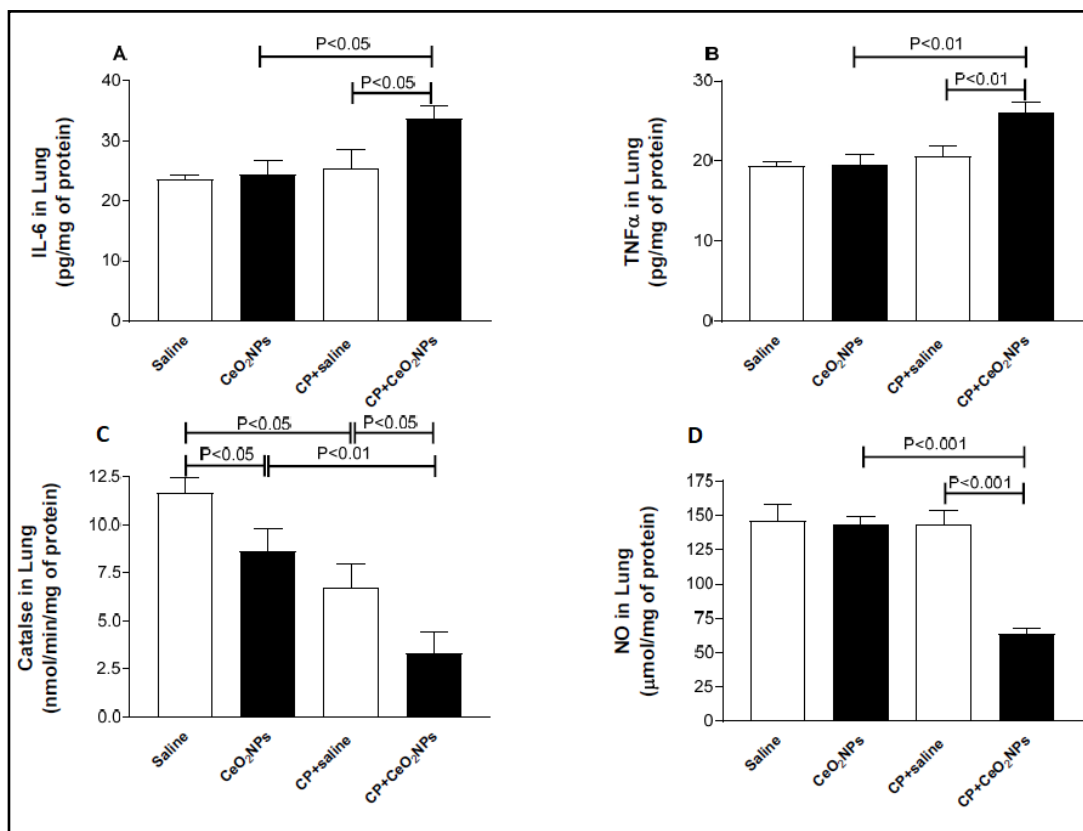
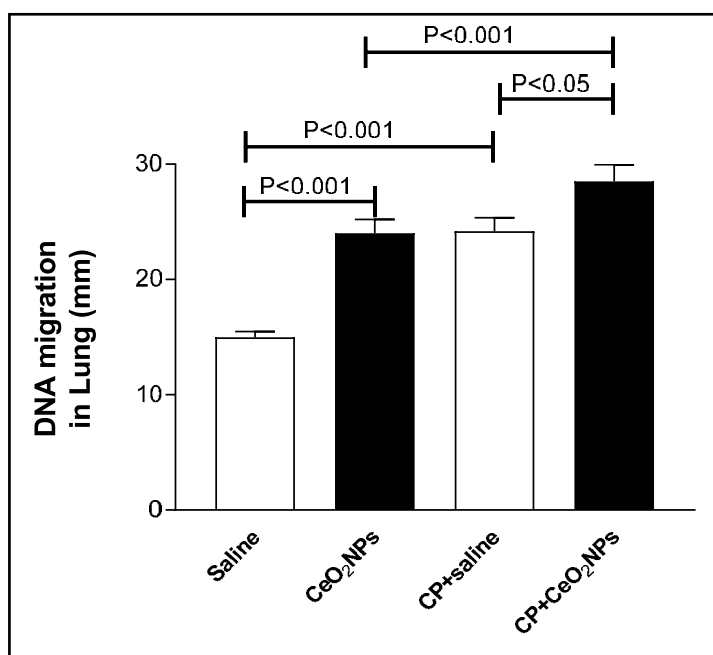


Fig. 7. interleukin 6 (IL-6, A), tumor necrosis factor α (TNF α , B), catalase (C) and total nitric oxide (NO, D) levels in lung homogenates of Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs) with or without cisplatin (CP) administration. Data are mean \pm SEM. IL-6: saline (n=6), CeO₂ NPs (n=6), CP+saline (n=6) and CP+ CeO₂ NPs (n=6). TNF α : saline (n=7), CeO₂ NPs (n=7), CP+saline (n=6) and CP+ CeO₂ NPs (n=6). Catalase: saline (n=6), CeO₂ NPs (n=5), CP+saline (n=5) and CP+ CeO₂ NPs (n=5). NO: saline (n=8), CeO₂ NPs (n=8), CP+saline (n=8) and CP+ CeO₂ NPs (n=8).

Fig. 8. DNA migration (mm) in the lung tissues evaluated by Comet assay, in Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CeO₂ NPs) with or without cisplatin (CP) administration. Data are mean \pm SEM. Saline (n=5), CeO₂ NPs (n=4), CP+saline (n=5) and CP+ CeO₂ NPs (n=6).



There is discrepancy in the literature regarding the effects of CeO₂ NPs. In fact, while some studies reported that these nanoparticles are capable of abrogating inflammation and oxidative stress *in vitro* and *in vivo* [8, 39], several others studies reported their ability to induce inflammation, oxidative stress, DNA damage and apoptosis in various organs [9, 10, 12, 13].

It has been shown that inhaled nanoparticles (<30 nm in diameter) can cross the alveolar capillary barrier and reach the blood [40-42]. The latter have been reported to be filtered and excreted by the kidneys and preferentially deposit at sites of vascular inflammation in both experimental animals and healthy subjects [40]. Moreover, we have recently showed that pulmonary exposure to CeO₂NPs induce inflammation, oxidative stress and DNA damage in different organs including the kidney [12, 13], thus, in the preset study, we thought it was important to assess if pulmonary exposure to CeO₂NPs is capable of aggravating acute renal injury induced by CP. This approach is particularly relevant since it is well-established that patients with renal disease develop states of increased inflammation and oxidative stress which can make them more susceptible to the effects of inhaled nanoparticles [43].

Our data show a decrease in body weight by around 17 % after CP administration and that the combination of CP and CeO₂ NPs further reduced it to 20% but the difference between CP + saline and CP + CeO₂ NPs groups was statistically insignificant. Moreover, we found that the relative kidney weight, water intake and urine volume were all increased in CP + saline compared with saline and that the concomitant administration of CP + CeO₂ NPs has aggravated these 3 variables compared with either CP + saline or CeO₂ NPs. The reduction of body weight and increase in kidney relative weight can be ascribed to the renal tubular damage, and subsequent deterioration of the capacity of tubular cells to reabsorb water, causing polyuria leading to dehydration, which can explain the ensuing increase of water intake. In the present study, the worsening of kidney function in rats exposed to CP and CeO₂ NPs was confirmed by the significant increase in the concentrations of creatinine and urea in plasma and significant decline of creatinine clearance compared with rats treated with either CP + saline or CeO₂ NPs. Such findings have not been reported before. It has been previously shown that the combination of CP + DEPs (1 mg/kg) aggravated the serum concentration of urea and creatinine compared with either CP + saline or DEPs [17]. However, no aggravating effect was observed for urine volume in CP + DEPs compared with either CP + saline or DEPs [17].

In order to investigate the mechanisms by which the combination of CP and CeO₂ NPs exerts their toxic effects, we measured, in kidney homogenates, several traditional and novel markers of kidney injury (KIM-1), inflammation (TNF α and IL-6) and oxidative stress (GSH). KIM-1 is very sensitive (95%) biomarker of early renal injury expressed by renal tubular epithelium and its expression has been reported to persist until kidney recovery [44]. The increase of KIM-1 has been reported in rats treated with CP [45]. Our data show that the concentration of KIM-1 was increased in kidney homogenate of rats given CP and saline compared with saline-treated ones. Interestingly, we found that the administration of both CP and CeO₂ NPs significantly elevated the concentrations of KIM-1 in kidney homogenates compared with rats given either CP and saline or CeO₂ NPs. Similarly, the concentrations of the pro-inflammatory cytokines IL-6 and TNF α were both significantly higher in rats treated with CP and CeO₂ NPs compared with those given either CP and saline or CeO₂ NPs. Along with the increase in KIM-1, IL-6 and TNF α , our data show that the levels of the antioxidant GSH were also significantly increased in CP + CeO₂ NPs compared with either CP + saline or CeO₂ NPs, indicating the occurrence of adaptive reaction in the kidney to counterbalance the potentially damaging action of oxygen radicals induced by the concomitant administration of CP and CeO₂ NPs. The latter findings suggest the aggravating effects of CP and CeO₂ NPs involve inflammation and oxidative stress. It has been reported that oxidative stress and inflammation are considered to be the driving factors in CP-induced nephrotoxicity and the use of natural antioxidants and anti-inflammatory agents such as emodin or ellagic acid alleviate these effects [20, 45]. We have recently demonstrated in mice that pulmonary exposure to CeO₂ NPs induces oxidative stress and DNA injury in the kidney [13]. Here, we

confirmed the occurrence of DNA damage in rats given CeO₂ NPs and those treated with both CP and saline. Interestingly, we found that the DNA damage is exacerbated in rats given both CP and CeO₂ NPs compared with either CP + saline group or CeO₂ NPs group. The latter result could be explained by the potentiation of injury, inflammation and oxidative stress exerted by the combination of CP and CeO₂ NPs. Histopathological analysis of the kidneys of rats given CeO₂ revealed the presence of focal areas of acute tubular injury (7% of total examined areas; score 1) involving proximal convoluted tubules. The treatment with CP and saline induced significantly more acute tubular injury (37% of total examined areas; score 2). Remarkably, the association of CP and CeO₂ NPs caused a much higher increase in extent of acute tubular injury (58% of total examined areas; score 3). Previously, in a rat model of AKI induced by CP administration, we showed no effect on kidney histology of pulmonary exposure to DEPs (1 mg/kg), and the administration of CP + saline or CP + DEPs induced comparable degree of acute tubular necrosis [17]. Thus, the current data suggest that at the same dose, i.e. 1 mg/kg, CeO₂ NPs alone or in combination with CP has more nephrotoxic effects than DEP alone or in combination with CP. The latter could be explained by the small homogenous size of CeO₂ NPs (20 nm) as compared with DEP (geometric mean aerodynamic diameter of 215 nm) [46]. The small size and large surface area of CeO₂ NPs allow them to cross different biological barriers and exert more toxicity [2, 47, 48].

Various studies have shown the presence of a close association between kidney injury and lung diseases [14]. This relationship takes place as a result of the occurrence of systemic inflammation and oxidative stress which subsequently trigger lung damage [14]. Primary kidney disease can induce inflammation and oxidative stress which can affect the lung, and vice versa kidney dysfunction can happen as a result of lung injury and the release of mediators of inflammation and reactive oxygen species in the blood which can reach the kidneys [14, 49]. Our lung histology data show that compared with saline group, the rats given either CeO₂ NPs alone or CP + saline displayed the presence of lung inflammation. Moreover, rats given both CP + CeO₂ NPs revealed a substantial potentiation of pulmonary inflammation. Likewise, the concentrations of markers of inflammation including IL-6 and TNF α in lung homogenates were significantly elevated by the combination of CP and CeO₂ NPs compared with either CP + saline or CeO₂ NPs. It has been shown that oxidative stress induces a decrease of bioactivity of NO [50]. Here, we show that the levels of NO were significantly decreased in lung homogenates of CP + CeO₂ NPs compared with either CP + saline or CeO₂ NPs. Moreover, the measurement of the antioxidant enzyme catalase exhibited its depletion in CeO₂ NPs-treated group and CP + saline group compared with saline group. A significantly higher decline in catalase activity was seen in rats given both CP and CeO₂ NPs. The latter finding indicates that this antioxidant has been consumed as a result of oxidative stress [51]. Additionally, compared with saline group, we found the presence of lung DNA damage in rats treated CeO₂ NPs and those given both CP and saline. Remarkably, the DNA damage was found to be worsened in rats given both CP and CeO₂ NPs. Such finding has never been reported before. Lung DNA damage has been reported in adenine-induced chronic kidney disease in mice, and was explained by the occurrence of oxidative stress [51]. The results obtained presently in the lung confirm previous clinical and experimental studies that kidney injury cause pulmonary pathophysiological effects [14, 17, 43, 51], and hence indicate that pulmonary exposure to CeO₂ NPs exacerbates the kidney injury.

Conclusion

Our data provide novel experimental evidence that pulmonary exposure to CeO₂ NPs in animal model of renal failure exacerbates both kidney and lung injury, and highlight the importance of performing a thorough evaluation of the toxicity of nanoparticles, particularly in animal models of human diseases.

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Disclosure Statement

The authors declare to have no competing interests.

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