

Original Paper

Exacerbation of Coagulation and Cardiac Injury in Rats with Cisplatin-Induced Nephrotoxicity Following Intratracheal Instillation of Cerium Oxide Nanoparticles

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Key Words

Cerium oxide nanoparticle • Heart injury • Acute kidney injury • Coagulation • Inflammation • Oxidative stress

Abstract

Background/Aims: Exposure to particulate air pollution is associated with increased cardiovascular morbidity and mortality. These effects are particularly aggravated in patients with pre-existing kidney diseases. Cerium oxide nanoparticles (CNPs), used as diesel fuel additives, are emitted in vehicle exhaust and affect humans when inhaled. However, thrombotic and cardiac injury resulting from pulmonary exposure to CNPs in experimental acute kidney injury (AKI) is not fully understood. The objective of the present study was to evaluate the thrombotic and cardiac injury effects of CNPs in a rat model of AKI. **Methods:** AKI was induced in rats by a single intraperitoneal injection of cisplatin (CDDP, 6 mg/kg). Six days after injection, rats were intratracheally (i.t.) instilled with either CNPs (1 mg/kg) or saline (control), and various cardiovascular variables and markers of inflammation, oxidative stress and DNA injury were assessed by enzyme linked immunosorbent assay, colorimetric assay, single-cell gel electrophoresis assay and immunohistochemistry, the following day. **Results:** Compared with individual CDDP or CNPs treatments, the combined CDDP + CNPs treatment elevated significantly the coagulation function, relative heart weight, and troponin I, lactate dehydrogenase, interleukin-6 (IL-6), tumor necrosis factor α (TNF α), and total nitric oxide levels

in the plasma. In heart homogenates, the combination of CDDP and CNPs induced a significant increase in IL-6, TNF α , catalase, and glutathione. Furthermore, significantly more DNA damage was observed in this group than in the CDDP or CNPs groups. Immunohistochemical analysis of the heart revealed that expression of nuclear factor erythroid-derived 2-like 2 (Nrf2) and glutathione peroxidase by cardiac myocytes and endothelial cells was increased in the CDDP + CNPs group more than in either CDDP or CNPs group. **Conclusion:** I.t. administration of CNPs in rats with AKI exacerbated systemic inflammation, oxidative stress, and coagulation events. It also aggravated cardiac inflammation, DNA damage, and Nrf2 expression.

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Introduction

Ambient levels of particulate air pollution have been linked to augmented morbidity, hospital admission, and cardiovascular and respiratory disease-induced mortality [1]. Inhaled particles induce lung inflammation and can cross the alveolar capillary barrier, causing systemic inflammation and oxidative stress and thus affecting distant organs such as the kidneys and heart [1-3]. Several epidemiological and clinical studies have reported that inhaling particulate air pollution may be principally detrimental to high-risk groups such as patients with diabetes, hypertension, or renal diseases [1-4]. Additionally, it has been shown that living near a major roadway (which increases the risk of exposure to air pollution) contributes to deterioration in kidney function [3-5].

Cerium oxide nanoparticles (CNPs) have been used in several countries as a diesel fuel-borne catalyst, increasing fuel efficiency and diminishing particle emanation by diesel engines through vehicle exhausts [6]. Nevertheless, this application of CNPs has been shown to result in the release of CNPs into the atmosphere. Humans are subsequently exposed to CNPs via inhalation, posing serious health concerns [7].

Experimental studies have shown that pulmonary exposure to CNPs induces lung inflammation and injury [8-12]. Given their small size, these nanoparticles can translocate across the alveolar capillary barrier and reach the blood, subsequently getting distributed in distant organs such as the kidneys and heart [8-12]. In these organs they can induce inflammation, oxidative stress, and DNA damage [8-12].

It is well established that effects of particulate air pollution are exacerbated in patients with pre-existing renal disease [3-5]. We have recently demonstrated that pulmonary exposure to CNPs in a rat model of acute kidney injury (AKI), induced by cisplatin (CDDP) administration, exacerbated kidney and lung injury and induced aortic oxidative stress, inflammation, and DNA damage [13, 14]. Inhaled nanoparticles have detrimental effects on heart physiology, and may cause thrombosis [15-17]. Kidney and heart functions are closely associated under physiological and pathophysiological conditions [18]. Therefore, we hypothesized here that the effects of CNPs on thrombosis and cardiac function would be aggravated in rats with AKI. This aspect has not been previously assessed, as far as we are aware. Our aim in this study was to explore the cardiovascular responses and mechanism of pulmonary exposure to CNPs during CDDP-induced AKI. This was conducted by measuring renal and cardiovascular endpoints, including coagulation function, systemic and cardiac inflammation, oxidative stress, and DNA damage.

Materials and Methods

Particles

CNPs 10 wt% in water (average diameter of 20 nm) were purchased from Sigma-Aldrich (St Louis, MO, USA). CNPs samples were diluted in saline. To reduce particle aggregation, CNPs suspensions were sonicated for five min (Clifton Ultrasonic Bath, Clifton, NJ, USA). CNPs suspensions were prepared immediately prior to their use and were vortexed comprehensively before their administration. Identical particles from the same source were thoroughly characterized and utilized recently by ourselves and Ma et al. [9, 11, 14, 19].

The concentration of endotoxin of the CNPs and saline was measured according to manufacturer's instructions by a chromogenic Limulus Amebocyte Lysate (Pierce, Rockford, IL, USA) test. In the saline and CNPs solutions, the concentrations of endotoxin measured were inferior to the detection limit (0.1 EU/mL).

Animals and intratracheal instillation

This project was evaluated and accepted by the Institutional Review Board of the United Arab Emirates University.

Male Wistar rats bred in the Animal Research Facility of the College of Medicine and Health Sciences, United Arab Emirates University were used in the present study. They were 10–12 weeks of age and weighing at the start 244 ± 17 g. Rats were fed with standard laboratory chow and water *ad libitum*. They were indiscriminately separated into 4 groups and separately kept in metabolic cages to allow urine collection at a relative humidity of 50–60%, temperature of $22 \pm 1^\circ\text{C}$ and a 12hrs light-dark cycle. The animals were permitted an adaptation period of 4 days prior any experimentation. The animals were weighed at the start of the experiment and immediately prior sacrifice.

Treatments

AKI was achieved in the rats by administering CDDP (Tocris Cookson Ltd., Bristol, Avon, UK) at a single intraperitoneal (i.p.) dose of 6 mg/kg [20, 21]. Control rats were given the same volume of normal saline i.p. On day 5 post CDDP treatment (viz. on day 6), the rats were anesthetized by isoflurane inhalation and positioned supine with an extended neck on an angled board. A cannula (18 Gauge; BD Biosciences, Franklin Lakes, NJ) was introduced via the mouth into the trachea. CNPs or saline-only (200 μL) suspensions were instilled intratracheally (i.t.) via a sterile syringe, followed by a 200 μL air bolus.

The 4 groups received the following treatment:

Group 1: Normal saline (control, 500 $\mu\text{L}/\text{rat}$) was administered i.p., and 5 days after, one dose of saline (200 μL per rat) was administered i.t.;

Group 2: Similar to group 1, apart from that the saline given i.t. was substituted with an i.t. instillation of CNPs (1 mg/kg);

Group 3: One dose of CDDP (6 mg/kg) was injected i.p., and 5 days after, a single dose of saline (200 μL per rat) was given i.t.;

Group 4: Similar to group 3, apart from that the saline given i.t. was substituted with an i.t. instillation of CNPs (1 mg/kg).

On day 6, animals were put in metabolic cages straightaway following i.t. instillation of saline or CNPs. The urine of each animal was obtained over a 24 h period, and the urine volume quantified.

Platelet aggregation in rat whole blood

The platelet aggregation assay in whole blood was performed as previously described [22, 23].

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) measurement in plasma in vitro

The PT and aPTT were measured according to previously reported techniques using plasma samples of rats i.t. administered with either saline or CNPs, with or without CDDP administration [24, 25].

Biochemical analysis, histology, and immunohistochemistry

Animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and blood was collected from the inferior vena cava in 4% of EDTA. An ABX Micros 60 counter was used for blood cell counts (ABX Diagnostics, Montpellier, France). The residual blood was centrifuged at $900 \times g$ (at 4°C for 15 min), and the plasma samples were kept at -80°C awaiting analysis.

The concentrations of urea in plasma and creatinine in urine and plasma were quantified using commercial kits (Roche Diagnostics, Indianapolis, IN, USA), and that of interleukin (IL)-6 and tumour necrosis factor α (TNF α) in plasma were assessed using ELISA kits (Duo Set, R&D systems, Minneapolis, MN, USA). The concentration of troponin I and activity lactate dehydrogenase (LDH) were assessed using commercial kits (Roche, Basel, Switzerland). The quantification of nitric oxide (NO) was achieved with a total NO assay kit (R&D systems, MN, USA), which quantifies the comparatively stable NO metabolites NO_2^- and NO_3^- .

The rats were sacrificed with an overdose of sodium pentobarbital. The hearts were removed, washed with ice-cold saline, blotted with filter paper, and weighed. Half of the hearts were fixed (10% buffered formalin). They were then sectioned, casseted, dehydrated in cumulative concentrations of ethanol, cleared with xylene, and embedded with paraffin wax. Sections (3 μm) were made from the paraffin blocks and stained with hematoxylin and eosin. The stained sections were blindly assessed by light microscopy. For immunohistochemistry, heart sections (5 μm) were made and mounted on aminopropyltriethoxysilane (APES)-coated slides. Following dewaxing with xylene and rehydrating with graded alcohol, slides were put in 0.01 M citrate buffer solution (pH=6.0). Pretreatment procedures were performed to unmask the antigens in a water-bath (95°C for 30 min). After that, sections were treated with peroxidase block (60 min), followed by protein block for an additional 60 min. The sections were incubated with either anti-nuclear factor erythroid 2-related factor 2 (Nrf2) (Rabbit Polyclonal, 1:100, Abcam, Cambridge, UK) or anti-glutathione peroxidase (Rabbit Polyclonal, 1:300, Abcam, Cambridge, UK) at room temperature for 60 min. Following conjugation with primary antibodies, sections were incubated with secondary antibody (EnVision, DAKO, Agilent, USA) at room temperature for 20 min, followed by a DAB chromogen addition (EnVision, DAKO, Agilent, USA) and counterstaining with hematoxylin. Suitable positive controls were utilized. Primary antibody was not added to the sections of the negative control group. Both negative and positive controls were utilized in each batch of stained slides (not shown in figures). The immunohistochemical staining of heart tissue was assessed blindly and semi-quantitatively in 4 slides of each specimen. Four equal coronal slices of the heart were present in each slide. Using the ImageJ software (<http://rsbweb.nih.gov/ij/>), the number of positively-stained cells were quantified in 10 randomly selected high-power fields (HPF) in the heart sections. The mean numbers of positively-stained cells were then changed from per HPF to per mm^2 ($1\text{mm}^2=4\text{HPF}$) [26, 27].

The other halves of the hearts were rapidly rinsed with ice-cold PBS (pH 7.4) prior to homogenization, as described before [11]. The homogenates were centrifuged at 3000 g for 10 min at 4°C to eliminate cellular debris, and the supernatants were utilized for additional analysis [11]. Bradford's method was used to quantify the cardiac protein content. We also measured in the heart homogenates the concentrations of IL-6, TNF α , glutathione (Sigma-Aldrich Fine Chemicals, St Louis, MO, USA) as well as the activity of catalase (CAT) (Cayman Chemicals Company, Ann Arbor, MI, USA).

DNA damage assessment by comet assay

In separate set of animals, the heart DNA injury assessment was quantified by comet (single-cell gel electrophoresis) assay according to previously reported technique [11, 14].

Statistical analysis

The data were analyzed with GraphPad Prism Version 4 (Graphpad Software Inc., San Diego, CA, USA). Data were analyzed for normal distribution with the Shapiro-Wilk normality test. Data are expressed as means \pm SEM. Comparisons between groups were achieved by one-way analysis of variance (ANOVA), followed by the Newman-Keuls test. P values < 0.05 were considered significant.

Results

Creatinine clearance and plasma concentrations of urea and creatinine

Creatinine clearance and the plasma concentrations of urea and creatinine were measured in the four groups of rats administered i.t. with either saline or CNPs, with or without CDDP administration (n=5–7 per group). The plasma concentration of urea did not differ between the saline (7.7 ± 0.6 mmol/L) and CNPs (8.2 ± 0.5 mmol/L) groups. However, the concentration was significantly elevated in the group treated with CDDP + CNPs (37.0 ± 9.6 mmol/L) when compared with those treated with either CDDP + saline (18.5 ± 3.7 mmol/L) (P < 0.05) or CNPs alone (P < 0.01).

The plasma creatinine concentration was comparable in the saline ($16.1 \pm 0.6 \mu\text{mol/L}$) and CNPs ($16.7 \pm 0.4 \mu\text{mol/L}$) groups. The plasma creatinine concentration was significantly higher in the group treated with CDDP + CNPs ($109.9 \pm 35.2 \mu\text{mol/L}$) when compared with those treated with either CDDP + saline ($52.3 \pm 10.2 \mu\text{mol/L}$) ($P < 0.05$) or CNPs alone ($P < 0.01$).

Similarly, there was no difference in creatinine clearance between the saline ($1.5 \pm 0.1 \text{ mL/min}$) and CNPs ($1.7 \pm 0.1 \text{ mL/min}$) groups. Clearance was significantly lower in the group treated with CDDP + CNPs ($0.2 \pm 0.05 \text{ mL/min}$) when compared with those treated with either CDDP + saline ($0.6 \pm 0.09 \text{ mL/min}$) ($P < 0.05$) or CNPs alone ($P < 0.0001$).

Coagulation function

Fig. 1A shows that the number of circulating platelets was significantly reduced in the CDDP + CNPs-treated group when compared with those treated with CDDP + saline ($P < 0.01$) or CNPs alone ($P < 0.01$).

Fig. 1B illustrates the effect of i.t. saline or CNPs administration, with or without CDDP administration, on platelet aggregation in whole blood. Whole blood obtained from rats exposed to CNPs and incubated with ADP ($0.1 \mu\text{M}$) showed significantly more platelet aggregation than that collected from rats exposed to saline ($P < 0.01$). Likewise, there was a significant increase in platelet aggregation in the CDDP + saline-treated group when compared with the saline-treated group ($P < 0.0001$). Interestingly, platelet aggregation was significantly increased in the CDDP + CNPs-treated group when compared with the CDDP + saline ($P < 0.01$) or CNPs groups ($P < 0.001$).

Fig. 2 shows that, compared with the saline-treated group, both the PT and aPTT were slightly but significantly shorter in the CNPs and CDDP + saline-treated groups. Likewise, PT and aPTT for the CDDP + CNPs-treated group were significantly shorter when compared with the CDDP + saline or CNPs-treated groups.

Relative heart weight and plasma concentrations of troponin I and lactate dehydrogenase

Fig. 3 illustrates that rats treated with CDDP + CNPs had significantly increased relative heart weight (Fig. 3A), plasma concentrations of troponin I (Fig. 3B), and LDH (Fig. 3C) when compared with the CDDP + saline and CNPs-treated groups.

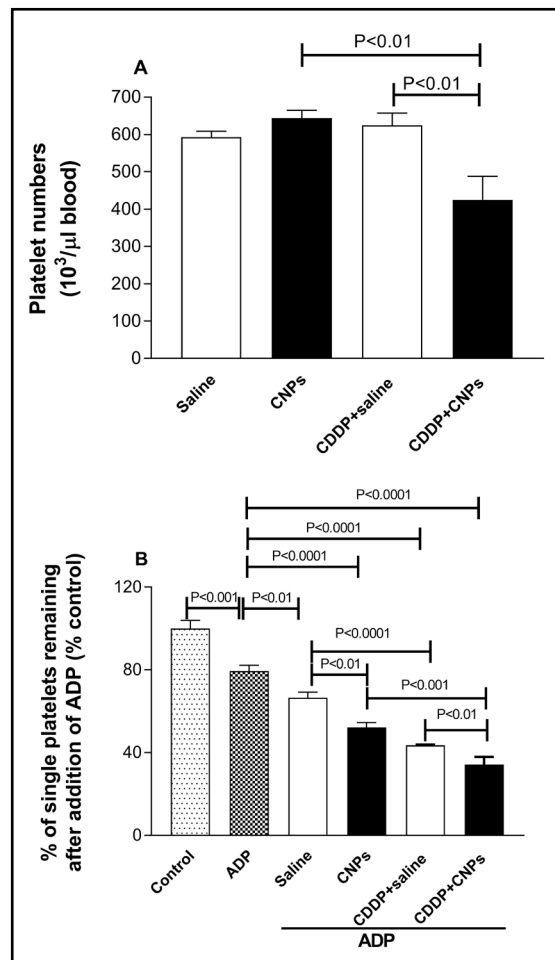


Fig. 1. (A) Circulating platelet numbers in rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. (B) Platelet aggregation in whole blood obtained from either untreated control rats with or without the addition of adenosine diphosphate (ADP; $0.1 \mu\text{M}$) or rats intratracheally instilled with either saline or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. Data are presented as means \pm SEM ($n=5-8$).

Total NO activity and plasma concentrations of IL-6 and TNF α

The plasma concentrations of IL-6 (Fig. 4A) and TNF α (Fig. 4B) and the total NO activity (Fig. 4C) were significantly augmented in the CDDP + CNPs-treated group when compared with the CDDP + saline and CNPs-treated groups ($P < 0.05$ – 0.001).

Heart homogenate concentrations of IL-6, TNF α and GSH, and CAT activity

The IL-6 concentration in heart homogenate was significantly increased in the CNPs-treated group compared with the saline-treated group ($P < 0.05$). Compared with the latter group, concentrations of IL-6 ($P < 0.0001$) and TNF α ($P < 0.0001$) in heart homogenates were significantly increased in the CDDP + saline-treated group (Fig. 5A–B). Moreover, the concentrations of IL-6 and TNF α were significantly increased in the CDDP + CNPs-treated group compared with the CDDP + saline ($P < 0.0001$) and CNPs-treated groups ($P < 0.0001$) (Fig. 5A–B).

CAT activity in heart homogenates was significantly increased in the CNPs-treated group when compared with the saline-treated group ($P < 0.05$). This was also true for the CDDP + saline-treated group when compared with the saline-treated group ($P < 0.0001$) (Fig. 5C). Furthermore, CAT activity was significantly increased in the CDDP + CNPs group when compared with the CDDP + saline ($P < 0.0001$) and CNPs-treated groups ($P < 0.0001$) (Fig. 5C). The concentration of GSH in heart homogenates was significantly augmented in the CDDP + CNPs group when compared the CDDP + saline ($P < 0.0001$) and CNPs-treated groups ($P < 0.0001$) (Fig. 5D).

DNA damage in the heart

Fig. 6 depicts the evaluation of DNA injury in the heart tissue via comet assay. Compared with the saline-treated group, i.e. administration of CNPs induced a significant increase in DNA damage ($P < 0.01$). DNA damage was significantly elevated in the CDDP + saline-treated group when compared with the group treated with saline only ($P < 0.001$). Additionally, the observed DNA damage increased significantly in the CNPs + CDDP-treated group when compared with the CDDP + saline ($P < 0.05$) or CNPs-only ($P < 0.05$) groups.

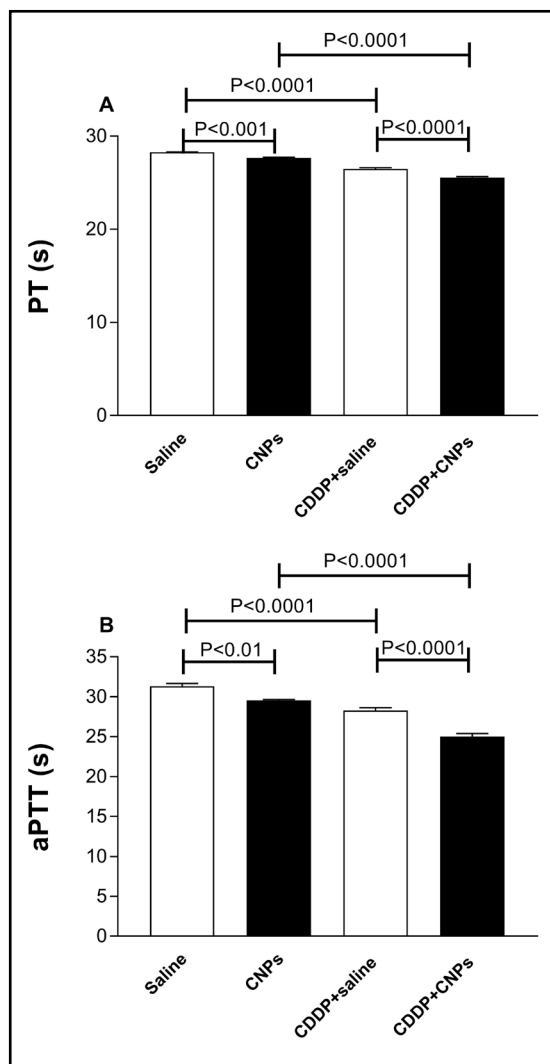


Fig. 2. (A) Prothrombin time (PT) and (B) activated partial thromboplastin time (aPTT) in plasma of rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. Data are presented as means \pm SEM ($n=5$).

Heart histopathology and immuno-histochemistry

There were no detectable morphological changes in the H&E-stained heart sections obtained from rats instilled i.t. with either saline or CNPs, with or without CDDP administration (Fig. 7).

Fig. 8A, 8C, 8E, 8G, and 8I reveal that there was nuclear and cytoplasmic Nrf2 expression by cardiac myocytes and endothelial cells in the heart sections of the studied groups. The expression was of various intensities and distributions. Nuclear Nrf2 expression by cardiac myocytes and endothelial cells was significantly increased in the CNPs-treated group when compared with the saline group ($P < 0.0001$), and in the CDDP + saline-treated group compared with the saline-only group ($P < 0.0001$). Furthermore, nuclear Nrf2 expression was significantly increased in the CDDP + CNPs-treated group when compared with the CDDP + saline ($P < 0.0001$) or CNPs ($P < 0.0001$) groups.

Fig. 8B, 8D, 8F, 8H, and 8J demonstrate various intensities and distributions of cytoplasmic glutathione peroxidase expression by cardiac myocytes in the heart sections of the four groups. Glutathione peroxidase expression was significantly increased in the CNPs-treated group when compared with the saline-treated group ($P < 0.01$), and in the CDDP + saline-treated group when compared with the saline-treated group ($P < 0.01$). Moreover, cytoplasmic glutathione peroxidase expression in cardiac myocytes was significantly increased in the CDDP + CNPs-treated group when compared with the CDDP + saline ($P < 0.05$) or CNPs-treated ($P < 0.05$) groups.

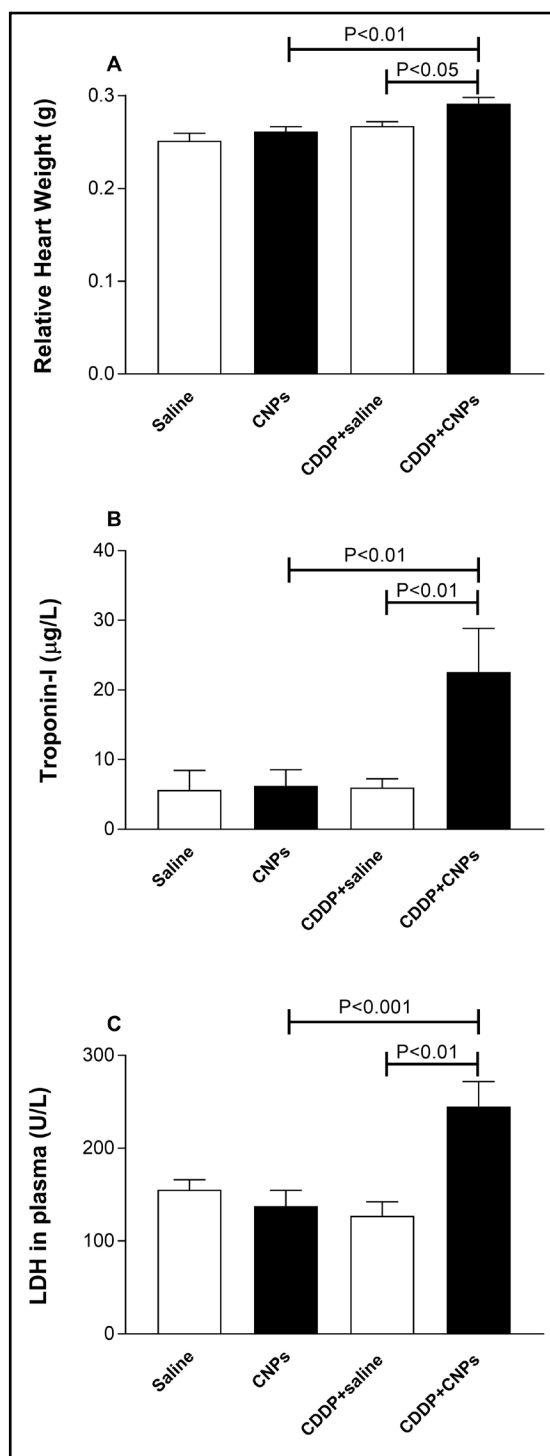
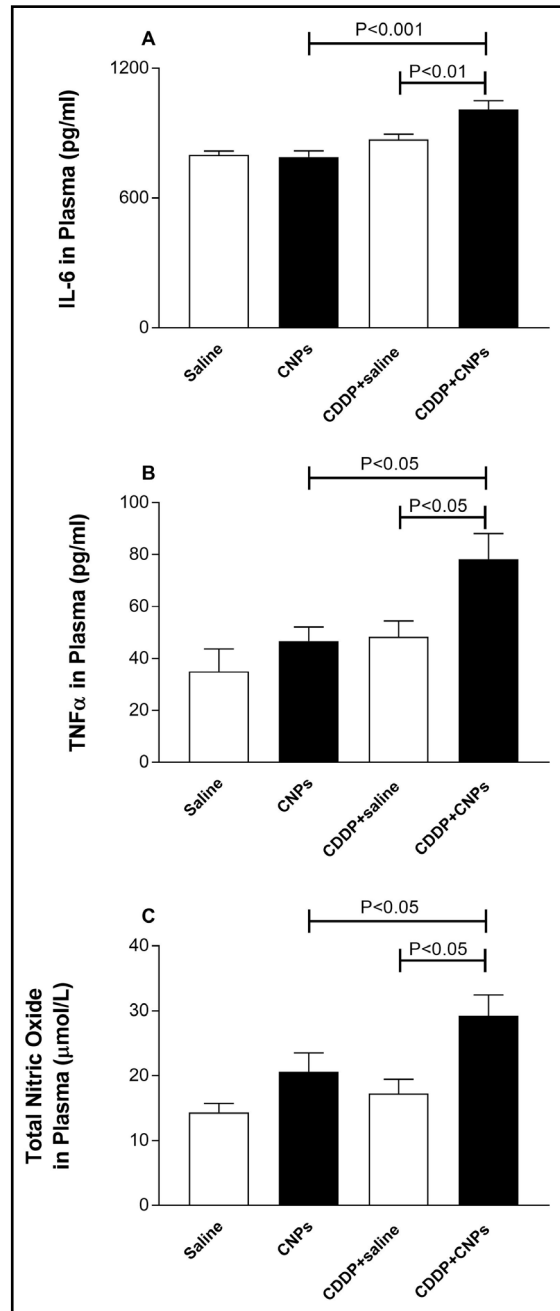


Fig. 3. (A) Relative heart weight and (B) plasma levels of troponin I and (C) lactate dehydrogenase (LDH) in rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. Data are presented as means \pm SEM (n=6-8).

Fig. 4. Plasma concentrations of (A) interleukin-6 (IL-6) and (B) tumour necrosis factor α (TNF α) and (C) activity of total nitric oxide (NO) in rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. Data are presented as means \pm SEM (n=6–8).



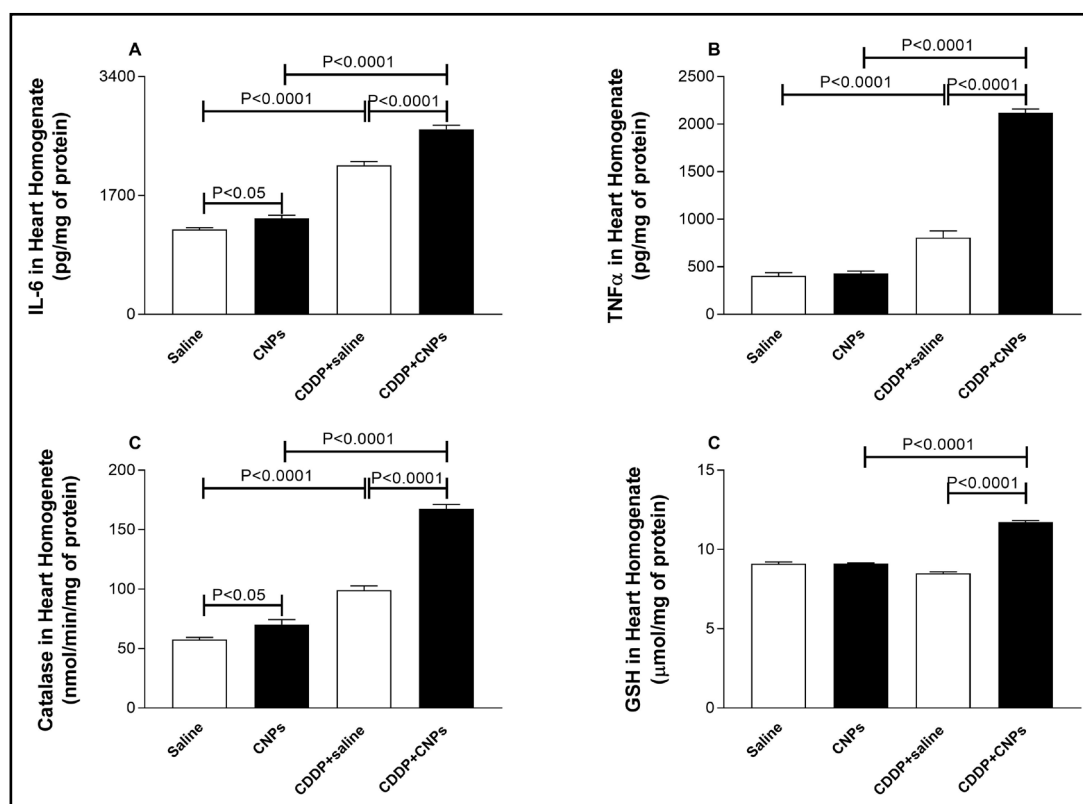
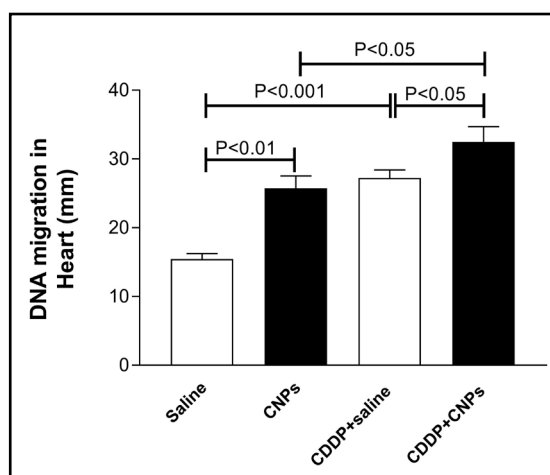


Fig. 5. Heart homogenate levels of (A) interleukin-6 (IL-6), (B) tumour necrosis factor α (TNF α), (C) catalase, and (D) glutathione (GSH) in rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. Data are presented as means \pm SEM (n=6–8).

Fig. 6. DNA migration (mm) evaluated by comet assay in the heart tissues obtained from rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. Data are presented as means \pm SEM (n=4–5).



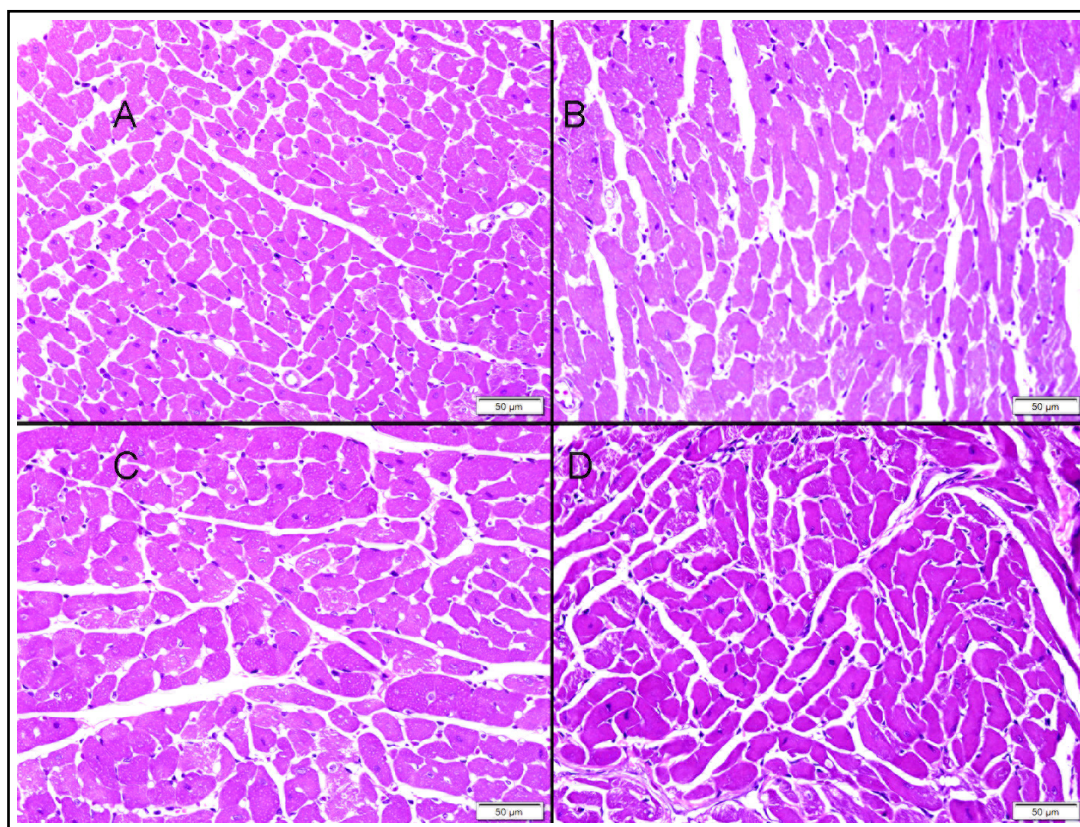


Fig. 7. A–D. Representative light microscopy sections of hematoxylin and eosin-stained heart tissues of Wistar rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. (A) Representative heart section obtained from saline-treated rats showing normal heart with unremarkable changes. (B) Representative heart section obtained from CDDP + saline-treated rats showing heart with unremarkable changes. (C) Representative heart sections obtained from CNP-treated rats showing heart with unremarkable changes. (D) Representative heart section obtained from CDDP + CNP-treated rats showing heart with unremarkable changes. Scale bars: 50 µm.

Discussion

In this study, we showed that i.t. administration of CNPs in rats with AKI exacerbated systemic inflammation, oxidative stress, and coagulation events. It also aggravated cardiac inflammation, DNA damage, and Nrf2 expression.

Kidney disease is a global public health problem affecting over 750 million people worldwide [28]. The prevalence of AKI is also rising globally, resulting in significant morbidity and mortality. AKI induced by nephrotoxins, traditional medicines, infectious agents, or hospitalization contributes to increased risk of mortality and the development of chronic kidney disease [28]. It is also well established that the adverse health effects of particulate air pollution are more pronounced in vulnerable subjects, such as those with kidney diseases [4].

CDDP is used in the treatment of several types of cancer. Though its effectiveness increases with dose, high doses have been reported to cause several adverse effects, principally nephrotoxicity [29]. This process involves inflammation, oxidative stress apoptosis, and proximal tubular injury [29, 30]. Numerous experimental studies have acknowledged AKI development in rats administered with a single i.p. dose of CDDP at 6 mg/kg [14, 31].

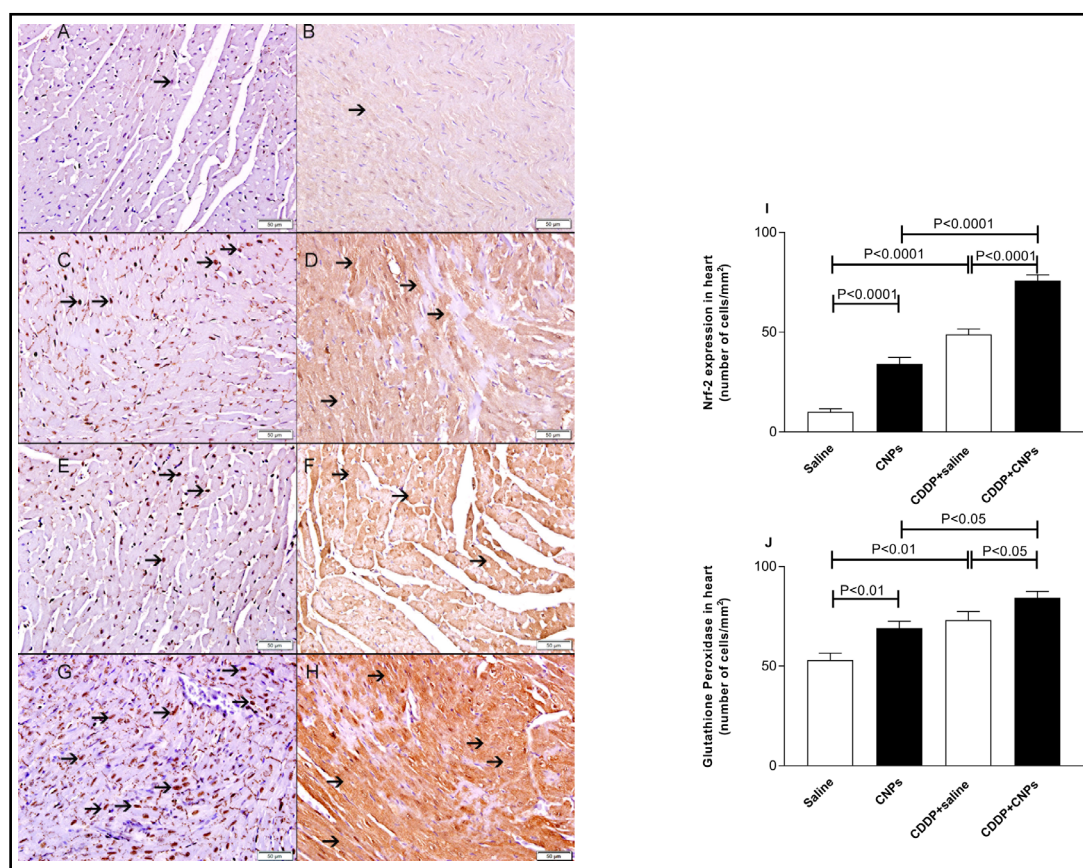


Fig. 8. A–H. Immunohistochemical analysis of the heart tissue sections for the detection of nuclear factor erythroid-derived 2-like 2 (Nrf2) and glutathione peroxidase in rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration. (A) Representative heart section obtained from saline-treated rats showing normal heart with Nrf2 nuclear staining of few cardiomyocytes (arrow). (B) Representative heart section obtained from saline-treated rats showing normal heart with glutathione peroxidase cytoplasmic staining of few cardiomyocytes (arrow). (C) Representative heart section obtained from CDDP + saline-treated rats showing increased number of nuclei of cardiomyocytes stained with Nrf2 (arrow). (D) Representative heart section obtained from CDDP + saline-treated rats showing increased number of cardiomyocytes stained with glutathione peroxidase (arrow). (E) Representative heart section obtained from CNPs-treated rats showing increased number of nuclei of cardiomyocytes stained with Nrf2 (arrow). (F) Representative heart section obtained from CNPs-treated rats showing increased number of cardiomyocytes stained with glutathione peroxidase (arrow). (G) Representative heart section obtained from CDDP + CNPs-treated rats showing increased number of nuclei of cardiomyocytes stained with Nrf2 (arrow). (H) Representative heart section obtained from CDDP + CNPs-treated rats showing increased number of cardiomyocytes stained with glutathione peroxidase (arrow). (I) Quantitative evaluation of Nrf2 nuclear staining of cardiomyocytes of hearts obtained from rats intratracheally instilled with either saline (control) or cerium oxide nanoparticles (CNPs), with or without cisplatin (CDDP) administration (n=8). (J) Quantitative evaluation of glutathione peroxidase staining of cardiomyocytes of hearts obtained from rats intratracheally instilled with either saline or CNPs, with or without CDDP administration (n=7–8). Scale bars: 50 μ m.

Recently, in a rat AKI model induced by CDDP administration, pulmonary exposure to CNPs exacerbated kidney and lung injury [14]. Moreover, we have also reported an aggravation of aortic oxidative stress, inflammation, and DNA damage following pulmonary exposure to CNPs in rats treated with CDDP [13]. However, the thrombotic and cardiac effects of CNPs in experimental AKI have not yet been reported. In the present study, we therefore continued to use the same rat model of AKI and the same dose of CNPs (1 mg/kg). The dose was deposited in the lung by i.t. instillation [13, 14]. A similar dose of CNPs has previously been used by others in mice and rats to assess pulmonary toxicity or ischemia-reperfusion injury [9-11, 32].

The impact of pulmonary exposure of CNPs in experimental AKI on the lung and kidney has been recently reported [14]. The present study was specifically designed to investigate the consequences of such an interaction on the cardiovascular system. As reported earlier [14], we confirmed in the present work that compared with values from rats treated with CDDP + saline or CNPs, co-administration of CDDP + CNPs resulted in a substantial elevation in the plasma concentrations of creatinine and urea and decreased creatinine clearance.

Along with other researchers, we recently reported that the *in vitro* incubation of mouse or human whole blood with CNPs neither caused platelet aggregation nor affected either PT or aPTT [10, 33]. However, in the same study, we also showed that *in vivo* pulmonary exposure to CNPs in mice caused thrombotic events in pial microvessels [10]. In the current study, blood was collected from rats previously instilled i.t. with either saline or CNPs, with or without CDDP administration. We measured the number of circulating platelets, platelet aggregation in the presence of ADP, and the PT and aPTT. Our data show a significant decrease in the number of circulating platelets in rats treated with CDDP + CNPs compared with those that received either CDDP + saline or CNPs. The decrease in platelets is suggestive of platelet activation *in vivo*. Both experimental murine and clinical studies have previously described a reduction in circulating platelet numbers after pulmonary exposure to particulate air pollution [34, 35]. Our data show that in CNPs group, platelet aggregation, PT, and aPTT were significantly affected compared with the saline-treated group. This was also true for the CDDP + saline group compared with the saline-treated group. Interestingly, the latter endpoints were all significantly affected in rats treated concomitantly by CDDP + CNPs compared with the CDDP + saline or CNPs treated-groups. This demonstrated a worsening of the thrombotic effects of CNPs in rats with AKI.

Since it is possible that the observed thrombotic effects may have resulted from systemic inflammation and oxidative stress caused by pulmonary deposited CNPs, we measured several markers of cytotoxicity, inflammation, and oxidative and nitrosative stress in the plasma. Our data show that relative heart weights, troponin I, and LDH were significantly increased in CDDP + CNPs-treated rats compared with those administered with either CDDP + saline or CNPs. The increase in relative heart weight is indicative of cardiac hypertrophy, and the augmentation of troponin I is suggestive of myocardial injury. It is well known that troponin I is a reliable biomarker of myocardial injury in cardiotoxicity [36, 37]. The increase of LDH in plasma is indicative of cytolysis [38]. As troponin was also increased in plasma, we can conclude that increased LDH could have resulted at least partly from the cardiotoxicity observed in rats exposed to CDDP + CNPs. Furthermore, our data exhibit that the levels of IL-6, TNF α , and total NO were significantly increased in the CDDP + CNP group compared with those administered with either CDDP + saline or CNPs. This result indicates the occurrence of inflammation and oxidative stress in rats with AKI exposed to CNPs. It is well known that patients with kidney diseases develop conditions of amplified inflammation and oxidative stress, thus making them more prone to the adverse effects of inhaled nanoparticles [4]. The observed increase in systemic inflammation and oxidative stress may explain the increased coagulation disturbances observed in the CDDP + CNPs group. In fact, both experimental and clinical studies have reported a close relationship between thrombosis, inflammation, and oxidative stress [39].

Markers of inflammation, oxidative and nitrosative stress, and cardiac injury were significantly increased in the plasma of CDDP + CNPs group. We therefore wanted to verify

the occurrence of cardiotoxicity and assess its mechanisms by evaluating inflammation, oxidative stress, and DNA damage in heart tissue. Using heart homogenates, we found that IL-6 and CAT levels were significantly increased in the CNPs group when compared with the saline group. We also found that IL-6, TNF α , and CAT levels were increased in the CDDP + saline group when compared with the saline group. This confirms the damaging effect of CDDP on the heart and corroborates the findings of a recent study reporting the cardiotoxicity of CDDP [40]. This study also demonstrated that the use of zingerone, a natural polyphenol with anti-inflammatory and antioxidant properties, alleviates this effect [40]. Remarkably, we presently demonstrated that compared with either CNPs or CDDP + saline, the combination of CDDP + CNPs significantly increased levels of IL-6, TNF α , CAT, and GSH in heart homogenates. It is well known that oxidative stress and inflammation, which mutually amplify each other, can lead to cell membrane damage or DNA injury [39]. We have previously reported that pulmonary exposure to CNPs in healthy mice induces oxidative stress, inflammation, and DNA damage in the heart [11]. More recently, we have documented the potentiation of inflammation, oxidative stress, and DNA damage in the kidney and lungs of rats exposed to CDDP + CNPs when compared with effects of CNPs or CDDP + saline treatment [14]. However, as far as we are aware, the impact of CDDP + CNPs on heart DNA damage has not been previously reported. Here we show that DNA damage was significantly increased in both CNPs and CDDP + saline groups when compared with saline only group, and that the association of CDDP + CNPs significantly potentiated this effect. The latter could be explained by the aggravation of inflammation and oxidative stress in the hearts of rats treated with both CDDP and CNPs.

It has been reported that a single high dose of CDDP (10 mg/kg, i.p.) or repeated administration of CDDP to rats at 4 mg/kg/week for 4 weeks caused alteration of the heart morphology [41, 42]. Here, the histological analysis of the heart did not show any detectable morphological changes following either CDDP + saline or CDDP + CNPs administration. This could be related to the fact that we used a single (i.p.) and relatively low dose of CDDP (6 mg/kg, i.p.) and assessed acute pulmonary exposure to single dose of CNPs as compared with repeated and chronic exposure to CNPs. However, our data did clearly show significant increases in the levels of troponin I and LDH in the plasma, and inflammation and oxidative stress in heart homogenates. In addition, the immunohistochemical analysis of the heart tissue revealed a concomitant and significant increase in Nrf2 expression and glutathione peroxidase in both CNPs and CDDP + saline, when compared with the saline-only group. Moreover, in concordance with the results seen in heart homogenates, we found a significant potentiation of Nrf2 and glutathione peroxidase in the CDDP + CNPs group compared with either CNPs or CDDP + saline. Nrf2 is a transcription factor and can be triggered by oxidative stress. It is implicated in the regulation of the expression of various cytoprotective genes encoding antioxidant proteins [43]. Significant intensification of Nrf2 and glutathione peroxidase expression was observed in the CDDP + CNPs group, suggesting that this treatment potentially prompted an adaptive reaction counterbalancing the potentially damaging action of CDDP + CNPs-induced oxygen radicals. An increase of Nrf2 expression has been reported in the hearts of mice exposed to waterpipe smoke and in lipopolysaccharide-induced lung injury in mice [44, 45].

The findings of the present work as well as numerous other previously published studies provided considerable and clear evidence that pulmonary exposure to CNPs induces not only lung toxicity characterized by inflammation, oxidative stress and DNA damage, but also systemic toxicity in various organs comprising the kidney and heart [9-11, 14, 32, 46-48]. Using different route of exposure, i.e. intraperitoneal administration, it has been reported that CNPs exert protective effects on CDDP-induced nephrotoxicity in mice and doxorubicin-induced liver injury in rats [49, 50]. The exact reason for these discrepancies is not clear, but it could be related to the route of exposure, the difference in the morphological characteristics of the nanoparticles, the biological environment used or other unknown factors. Further studies are needed to clarify this issue.

Conclusion

We conclude that i.t. administration of CNPs in rats with AKI impaired coagulation, systemic inflammation, and oxidative stress. It also exacerbated cardiac inflammation, oxidative stress and DNA damage, and Nrf2 expression. Our study suggests that the adverse cardiovascular effects of inhaled CNPs are expected to be the greatest in patients with kidney injury. Further studies are needed to assess the renal and cardiovascular effects of CNPs in animal model of chronic renal disease.

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All authors have read and approved the manuscript.

Author Contributions

AN designed, planned, and supervised all experiments and wrote the article. SA performed histological analysis. SN, MK, FM, SB, PY, and JY performed the experiments. BHA contributed to the design of the study and wrote the article.

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Disclosure Statement

No conflicts of interest to disclose.

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