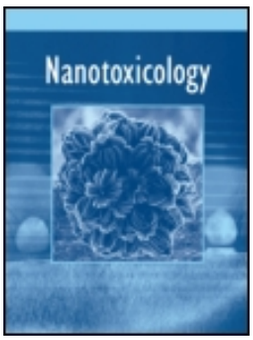

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ORIGINAL ARTICLE

The effects of palladium nanoparticles on the renal function of female Wistar rats

Luca Fontana[1], Veruscka Leso[1], Alessandro Marinaccio[2], Giovanna Cenacchi[3], Valentina Papa[3], Kerstin Leopold[4], Roland Schindl[4], Beatrice Bocca[5], Alessandro Alimonti[5], and Ivo Iavicoli[1]

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Abstract

A number of studies have shown that palladium nanoparticles are able to exert some adverse health effects, such as concentration-dependent cytotoxicity, induction of apoptosis and alterations of the release and expression of numerous cytokines. Nevertheless, our current knowledge of the potential toxic effects induced by exposure to these nanoparticles is far from being complete. For this reason, the present study assessed the possible renal toxicity of palladium nanoparticles by investigating urinary excretion of retinol binding protein, β_2-microglobulin and albumin in female Wistar rats intravenously exposed to different nanoparticle concentrations (0.012, 0.12, 1.2 and 12 μg/kg) and by carrying out a morphological observation of the kidneys of treated animals. Results showed a significant increase in urinary retinol binding protein and β_2-microglobulin levels in rats that were administered 12 μg/kg compared to controls. Moreover, an ultrastructural study of the kidneys revealed significant alterations in the proximal and distal tubular epithelium were observed, with a range of severity, in all experimental conditions. Collectively, our findings suggest that exposure to palladium nanoparticles is able to induce a significant renal tubular dysfunction, whereas it does not seem to affect kidney glomerular filtration. However, further studies are needed to confirm our results, to understand the molecular mechanisms of toxic action and to evaluate the potential adverse effects of these nanoparticles also on the glomerular section of the kidney.

Keywords

β_2-Microglobulin, albumin, kidney, rats, retinol binding protein

History

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Introduction

Palladium (Pd) is a noble and precious metal that together with iridium (Ir), platinum, rhodium (Rh), ruthenium and osmium, belongs to the platinum group elements (PGEs). Although this metal is used in many industrial sectors, by far its most extensive use is for production of automobile catalysts (Iavicoli et al., 2008a, 2010). These devices are able to further reduce carbon monoxide (CO), unburned hydrocarbons (HCs) and nitrogen oxides (NO*x*) emissions in the exhaust of lean-burn engines (Ravindra et al., 2004). However, surface abrasion and deterioration of catalytic converters release Pd into the environment, causing a constant and progressive increase of its level in road dust, soil, airborne particulate and groundwater tables (Iavicoli et al., 2011a; Ravindra et al., 2004).

Exposure to this metal can cause acute toxicity or hypersensitivity with respiratory symptoms, urticaria and, less frequently, contact dermatitis (Cristaudo et al., 2005; Daenen et al., 1999; Garau et al., 2005; Goossens et al., 2006; Jappe et al., 1999; Katoh et al., 1999; Murdoch et al., 1986; Van Joost & Roesyanto-Mahadi, 1990; Van Ketel & Niebber, 1981). Moreover, Pd is able to exert a significant effect on the production and release of a number of cytokines (Boscolo et al., 2004; Iavicoli et al., 2006, 2008b; Schmalz et al., 2000). Effects on the immune system currently represent the most important health hazard to humans even if the capacity of Pd ions to elicit a series of *in vitro* cytotoxic effects by stimulating free radical production (Botrè et al., 2007) has given rise to concern about other health effects. Wiseman & Zereini (2009) recently expressed disagreement with the viewpoint that PGE exposure represents a minimal health risk. They claimed that these elements are indeed a human health concern and pose a greater health risk than once thought for a variety of reasons. In fact, a significant proportion of PGEs is present in the finer particle size fractions of airborne particulate matter (PM) found to be associated with increases in morbidity and mortality (Bell et al., 2008; Dominici et al., 2006; Katsouyanni et al., 2001; Samet et al., 2000). In general, PGEs pose a potential health risk due to the fact that 30 to 57% of the total measured PGEs in air samples has been found to be present in PM with an aerodynamic diameter of less than 10 μm, especially the $PM_{2.5}$ fraction (Artelt et al., 1999; Gomez et al., 2002; Zereini et al., 2004). If we focus our attention on Pd, evidence suggests that this metal is more commonly associated with finer particle size fractions (Kanitsar et al., 2003; Zereini et al., 2004). Unfortunately, to our knowledge, there are no studies in the literature that have investigated Pd concentrations in ultrafine PM particles (UFPs, smaller than 100 nm) however,

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considering that vehicle traffic has been found to be one of the most important sources of UFP emissions (Morawska et al., 2008; Shi et al., 1999), it is plausible to assume that a significant proportion of Pd, released into the environment by automobile catalysts, may be present in UFPs. In fact, the coarse particles emitted by car catalytic converters consist of alumina or silica that carry dispersed PGE particles with diameters of a few nanometers (Artelt et al., 1999; Rauch et al., 2002).

The term UFPs has traditionally been used to describe airborne particles that are less than 100 nm in diameter and is frequently employed in connection with nanometer-diameter particles that are incidentally produced by secondary sources, such as technical products and byproducts of various industrial or combustion processes (Iavicoli et al., 2013). In contrast, engineered nanoparticles (NPs) are intentionally produced and designed with very specific properties or compositions. However, it is currently unclear whether the use of source-based definitions of NPs and UFPs is justified from a safety and health perspective (NIOSH, 2009). In fact, both NPs and UFPs are characterized by particular physico-chemical properties that make them more harmful from a health perspective than larger particles composed of the same materials (Nel et al., 2006).

A number of recent studies have investigated the potential toxicity of Pd nanoparticles (Pd-NPs) and have demonstrated their ability to cause adverse health effects, such as concentration-dependent cytotoxicity, induction of apoptosis and alterations of the release and expression of numerous cytokines (Adams et al., 2014; Boscolo et al., 2010; Hildebrand et al., 2010; Reale et al., 2011; Speranza et al., 2010; Wilkinson et al., 2011). However, despite these recent efforts, overall understanding of the biological effects of Pd-NP exposure is far from complete. Therefore, in order to gain a deeper knowledge of Pd-NP toxicity, the present study aims to evaluate the renal toxicity of Pd-NPs, by investigating urinary excretion of retinol binding protein (RBP), β_2-microglobulin and albumin. In fact, the assessment of the toxicological effects of Pd-NPs on the renal system appears to be highly relevant, considering that after 14- and 90-day exposure, the highest Pd concentrations were observed in the kidneys (Iavicoli et al., 2009, 2010) and that other PGEs, such as Ir and Rh have been shown to exert a nephrotoxic effect (Iavicoli et al., 2011b, 2014).

Materials and methods

Animal husbandry

Twenty-five 3-month-old female Wistar rats were supplied from the Experimental Animal Production Plant of the Catholic University of the Sacred Heart (Rome, Italy) and allowed to acclimatize for two weeks before starting the experiment. Throughout the experiment, the animals were kept in Makrolon cages (model 1291, with overall dimensions of $425 \times 266 \times 185$ mm and floor area of 800 cm^2) (Tecniplast S.p.A., Buguggiate, Italy) containing a wooden dust-free bedding (model Scobis Uno, Mucedola s.r.l., Settimo Milanese, Italy), at a room temperature of 23.1 °C, a relative humidity of 55% and a 12-h light/dark cycle. The animals had a mean weight of 271 ± 16 g and were fed with the solid ''R'' maintenance diet for rats (Altromin Rieper A. S.p.A., Vandoies, Italy). The animals were provided *ad libitum* with diet and purified water. No significant changes in body weight were observed during and at the end of the experiments.

Chemicals

The experimental protocol of this study required the use of the following chemicals: sodium borohydride, palladium (II) nitrate [$Pd(NO_3)_2$] and nitric acid (HNO_3) for the preparation and characterization of uncoated palladium nanoparticle hydrosol; HNO_3, sodium azide, medetomine and ketamine for the sampling of biological materials and the sacrifice of rats; HNO_3, Pd, indium, certified reference materials Clincheck® Urine Control Level I and Level II for analysis of Pd in urine and serum. Assay buffer, anti-albumin antibody (Shibayagi Co., Ltd., Gunma, Japan), HRP-conjugated antibody, chromogenic substrate solution and stopper reaction were used in the ELISA kit for the determination of urinary albumin; sample diluent, anti-rat β_2-microglobulin antibody (Mitsubishi Chemical Medience Corporation, Tokyo, Japan), enzyme-labeled antibody solution, chromogenic substrate solution and stop solution were employed in the ELISA kit for the determination of urinary β_2-microglobulin; assay buffer, DetectXs RBP-peroxidase conjugate, DetectXs RBP antibody solution (Arbor Assay, Ann Arbor, MI), TMB substrate solution and stop solution were used in the ELISA kit for RBP urinary determination; buffer solutions were employed to calibrate the instrument used to quantify urinary pH; glutaraldheyde, cacodylate buffer, osmium tetroxide (OsO_4), veronal acetate buffer, ethanol, araldite, uranyl acetate and lead citrate were used to prepare kidney tissues for ultrastructural observation.

Preparation and characterization of uncoated palladium nanoparticle hydrosol

As a first step, 300 µL of a freshly prepared 0.029 molar sodium borohydride solution, obtained by dissolution of 11 mg of sodium borohydride (p.a., Merck, Darmstadt, Germany) in 10 mL of ultrapure water, were diluted in 100 mL of ultrapure water. 500 µL of a Pd stock standard solution (1000 mg/L, $Pd(NO_3)_2$ in 0.5 mol/L HNO_3, Merck, Darmstadt, Germany) were then added and the mixture was shaken thoroughly. The immediate color change from transparent to dark grey indicated the formation of Pd-NPs. The mixture was kept in the dark at room temperature for 12 h to complete the reaction.

The Pd-NP hydrosol obtained was characterized by continuum source - graphite furnace atomic absorption spectrometry (CS-GFAAS; contrAA 600, Analytik Jena, Jena, Germany) and transmission electron microscopy (TEM; Zeiss EM 10, Carl Zeiss Microscopy GmbH, Jena, Germany) operating at 80 kV. Pd concentration of the stock hydrosol was determined in a 100-fold dilution of the stock hydrosol in ultra pure water by means of CS-GFAAS using the spectral line at 244.791 nm. Calibration was performed in a concentration range from 20 to 80 µg Pd/L by applying adequate dilutions of a Pd stock standard solution (1000 mg/L, $Pd(NO_3)_2$ in 0.5 mol/L HNO_3, traceable to Standard Reference Materials from the National Institute of Standards and Technology, Merck, Darmstadt, Germany), thus producing a linear calibration function with a correlation coefficient of 0.986. The stock hydrosol Pd concentration was found to be 4.71 ± 0.05 mg/L. Size distribution of the particles was determined to be 10 ± 6 nm (Figures S1 and S2), by measuring 500 individual particles depicted by TEM images using ImageJ software (National Institutes of Health, Bethesda, MD). The hydrosol served as a stock solution for all experiments and is stable for at least 2 weeks when stored in refrigerators at 4 °C. Before use, the stored Pd-NP hydrosol was homogenized by shaking vigorously. Finally, aliquots of the stock solution were diluted in ultrapure water to obtain the final concentrations used in the experiments.

Animal administration and sampling of biological material

The experimental protocol as described below was approved by the Ethical Committee of the Catholic University of the Sacred Heart (Rome, Italy). The twenty-five female Wistar rats were

randomly divided into four exposure groups and one control group, with five rats per group. Rats were given a single injection of vehicle (control group) and different concentrations of Pd-NPs (0.012, 0.12, 1.2 and 12 μg/kg) via the tail vein. Urine samples were collected over a 24-h period in individual metabolic cages (metabolic cages for rats with overall dimensions of $265 \times 265 \times 380$ mm and floor area of 397.4 cm^2) (Tecniplast S.p.A., Buguggiate, Italy) on day 1, 7 and 14. During the collection of urine, no food was supplied, as the experimental protocol scheduled availability of drinking water only, in the 24-h period prior to collection. Urine samples were collected in 12 mL polyethylene tubes (BD Biosciences, Durham, NC) containing sodium azide (10 mg/100 mL) as preservative and were frozen at −20 °C within 2 h of collection. The polyethylene tubes were decontaminated by an overnight soaking with 10% HNO_3 (Suprapur grade, Merck, Darmstadt, Germany) and then rinsing with high-purity deionized water (Idrolab-a-System, Idron, Rome, Italy). Fourteen days after exposure, rats were anesthetized with 0.5 mg of medetomine and 75 mg of ketamine per kg body weight. Subsequently, blood from each animal was drawn by cardiac puncture and collected in a 1.5 mL vial (Eppendorf srl, Milan, Italy). Serum samples were obtained from blood by centrifugation (3500 rpm per 15 min) and stored at −28 °C until analysis. After the blood sampling, rats were euthanized by exsanguinations, cutting both the abdominal aorta and vena cava, and then kidneys were collected from each rat.

Analysis of Pd in urine and serum

Urine and serum were directly analyzed after 1:10 dilution with water (Barnstead EASYpure UV). A 1% of HNO_3 (Normatom ultrapure grade) was added to urine samples in order to dissolve any remaining sediment. Pd measurements (standard by CPA Chem, Chebios, Rome, Italy) were performed by the sector-field inductively coupled plasma mass spectrometer (SF-ICP-MS) (Element2, Thermo Fischer, Bremen, Germany), whose instrumental characteristics and settings have been previously described by Iavicoli et al. (2009). The instrument worked at the medium resolution of 4000 m/Δm and the mass ^{106}Pd was chosen for quantification. The additional calibration approach and internal standardization with indium (CPA Chem, Chebios, Rome, Italy) were adopted to correct for possible instrumental drifts or non-spectral interferences. The limit of detection (LoD) was 0.008 μg/L in the matrices. Recoveries of Pd on two certified reference materials, the Clincheck® Urine Control Level I and Level II (Recipe, Munich, Germany), were 95 and 99%, respectively, and precision lower than 10%.

Analysis of urinary proteins

Albumin, β_2-microglobulin and RBP were determined in the urine samples collected 14 days after exposure using three different enzyme-linked immunosorbent assay (ELISA) systems and following the procedures previously described in greater detail elsewhere (Iavicoli et al., 2011b, 2014). ELISA kits for the analyses of urinary albumin, β_2-microglobulin and RBP were provided by Shibayagi Co. Ltd. (Gunma, Japan), Mitsubishi Chemical Medience Corporation (Tokyo, Japan) and Arbor Assay (Ann Arbor, MI), respectively. All reagents and samples were brought to room temperature before use. Absorbance was measured at 450 nm with the multilabel counter Wallac Victor3 1420 (PerkinElmer, Turku, Finland). Protein concentrations were given in μg/mL after proper calibration.

Quantification of urinary pH

The pH of urinary samples collected from exposed animals and controls was determined using a PHM 92 Lab pH Meter from Radiometer Copenhagen (Brønshøj, Denmark) after calibration of the instrument with two different buffer solutions (Radiometer-Analytical, Hach Lange, Berlin, Germany) with standard pH reference values (4.005 and 7.000).

Ultrastructural observations of the kidneys

Cortical kidneys were immediately processed for ultrastructural study using the routine protocol: 2 mm^3 kidney specimens were fixed by immersion in 2.5% glutaraldheyde (JT Baker, Deventer, Holland) in cacodylate buffer 0.1 M and post-fixed in 1% OsO_4 (EMS, Washington, PA) in veronal acetate buffer 0.1 M. Specimens were dehydrated in ascendant ethanol and embedded in Araldite (Serva, Heidelberg-D, Heidelberg, Germany). Thin sections, stained with uranyl acetate and lead citrate were studied using a Philips 410 LS TEM (Philips/FEI Corporation, Eindhoven, Holland).

Statistical methods

Statistical analysis was carried out by IBM SPSS statistics software (IBM Statistical Package for Social Sciences for Windows, Version 22.0. Armonk, NY). We assessed the responses to four different concentrations (0.012, 0.12, 1.2 and 12 μg/kg) of Pd-NPs and compared urine and serum Pd levels with those of the control animals. On day 14, levels of urinary proteins (albumin, β_2-microglobulin and RBP) were measured after exposure to the four different concentrations. The normal distribution of observed values was checked using the non-parametric Kolmogorov–Smirnov *Z*-test and variance homogeneity was tested by the Levene test. The one-way analysis of variance (ANOVA) was then performed to test the significance of differences in parameter means in the exposed and control rat groups. The Dunnett *post-hoc* multiple comparison test was used to test the significance (p value of Dunnett's t test <0.05) of differences in values for each parameter at different exposure levels against the control group. Box-plot or linear graphs were obtained for all analyzed parameters at different exposure levels.

Results

Urinary and serum Pd concentrations and levels of urinary proteins in Wistar female rats exposed to different concentrations of Pd-NPs are shown in Table 1 and Figures 1–3. In the different exposure groups, mean urinary pH ranged from 6.23 to 6.51 with a mean value of 6.32. Results showed an increasing trend in urinary Pd levels demonstrating that Pd urinary concentrations are directly associated with the administered doses. The dose-dependent increase in urinary Pd levels was particularly evident in urine samples collected on day 1 and 7. In fact, the highest levels of exposure, 1.2 and 12 μg/kg, induced mean values of Pd ± standard deviation (SD) of 3.92 ± 1.65 and 73.9 ± 32.6 and of 0.32 ± 0.08 and 1.14 ± 0.34 μg/L on day 1 and 7, respectively. These levels were significantly higher than the mean Pd content observed in urinary samples of the control group. In the urine samples collected 14 days after the intravenous administration of Pd-NPs, the urinary Pd levels of exposed animals were quite similar to those of controls with the exception of rats exposed to 12 μg/kg whose urinary Pd concentrations were higher (0.5 μg/L). At the end of the experiment, 14 days after the single injection of different concentrations of Pd-NPs via the tail vein, the mean serum Pd levels of exposed groups were in the same order of magnitude as those observed in the control group.

With regard to renal function parameters, the mean urinary albumin concentration was significantly higher than in the control group (44.09 ± 22.16 μg/mL) only at the lowest dose of treatment (80.72 ± 11.79 μg/mL), while exposure to increasing Pd-NP

Table 1. Mean levels ± SD of urinary and serum Pd and albumin, RBP and β_2-microglobulin urinary levels in control rats and in four groups of female Wistar rats exposed to different levels of Pd-NPs (0.012, 0.12, 1.2 and 12 μg/kg).

	Exposure doses (μg/kg)					
Parameters	Controls	0.012	0.12	1.2	12	ANOVA *F* test (*p* value)
1 d	–	0.27 ± 0.05	0.49 ± 0.08	3.92 ± 1.65	73.9 ± 32.6	<0.001
7 d	–	0.19 ± 0.07	0.32 ± 0.08	0.32 ± 0.08	1.14 ± 0.34	<0.001
14 d	0.09 ± 0.08	0.04 ± 0.05	0.01 ± 0.03	0.01 ± 0.009	0.50 ± 0.28	<0.001
Serum Pd levels (μg/L)	0.06 ± 0.03	0.07 ± 0.04	0.07 ± 0.01	0.06 ± 0.04	0.08 ± 0.01	ns
Albumin (μg/mL)	44.09 ± 22.16	80.72 ± 11.79	75.31 ± 35.49	35.13 ± 17.61	43.39 ± 10.18	0.008
RBP (μg/mL)	0.11 ± 0.03	0.08 ± 0.04	0.10 ± 0.04	0.07 ± 0.04	0.23 ± 0.07	<0.001
β_2-microglobulin (μg/mL)	0.40 ± 0.07	0.37 ± 0.13	0.79 ± 0.14	0.45 ± 0.20	0.97 ± 0.23	<0.001

ANOVA test and statistical significance (*p* value ANOVA).

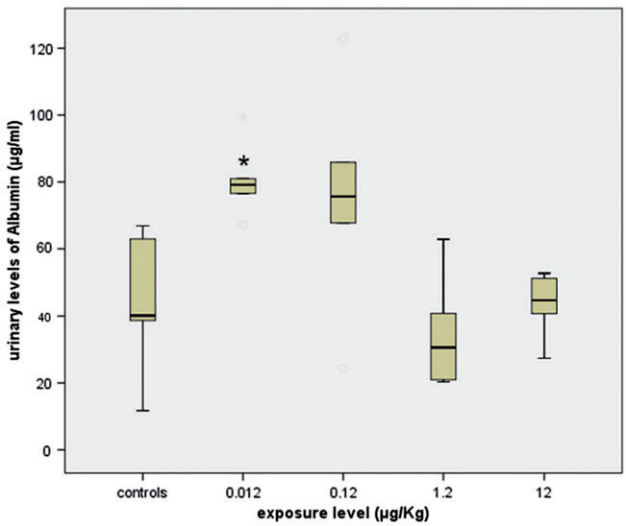

Figure 1. Urinary levels of albumin (μg/mL) in control rats and in four groups of female Wistar rats exposed to different levels of Pd-NPs (0.012, 0.12, 1.2 and 12 μg/kg). *Mean value significantly higher than the control group ($p < 0.05$).

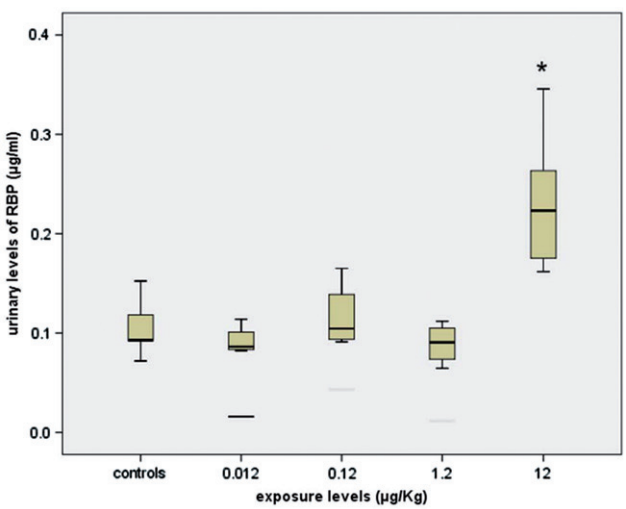

Figure 2. Urinary levels of RBP (μg/mL) in control rats and in four groups of female Wistar rats exposed to different levels of Pd-NPs (0.012, 0.12, 1.2 and 12 μg/kg). *Mean value significantly higher than the control group ($p < 0.05$).

concentrations did not induce significant alterations in its urinary levels, specifically at 1.2 (35.13 ± 17.61 μg/mL) and 12 (43.39 ± 10.18 μg/mL) μg/kg (Figure 1 and Table 1). On the contrary, an important increase in urinary RBP levels was

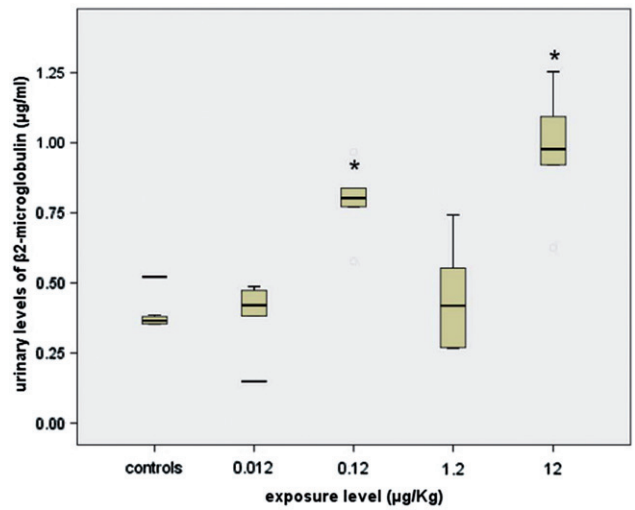

Figure 3. Urinary levels of β_2-microglobulin (μg/mL) in control rats and in four groups of female Wistar rats exposed to different levels of Pd-NPs (0.012, 0.12, 1.2 and 12 μg/kg). *Mean value significantly higher than the control group ($p < 0.05$).

observed in rats exposed to 12 μg/kg compared to controls (Figure 2 and Table 1). In fact, at this exposure concentration, a greater mean (±SD) RBP value (0.23 ± 0.07 μg/mL) was determined compared to the RBP level obtained in non-exposed animals (0.11 ± 0.03 μg/mL). Similarly, at the same dose, there was a statistically significant increase in β_2-microglobulin value (Figure 3 and Table 1). Indeed, this higher level of exposure induced a mean (±SD) value of β_2-microglobulin of 0.97 ± 0.23 μg/mL significantly greater than the 0.40 ± 0.07 μg/mL value detected in the control group. These results suggested that exposure of rats to 12 μg/kg of Pd-NPs is able to exert an important nephrotoxic action at tubular level.

This finding was also confirmed by morphological observations of the kidneys at electron microscope (Figures 4 and 5). In fact, on comparison with the control specimens, ultrastructural study revealed that alterations are visible in kidney specimens under all experimental conditions ranging in dose severity from a low Pd percentage (0.012 μg/kg) to the highest at 12 μg/kg (Table 2). The main target of subcellular alterations was the proximal and distal tubular epithelium. In particular, at the highest dose of Pd-NPs there was a diffuse and subtotal loss of superficial microvilli, which were sometimes replaced by the formation of pseudopodial expansions (Figure 4F). Other subcellular modifications consisted in diffuse and severe dilation of inter-epithelial spaces (Figure 4C–E) as well as the presence of exfoliated cells in the tubular lumina (Figure 4D). The same alterations were found to a lesser degree in the specimens treated with lower doses of Pd-NPs (Table 2). Some vacuolar structures with an electrondense

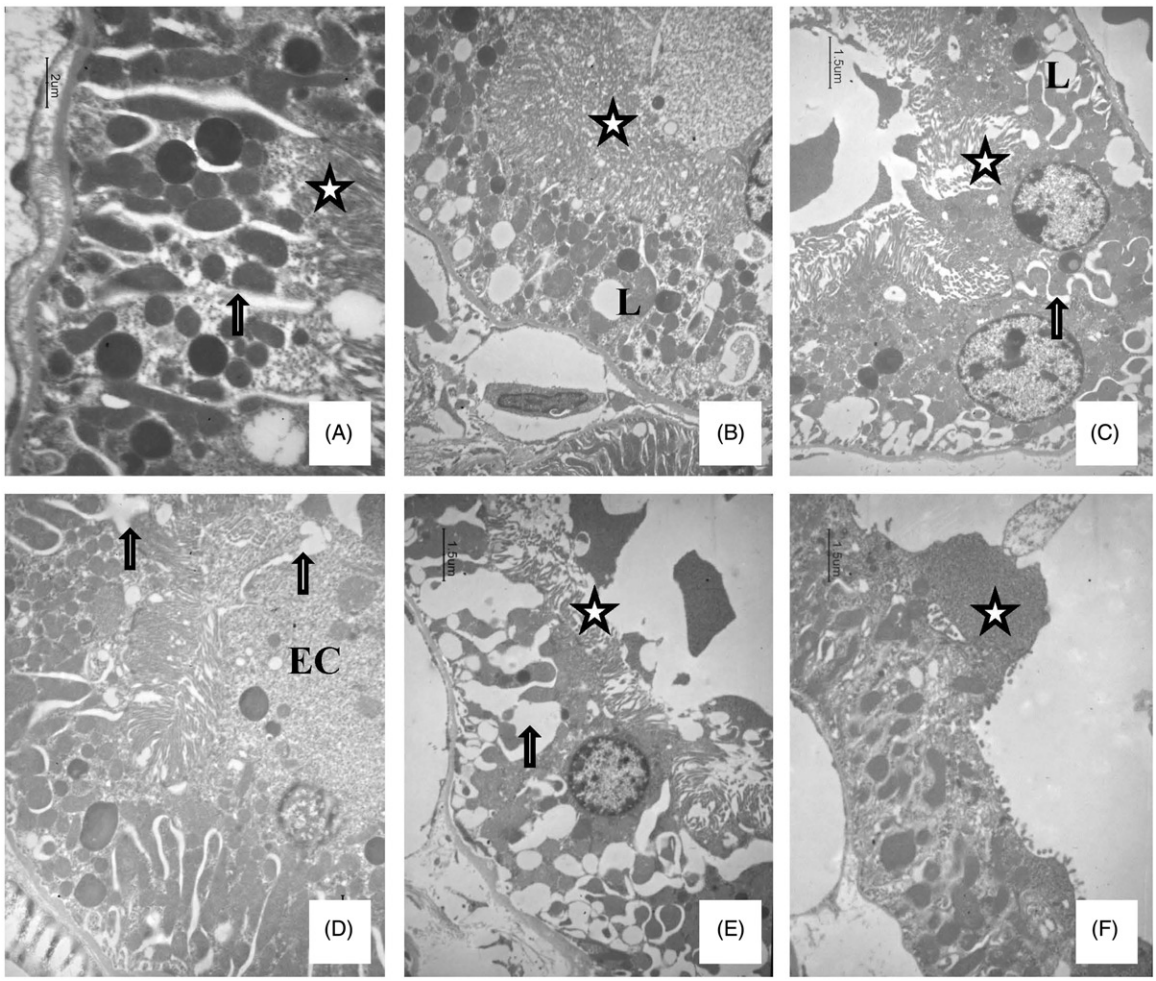

Figure 4. Transmission electron microscope (TEM observations were conducted on kidney specimens collected on the 14th day post-exposure, at the end of experiment). (A) The control kidney specimen shows a likely normal organization of tubules: rarely, intercellular widening can be observed (arrow); the superficial microvilli pattern is regular (asterisk); (B) after treatment with 0.012 μg/kg microvilli (asterisk) still maintain a normal organization and number, while some intracytoplasmic lipid vacuoles (L) can be observed; (C) after treatment with 0.12 μg/kg of palladium nanoparticles there is a mild and focal enlargement of inter-epithelial spaces (arrow) and a mild decrease in superficial microvilli (asterisk); (D) by increasing the dose to 1.2 μg/kg it is possible to observe intraluminal shedding of an epithelial cell (EC): the subcellular pattern seems similar to that of 0.12 μg/kg; mild and diffuse enlargement of intercellular spaces (arrow); (E) after 12 μg/kg the overall renal tubule architecture appears to be focally completely altered by the presence of severely dilated intercellular spaces (arrow); moreover, tubular epithelial cells contain only a few superficial microvilli (asterisk); (F) microvilli are sometimes substituted by large pseudopodial structures (asterisk). Bar = 1.5 μm.

matrix were observed in all specimens (0.012, 0.12, 1.2 and 12 μg/kg) including those of the controls (Figure 5). They were characterized by the presence of intramatriceal electrondense granular particles likely resembling ferritin particles: the vacuolar content appeared to have been phagocitosized by the tubular epithelial cells (Figure 5B). The glomerular structures were of normal appearance in all the experimental specimens.

Discussion

A significant increase in the industrial use of Pd in the past 20 years and emission of this metal from automobile catalytic converters have resulted in higher environmental Pd levels (Iavicoli et al., 2008a, 2011a; Leopold et al., 2008; Zereini et al., 2004, 2007). Although the weight distribution of PGE emission is largest in the micrometer range, catalysts also produce a nanoparticulate form of these metals (Kalavrouziotis & Koukoulakis, 2009; Wilkinson et al., 2011). Therefore, it is clear that in recent years there has been a notable increase in exposure to micrometer- and nanometer-sized Pd particles that has generated a growing concern regarding the possible adverse effects that Pd-UFPs might have on human health.

A number of recent studies have in fact demonstrated that Pd-NPs are able to exert different toxic effects. In kiwifruit pollen, this xenobiotic exhibited a significantly greater toxicity than palladium (II) chloride ($PdCl_2$) (Speranza et al., 2010), whereas only minor effects on the cell viability were observed in human and fish cell lines (Hildebrand et al., 2010). On the other hand, Wilkinson et al. (2011) showed that the treatment of lung cells with increasing concentrations of Pd-NPs resulted in concentration-dependent cytotoxicity, apoptosis and alterations in the secretion of biomarkers (IL-8 and PGE_2). Pd-NPs exerted modulatory effects on the release and expression of cytokines also in peripheral blood mononuclear cells (PBMC) of non-atopic and Pd-sensitized women (Boscolo et al., 2010; Reale et al., 2011). Finally, Adams et al. (2014) showed that Pd-NPs generally exert a much more inhibitory effect on *Staphylococcus aureus* than on *Escherichia coli*, demonstrating that NPs with a smaller diameter exhibit greater toxicity. Nevertheless, information currently available regarding the possible adverse health effects of Pd-NPs is still rather limited and there continues to be a shortage of data concerning the possible toxic effects of these particles on major organ systems (e.g. kidney). However, our previous research showed that administration of potassium

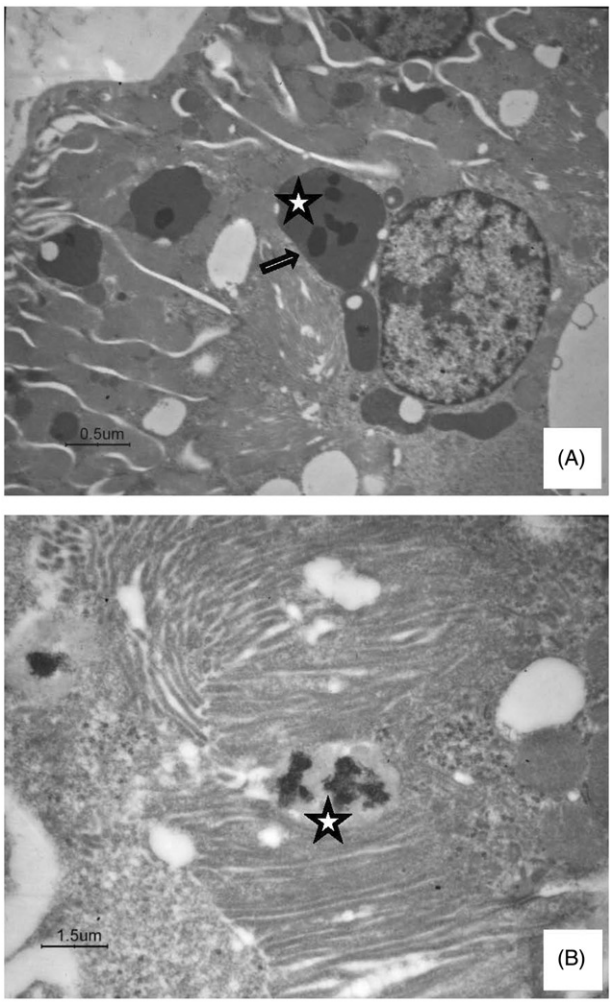

Figure 5. Transmission electron microscope (TEM observations were conducted on kidney specimens collected on the 14th day post-exposure, at the end of experiment). (A) In all samples (all dose treatment) several electrondense vacuoles are clearly visible in the cytoplasm of epithelial tubular cells (asterisk). Ferritin-resembling particles are identifiable in the vacuolar matrix (arrow): (B) a higher magnification picture shows ferritin-like particles (asterisk), contained in an amorphous matrix that seem to be phagocytosized by an epithelial cell. 0.012 μg/kg dose. Bar = 1.5 μm.

Table 2. TEM semi-quantitative assessment.

	Type of alteration				
Exposure doses (μg/kg)	Luminal EC	Microvilli loss	DIS	Lipid	E-dense vac.
Controls	–	–	–/+	–	+
0.012	–	–	–/+	+	+
0.12	–	+	+	+	+
1.2	+	+	++	+	+
12	++	+++	+++	++	+

EC, epithelial cell; DIS, dilated intercellular spaces; E-dense vac, electron-dense vacuoles.

–, normal; –/+, rarely; +, few and focal; ++ few and diffuse; +++, many and diffuse.

hexachloro-palladate to Wistar rats resulted in a significant Pd accumulation in this organ, suggesting that kidney could be a target tissue for the possible toxic effects of this metal (Iavicoli et al., 2009, 2010). Moreover, it has been demonstrated that Ir and Rh affect the tubular kidney function of Wistar rats, as demonstrated by the increased urinary concentrations of RBP and β_2-microglobulin (Iavicoli et al., 2011b, 2014). Increasing exposure to nanosized Pd particles and our previous findings would therefore seem to indicate that a toxicological assessment of the effects induced by Pd-NPs on the kidney is urgently required.

This study investigated the possible nephrotoxic effects of Pd-NPs administered to rats via the intravenous route of exposure. This administration route was chosen as it produces a worst case scenario of 100% bioavailability. The doses used to treat animals were selected to resemble possible occupational and/or environmental exposure scenarios. In fact, if we take into consideration the Pd airborne levels (highest mean level of $7.70 \pm 4.15\ \mu g/m^3$) recently reported in literature for an occupational setting (Cristaudo et al., 2007) and a human breathing rate of around $20\ m^3$/day (for a man with a mean weight of 70 kg), a potential occupational exposure to Pd via inhalation corresponds to an exposure dose of 2.20 μg/kg, which is in the range of doses used in our experiments. The higher exposure doses (1.2 and 12 μg/kg) simulated possible occupational exposure both under normal and accidental conditions during which re-exposure can occur. The lower doses (0.012 and 0.12 μg/kg) were used to investigate potential adverse effects at exposure levels closely resembling those of the general population and to establish a preliminary dose-response curve for defining the toxicological behavior of Pd-NPs. The results presented in this study suggest a nephrotoxic action of Pd-NPs as demonstrated by the increase in RBP and β_2-microglobulin in urine samples collected from rats intravenously treated with 12 μg/kg of Pd-NPs compared to the control group. In fact, these low molecular weight proteins are excreted in urine in small quantities when tubular function is normal, while an increase in their urinary excretion is indicative of renal tubular damage (Bernard, 2004; Johri et al., 2010; Piscator, 1991). The Pd-NPs were therefore able to affect renal function at tubular level. On the contrary, exposure of Wistar rats to this xenobiotic did not seem to have an adverse impact on kidney glomerular filtration. In fact, urinary levels of albumin, a comparatively large protein that is a biomarker for glomerular damage, were quite similar in control and exposed groups treated with the highest doses (1.2 and 12 μg/kg).

In order to confirm these findings, we also carried out a morphological study of kidneys collected at the end of experiment aimed to describe the qualitative changes at subcellular level by TEM. In accordance with the data of urinary excretion of albumin, RBP and β_2-microglobulin and as a confirmation of the accuracy of these results, ultrastructural analysis revealed the presence of significant alterations in kidney specimens of all treated animals compared with the controls. These modifications became particularly evident in the kidney of rats exposed to the highest dose of Pd-NPs since we observed a diffuse and subtotal loss of superficial microvilli, which were sometimes replaced by the formation of pseudopodial expansions. At lower Pd-NP doses we also observed qualitative changes in the same subcellular markers as reported for higher doses, although these were less extensive and severe (Table 2). The ultrastructural study of the kidneys therefore clearly indicated the presence of damage at tubular level suggesting that the main target of Pd-NP toxic action was the proximal and distal tubular epithelium, while glomerular structures, that appeared normal in all the experimental specimens, were unaffected by the treatment.

The nephrotoxic action of Pd-NPs seems somewhat similar to that exerted by Ir and Rh (Iavicoli et al., 2011b, 2014). Since these metals share comparable physico-chemical properties, it is plausible that the molecular mechanisms of action underlying the toxic effect on kidney are also quite similar. It is interesting to note that sub-chronic exposure to Ir also induced a dose-dependent increase in the albumin urinary levels, whereas its

concentrations were not enhanced by sub-acute treatment with Rh or Pd-NPs. These observations support the idea that sub-chronic/chronic exposure may damage not only tubular function, but also kidney glomerular filtration. Since, in the future, the general population and workers may be chronically exposed to increasing concentrations of this xenobiotic, it seems important to focus attention also on the long-term effects of Pd-NPs may have on renal function. The analysis of RBP and β_2-microglobulin urinary concentrations is widely used to detect early renal tubular dysfunction. In presence of acid urines the RBP/β_2-microglobulin ratio increase, while when the urinary pH is >6 there is a good correlation with a slope close to 1 (Bernard et al., 1987). In the present study, the pH value of urine samples ranged from 6 to 7.7, but interestingly the RBP/β_2-microglobulin ratio was lower than 1, indicating, particularly in the exposed rats, an increased excretion of β_2-microglobulin compared to RBP. In our previous studies (Iavicoli et al., 2011b, 2014), in the presence of similar urinary pH values, the RBP/β_2-microglobulin ratio was above 1. According to Bernard et al. (1987), in a small fraction of urine samples (with pH >6) a slight increase in the urinary excretion of β_2-microglobulin may be observed with minor or no changes in that of RBP and these results could represent an increased cell membrane turnover as a result of renal tissue destruction and cell regeneration. Therefore, considering the unique physico-chemical characteristics of NPs, it is possible to hypothesize that exposure to Pd-NPs is responsible for a greater renal damage that occurs with renal tissue destruction which in turn causes an increased excretion of β_2-microglobulin. Interestingly, the time course of urinary Pd levels observed in the different exposure groups (Figure S3) is quite similar to the data of Durbin (1960) and Moore et al. (1975), who reported a biphasic urinary excretion pattern with a first half-life of approximately 1 day followed by a second term of approximately 10 days. A linear relationship between treatment doses and urinary Pd concentrations was demonstrated at one day post-exposure, while this trend was less evident in the other two time points taken into consideration (7 and 14 days post-administration). Therefore, it can be argued that the initial fast elimination phase of the metal may be strongly dependent on the administered dose (as the linear relation confirms), whereas the subsequent gradual excretion of the remaining Pd-NPs could be significantly affected by interactions of NPs with biological materials (i.e. the protein corona phenomenon). The Pd-NP biological interactions may also affect the serum Pd concentrations. In this regard, it should also be noted that serum Pd levels observed in the treated groups were very similar to the value detected in the control group, thus suggesting that the Pd administered was almost completely eliminated after 14 days.

All in all, our results confirm the ability of NPs to exert significant adverse effects on the renal system. Recently, several studies have investigated potential renal nanotoxicity, demonstrating that different types of NPs are able to induce important toxic effects on kidneys. In this regard, several renal tubular alterations that were size-dependent and probably induced by increased generation of ROS, were observed in Wistar-Kyoto rats subacutely treated via intraperitoneal administration of gold NPs (Abdelhalim & Jarrar, 2011). A similar molecular mechanism of renal toxicity (oxidative and nitrosative stress) was hypothesized by Sarkar et al. (2011) in Swiss albino male mice treated with copper NPs (Cu-NPs) via oral gavage. The same type of NPs administered via oral gavage caused widespread renal proximal tubule necrosis in Wistar rats (Liao & Liu, 2012). Similar findings (reduction in renal glomerulus number, tissue necrosis or disorganization of renal tubules and production of ROS) were observed by Gui et al. (2013) in mice exposed to titanium dioxide NPs (TiO_2-NPs) by subchronic intragastric administration. In Kunming mice intraperitoneally exposed to iron oxide nanoparticles (Fe_3O_4-NPs), Ma et al. (2012) observed swelling of cytoplasm, fused or collapsed cells, an increase in ROS production and glutathione depletion. Similarly, a dose- and time-dependent induction of oxidative stress in renal tissue was also detected in albino rats intraperitoneally injected with aluminum oxide nanoparticles (Al_2O_3-NPs) (Morsy et al., 2013). Although further studies are needed to fully understand renal NP toxicity, the findings of the aforementioned studies suggest that oxidative stress is one of the major negative effects produced by NPs. This hypothesis is also supported by the results of several *in vitro* studies carried out on human IP15 mesangial and porcine LLC-PK_1 cells exposed to carbon and TiO_2-NPs (L'Azou et al., 2008), IP15 and Human Kidney (HK-2) epithelial cells treated with TiO_2, zinc oxide and cadmium sulfide NPs (Pujalté et al., 2011) and HK-2 and LLC-PK_1 cells exposed to silica NPs (Passagne et al., 2012).

Conclusions

To date, many studies have described the adverse health effects of a number of NPs on the lungs, while little attention has been given to kidneys which are considered to be a secondary target organ. However, in the past few years, several *in vitro* and *in vivo* studies have investigated the potential renal toxicity of different nanomaterials, demonstrating that NPs are able to exert important toxic effects on this organ. Nevertheless, to our knowledge, this is the first attempt to evaluate the nanotoxicity of Pd-NPs on kidney. The increased levels of urinary RBP and β_2-microglobulin observed in treated rats compared to controls demonstrated that exposure to Pd-NPs induced a significant renal tubular dysfunction that is also confirmed by the important histological alterations in the proximal and distal tubular epithelium. The kidney parameters investigated, particularly urinary RBP and β_2-microglobulin concentrations, are markers of early NP alterations induced on the renal system of sub-acutely exposed animals. Consequently, even if these are only the preliminary findings of a first attempt to investigate the potential toxic effects of Pd-NPs on the kidney, the significant increase in urinary protein excretion supports the hypothesis that Pd-NPs can act as a nephrotoxic agent. However, further investigations are needed to confirm the negative impact of these NPs on the kidney and to better understand the molecular mechanisms of toxic action (oxidative stress) and the cell death pathways (i.e. necrosis, apoptosis). Moreover, further research is required to assess potential adverse effects also on glomerular section of the kidney, particularly after sub-chronic/chronic exposure.

Lastly, as potential new indicators of effect, RBP and β_2-microglobulin could be extremely useful for risk assessment and management in conditions of occupational exposure. In fact, when a very early detection of tubular injury is desirable (e.g. in occupational or environmental medicine), it may be useful to measure both proteins.

Declaration of interest

The authors of the present manuscript report no conflicts of interest regarding any financial, consulting and personal relationships with other people or organizations that could influence (bias) the author's work. The authors alone are responsible for the content and writing of the manuscript.

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Supplementary material available online

Supplementary Figures S1, S2 and S3.