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Dietary exposure to silver nanoparticles in Sprague–Dawley rats: Effects on oxidative stress and inflammation

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ABSTRACT

Due to undesirable hazardous interactions with biological systems, we evaluated the effect of silver nanoparticles (AgNPs) intake on oxidative stress and inflammation.

Rats received for 81 days a standard diet (Controls) or a standard diet plus 500 mg/d/kg BW AgNPs. We assayed plasma lipids, and oxidative stress was assessed by measuring liver and heart superoxide anion production ($O_2^{\cdot-}$) and liver malondialdehyde levels (MDA). Antioxidant status was appraised using plasma paraoxonase activity (PON), plasma antioxidant capacity (PAC) and liver superoxide dismutase activity (SOD). Liver inflammatory cytokines TNFα and IL-6 levels and plasma alanine aminotransferase (ALT) were assayed.

Compared with Controls, AgNPs raised cholesterolemia (9.5%), LDL-cholesterol (30%), and lowered triglycerides (41%). They also increased liver (30%) and cardiac (41%) $O_2^{\cdot-}$ production, reduced PON activity (15%) and raised liver TNFα (9%) and IL-6 (~12%). Plasma ALT activity rose (12%) after treatment with AgNPs. However, PAC and liver MDA and SOD activity were unchanged.

These features indicate that exposure to 500 mg/d/kg BW of AgNPs results in liver damage by a dysregulation of lipid metabolism, highlighting liver and heart as the most sensitive organs to the deleterious effects. Our findings also demonstrate for the first time the oxidative and inflammatory effects of dietary AgNPs.

1. Introduction

Additionally to potential advantages (antibiotic, antifungal and antiviral properties) (Wijnhoven et al., 2009), silver nanoparticles (AgNPs) may trigger undesirable hazardous interactions with biological systems with potential to generate toxicity (Nel et al., 2006; Skebo et al. 2007; Wallace et al., 2007). Silver nanoparticles are far most commonly used in consumer products and there is a widespread use of AgNPs in bedding, washing machines, water purification, toothpaste, shampoo and rinse, nipples and nursing bottles, fabrics, deodorants, filters, kitchen utensils, toys, and humidifiers (Kim et al., 2010). Despite such widespread use of AgNPs-containing products, *in vivo* toxicity data on orally ingested AgNPs remain rare. Toxicity study *in vivo* was conducted on Sprague–Dawley rats orally treated for 28 days with AgNPs by Kim et al. (2008) at doses of 30, 300 and 1000 mg/kg/day and no visible clinical signs were noted nor difference in food consumption between animal treated and control groups. There was no change in body weight or organ weights. The effects on blood biochemistry, alkaline phosphatase and cholesterol increased at high doses indicating a moderate hepatic toxicity. A more recent study by Kim et al. (2010) tested the oral toxicity of AgNPs over a period of 90 days in F344 rats. There was a decrease in the body weight of male rats after 4 weeks of exposure and dose-dependent changes were found in alkaline phosphatase and cholesterol, indicating that exposure to more than 125 mg/kg of AgNPs may result in slight liver damage. The target organ for the AgNPs was found to be the liver and a lowest observable adverse effect level of 125 mg/kg was suggested.

Elsewhere, nonspecific oxidative stress has been suggested as one of the greatest concern in nanoparticle-induced toxicity (Nel et al., 2006). Several toxic changes have been reported in nanoparticle-exposed fish and embryos, i.e. oxidative stress markers changes such as lipid oxidation, apoptosis and changes in gene expression (Oberdörster, 2004; Smith et al., 2007; Zhu et al., 2006). Moreover, some *in vivo* and *in vitro* studies on pulmonary inflammation and oxidative stress of various inhaled nanoparticles (NPs) have been published (Muller et al., 2008; Karlsson et al., 2008). However, oxidative stress and inflammation are closely linked by amplification loops. Indeed it has been shown that pro-inflammatory cytokines enhance expression and activation of

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Table 1
Nutritional parameters of rats fed a standard diet (Control) or a standard diet plus silver nanoparticles (Experimental)[A].

	Control	Experimental
Final body weight (g)	541 ± 13[a]	445 ± 16[b]
Weight gain (g/day)	5.53 ± 0.16[a]	4.38 ± 0.19[b]
Food intake (g/day)	28.1 ± 0.4[a]	23.1 ± 0.3[b]

[A] Values are the means ± SEM (n = 16). For each dietary treatment, mean values in a column with different superscript letters significantly differ, $p < 0.05$.

NOX/NADPH oxidase family, resulting in massive free radicals production. In addition to the effect of inflammation on oxygen radicals, it is noteworthy that oxidants modulate inflammatory mediator release through activation of transcription factors including NF-κB, AP-1 and hypoxia inducible factor (Lander, 1997; Acker, 2005), leading to amplification loops between oxidative stress and inflammation. Thus, oxidative stress may exhibit an ambivalent role, as an "effector" (by oxidant release and induced toxicity) but also as a "modulator" (in regulating transcription factors) of chronic micro-inflammatory process. These amplification loops between inflammation and oxidative stress could be involved in NPs induced harmful effects.

Nowadays, microorganisms are becoming resistant to pharmaceutical antibiotics, diminishing their effectiveness. For this reason, colloidal silver is now in resurgence as an important alternative to the use of antibiotics; in addition to its proven effectiveness against a wide range of germs, due to its way of working, it prevents the creation of microorganism resistance. This is the reason why the use of silver as an antibacterial agent in medicine has been increasing lately. Collargol, a high quality silver colloid, is produced by Argenol Laboratories (Zaragoza, Spain) used for example as a silver-based intestinal astringent to prevent diarrhoeas. Today, it can be an important alternative in the treatment of intestinal problems, occurring mainly in underdeveloped countries through ingestion of contaminated food and water.

Given the potential oxidative and inflammatory effects of dietary AgNPs, we aimed to investigate for the first time the effects of a silver colloid such as Collargol following 81 days repeated oral exposure on some markers of oxidative stress and inflammation in rats liver and heart.

2. Materials and methods

2.1. Materials

The silver nanoparticles (20 nm) were purchased as Collargol from Argenol Laboratorios (Spain), and were at least 75% pure. According to the manufacturer, it contains silver nanoparticles in colloidal state, placed in a protein protective colloid as support (25%). Ag content, determined by acid digestion followed by ICP–AES, was 70–80% and the rest of the product was protein.

2.2. Animals

Thirty-two weanling male Sprague Dawley rats (Janvier, Le Genest-St-Isle, France) weighing 92 ± 3 g were randomly separated into two groups (Control and Experimental) of 16 animals each. Experimental rats were treated with Collargol (Argenol Laboratories) for 81 days. There were maintained in plastic cages in temperature-controlled environment (21 ± 1 °C) with a relative humidity 60% ± 4%, and subjected to a 12-h light/dark cycle from 7 p.m. to 7 a.m and allowed free access to both food and water.

Rats were handled in compliance with European Union rules and according to the guidelines of NIH (National Research Council, 1985) and the Committee on Animal Care at the University of Montpellier (France).

2.3. Diets and feeding procedures

Rats were fed *ad libitum* on a standard diet supplied by SSNIFF (Spezialdiaten GmbH, Soest, Germany) and consisting of 236 g/kg casein, 3.5 g/kg L-methionine, 300 g/kg corn starch, 30 g/kg maltodextrin 10, 290.5 g/kg sucrose, 50 g/kg cellulose, 45 g/kg vegetable oil (10.3% energy of the diet), 35 g/kg mineral mix and 10 mg/kg vitamin mix. Vitamin and mineral mixes were formulated according to AIN-93 guidelines (Reeves et al., 1993). Rats and uneaten food were weighed every 2 days. Rats of each group received daily by gavage either tap water (Control) or an aqueous solution of Collargol (Experimental) at a dose of 820 mg/kg BW (i.e. 500 mg silver nanoparticles/kg BW). Collargol powder was dissolved in ultra-pure water and sonicated for 60 s. The volume of the solutions fed was adjusted weekly to the weight of rats.

2.4. Analytical procedures

At the end of each experimental period, rats were deprived of food overnight and blood samples were collected under anaesthesia by cardiac puncture. Plasma was prepared by centrifugation at 4000 rpm for 10 min at 4 °C, then stored at −80 °C until analysis. Plasma concentrations of total cholesterol (TC), HDL cholesterol (HDL-C) and triglycerides (TG) were measured by commercially available enzymatic methods (respectively Nos. CH 200, CH 203 and TR 1697, Randox Laboratories LTD, Crumlin, UK). Plasma non HDL-C (∼LDL-C) was calculated from the difference between TC and HDL-C. Paraoxonase activity (PON) was determined using paraoxon as a substrate and measured by increases in the absorbance at 412 nm due to the formation of 4-nitrophenol, according to Jaouad et al. (2006). Briefly, the activity was measured at 25 °C, by adding 50 μL of plasma to 1 mL Tris/HCl buffer (100 mM, pH 8.0) containing 2 mM $CaCl_2$ and 5.5 mM paraoxon. The rate of generation of 4-nitrophenol was determined at 412 nm. Enzymatic activity was calculated using the molar extinction coefficient 17,100 M^{-1} cm^{-1}. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions. Plasma alanine aminotransferase (ALT) was measured by using a clinical commercial kit (AL 2360, Randox Laboratories LTD, Crumlin, UK). The antioxidant capacity of plasma (PAC) was measured using a commercial kit (NX2332, Randox Laboratories LTD, Crumlin, UK). This assay is based on the ability of 2,2′-azino-di-(3-ethylbenzothiazoline sulfonate) (ABTS) to produce the radical cation $ABTS^{\circ+}$ when incubated with peroxidase and hydrogen peroxide, producing a relatively stable blue–green color of which absorbance is measured at 600 nm. Antioxidants in the added plasma cause suppression of this color production to a degree proportional to their concentration. Trolox standard (1 mmol/L) is used as standard and plasma antioxidant power is expressed as Trolox equivalent (Miller et al., 1993).

Liver was homogenized in ice cold 0.1 mol/L potassium phosphate buffer (pH 7.4) and the homogenate was spun at 4000 rpm for 20 min at 4 °C. Superoxide dismutase (SOD) activity was assayed from the supernatant on an automat Pentra 400 (HORIBA ABX, Montpellier, France) using a commercial kit (Ransod kit No. SD 125, Randox Laboratories LTD, Crumlin, UK). For pro-inflammatory cytokine determination, liver tissue was homogenized in 10 mM Tris buffer (pH 7.4) containing 2 M NaCl, 1 mM EDTA, 0.01% Tween 80, 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 8500 rpm for 30 min at 4 °C. The resultant supernatant was used for cytokine determination. Liver tumour-necrosis-factor-alpha (TNFα) and interleukin-6 (IL-6) levels were quantified by ELISA using commercial kits from R&D Systems (Lille, France).

Liver and cardiac superoxide anion ($O_2^{\cdot-}$) production by NAD(P)H oxidase were evaluated by the intensity of lucigenin-enhanced chemiluminescence (10 μM lucigenin), measured with a luminometer (Perkin Elmer Wallac, Victor, Turku, Finland) on tissue homogenates, as previously described (Sutra et al., 2007). Results were expressed as relative light units (RLU/mg protein). The index of lipid peroxidation (MDA) was determined spectrofluorimetrically from the liver homogenate by measuring the condensation product formed of malondialdehyde (MDA) and 2-thiobarbituric acid, as described by Yagi (1984). Protein content of tissues was determined by using a commercial protein assay (Sigma, Saint Quentin Fallavier, France) according to the method of Smith et al. (1985) and using bovine serum albumin as standard.

2.5. Statistics

Data are shown as the mean ± SEM from 16 measurements. Statistical analysis of the data was carried out using the Stat View IV software (Abacus Concepts, Berkeley, CA) by one-way ANOVA followed by Fisher's protected least significant difference test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Food intake and body weight

Food intake and body weight gain were significantly lower in AgNPs-fed rats (∼18% and 20% respectively) (Table 1) while HDL-cholesterol was unchanged ($p = 0.0931$). No significant organ-weight changes were observed after 81 days (Table 2).

3.2. Plasma analysis

Rats treated with AgNPs produced a significant increase (9.5%, $p = 0.0133$) in plasma total cholesterol and LDL-cholesterol (30%, $p = 0.0028$) concentrations compared with those in Control group (Table 3). In contrast, triglyceride levels were strongly reduced (41%, $p = 0.0003$).

In the plasma, PON is localized in the HDL fraction. The AgNPs intake (Experimental group) reduced the ratio of PON activity to HDL by about 15% ($p = 0.0293$) in comparison to the standard group (Table 3). When expressed as Units/mL of plasma, this drop is similar (16%) but more significantly different ($p = 0.0032$). Supplying silver nanoparticles led to a rise in plasma ALT activity (12%, $p = 0.0390$) while plasma antioxidant capacity (PAC) was not modified.

3.3. Cardiac and liver biomarkers of oxidative stress

In the liver, $O_2^{\circ-}$ production increased 30% in Experimentals when compared to Controls ($p = 0.0067$), and 41% in the heart ($p = 0.0265$) (Fig. 1). However, liver malondialdehyde levels (MDA) and superoxide dismutase activity (SOD) did not change.

3.4. Liver inflammation

The concentrations of IL-6 and TNFα in the liver of Experimental rats were significantly increased by 12% ($p = 0.0344$) and 9% ($p = 0.0001$) respectively, in comparison with concentrations in Control rats (Fig. 2).

4. Discussion

The antibacterial effect of silver nanoparticles has resulted in their application in areas such as health, electronics and food. The increased use leads to an increase of the exposure of consumers and professionals raises questions in terms of risks to safety. However, the effects of AgNPs on humans are still poorly understood. Found in the literature mostly studies *in vitro* toxicity and inhalation or intravenous, subcutaneous and intraperitoneally silver nanoparticles. It has been demonstrated that AgNPs cause cytotoxicity *in vitro* on macrophages and generate free radical production (Schins and Knaapen, 2007). A size-dependent toxicity was produced by AgNPs (Carlson et al., 2008). Indeed, tests on the size of the nanoparticle (20, 80, 113 nm) revealed a higher toxicity for sizes lowest (Park et al., 2011).

One could wonder if significant aggregation/agglomeration of Collargol suspension happens during feeding, since we only sonicated the Collargol for 60 s in water. However, in any case, silver nanoparticles can be aggregated (irreversible bound of nanoparticles) in the stomach, and generally, the smaller sized AgNPs showed higher rates of aggregation and physical transformation than larger particles (Mwilu et al., 2013). This works also suggests that the larger particles and more durable particle coatings may survive transit through the stomach. Obviously, we have not been able to perform such analysis at the very high concentrations we used for feeding. Moreover, it should be kept in mind that too much sonication could induce the formation of free radicals that could influence the oxidative status of the animals.

Regarding the distribution in the body, the nanoparticles were found in all tissues after inhalation (Sung et al., 2009), injection (Xue et al., 2012) or gavage (Kim et al., 2008) with higher levels in the liver and lungs after inhalation and in the liver and kidneys after injection or gavage. Oral administration is a relevant route of exposure due to the use of AgNPs in products related to materials in contact with food. It is known that NPs can cross the intestinal barrier not only *via* M-cells in the Peyer's patches but also *via* enterocytes (Florence, 1997). But there are few studies on the harmful effects after oral administration and free radical production in an *in vivo* model. An oral toxicity study of silver

Table 2
Organ weights (as % body weight) of rats at the end of the experimental period. Control rats received daily water by gavage while experimental rats received Collargol at 500 mg/kg body weight.

	Control	Experimental
Liver	2.55 ± 0.06[a]	2.52 ± 0.05[a]
Heart	0.30 ± 0.06[a]	0.31 ± 0.04[a]
Spleen	0.24 ± 0.09[a]	0.26 ± 0.01[a]
Kidneys	0.61 ± 0.02[a]	0.63 ± 0.01[a]

Data are expressed as mean values ± SEM ($n = 16$). Mean values in a row with different superscripts are significantly different at $P < 0.05$.

Table 3
Effect of feeding a standard diet (Control) or a standard diet plus silver nanoparticles (Experimental) for 81 days on plasma lipids and alanine aminotransferase activity (ALT), plasma antioxidant markers, liver superoxide dismutase activity (SOD) and liver malondialdehyde (MDA)[A].

	Control	Experimental
Plasma lipids		
Total cholesterol (mmol/L)	2.11 ± 0.06[a]	2.31 ± 0.05[b]
HDL-cholesterol (mmol/L)	1.16 ± 0.04[a]	1.07 ± 0.04[a]
LDL-cholesterol (mmol/L)	0.95 ± 0.07[a]	1.24 ± 0.06[b]
LDL-C/HDL-C ratio	0.84 ± 0.08[a]	1.21 ± 0.09[b]
Triglycerides (mmol/L)	0.93 ± 0.07[a]	0.55 ± 0.05[b]
ALT (U/L)	35.7 ± 1.2[a]	40.0 ± 1.6[b]
Plasma antioxidants		
Paraoxonase (U/mL)	62.5 ± 1.5[a]	52.5 ± 2.9[b]
Paraoxonase/HDL-C	55.6 ± 2.0[a]	47.4 ± 3.1[b]
PAC (mmol/L)[B]	0.78 ± 0.02[a]	0.75 ± 0.0[a]
SOD (U/mg protein)	18.4 ± 1.3[a]	16.0 ± 0.9[a]
MDA (μM)	1.5 ± 0.1[a]	1.8 ± 0.1[a]

[A] Values are the means ± SEM ($n = 16$). For each dietary treatment, mean values in a column with different superscript letters significantly differ, $p < 0.05$.
[B] Plasma antioxidant capacity.

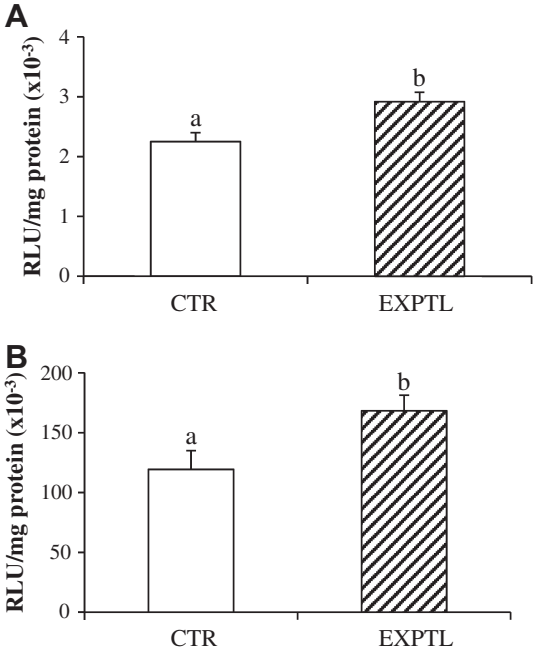

Fig. 1. Liver (A) and cardiac (B) superoxide anion production in rats fed with a standard diet (CTR) or a standard diet plus silver nanoparticles (EXPTL) at 500 mg/d/kg BW for 81 days. Values are expressed as mean ± SEM ($n = 16$). For each group, bars with different index letters differ ($p < 0.05$).

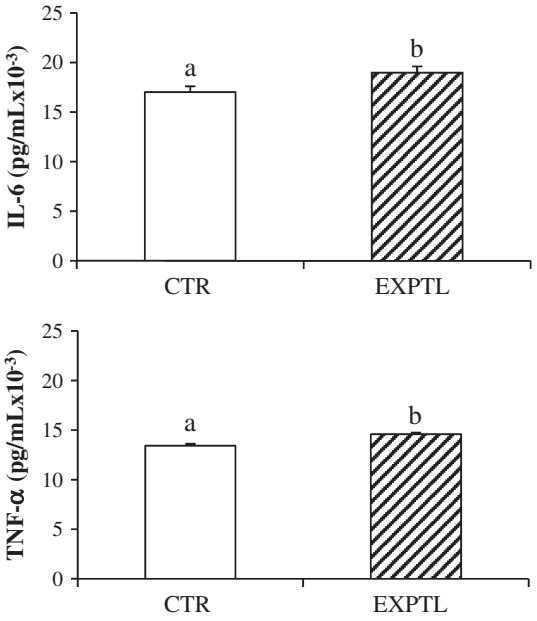

Fig. 2. Liver pro-inflammatory cytokine levels (IL-6 and TNF-α) expressed as pg/mL homogenate, in rats fed with a standard diet (CTR) or a standard diet plus silver nanoparticles (EXPTL) at 500 mg/d/kg BW for 81 days. Values are expressed as mean ± SEM ($n = 16$). For each group, bars with different index letters differ ($p < 0.05$).

nanoparticles at doses of 30 mg/kg, 300 mg/kg and 1000 mg/kg was conducted for 28 days in rats (Kim et al., 2008). Exposure to over 300 mg/kg resulted in alterations in lipid metabolism and tissue damage particularly on target organs such as the liver. To determine whether oxidative stress is the cause of the tissue damage, we conducted a research on 81-days orally-provided silver nanoparticles (20 nm) in Sprague–Dawley rats. We administered a daily dose of 500 mg/kg of silver nanoparticles to better visualize a potential deleterious effect while Kim et al. (2010) used an exposure time of 90 days. Nutritional parameters including food consumption and body weight decreased after the experiment. Ingestion of nanoparticle appears to reduce appetite and weight gain. These results are different from those obtained by Kim et al. (2010) where only a decrease in body weight was observed. This can be explained by the smaller size of AgNPs used in our study (20 nm) compared to those used (56 nm) by Kim et al. (2010). However, it is generally well known that toxicity of nanoparticles depends on many factors including size, shape, chemical composition, surface area, surface charge, and others. On the other hand, in our study and that of Kim et al. (2008), plasma total cholesterol level rises. This increase is due to an increase of cholesterol carried by LDL since HDL cholesterol was unchanged. Here, we also observed a decrease in plasma triglyceride level as reprorted by Kim et al. (2008), although these authors did not show a significant decrease compared with controls. Changes in levels of total cholesterol and triglycerides are difficult to explain because usually both vary in the same direction. Alanine aminotransferase (ALT) is a cytoplasmic in location and only largely released into circulation after hepatocyte structural integrity damage, thus, its activity is most commonly used as reliable marker for clinical monitoring of liver injury or liver function (Recknagel et al., 1989). Here, the activity of ALT increases in plasma signing an hepatic harmful effect which could partly explain the observed dyslipidemia. The liver is affected by the action of nanoparticles and cannot properly regulate the metabolism of triglycerides and cholesterol. This defense system is not only disturbed by increasing hepatic cytolysis, but also by the fall of paraoxonase activity. Indeed, serum paraoxonase (PON) is a glycoprotein of 43–45 kDa, synthesized primarily by the liver that circulates in the serum in conjunction with high-density lipoprotein (HDL) (Shamir et al., 2005). PON is a calcium-dependent esterase that hydrolyzes a wide range of substrates, particularly oxidized lipids (Aviram et al., 2000) and reduces oxidative stress in atherosclerotic lesions (Mackness et al., 2000). PON also protects against HDL oxidation of nanoparticles (Shamir et al., 2005). Its activity is a marker of oxidative stress. Here, the decreased PON activity in rats receiving the AgNPs is supported by increased production of superoxide anion, i.e. an activity of liver NADPH oxidase. While the antioxidant capacity of plasma (CAOP) does not vary, this is not contradictory variations of PON and NADPH oxidase, since CAOP represents the activity of plasma antioxidants such as vitamins E and C, glutathione or Coenzyme Q key. After 81 days, the oxidative stress observed could be at the level of initiation and the metabolism of these antioxidants is not too disturbed. This may also explain the SOD hepatic activities. Our results suggest that oxidative stress stimulated by silver nanoparticles is an important factor in their harmfulness. Inflammation is a physiologic response to an aggression, which can be not only a microorganism but also any soluble or not soluble product. Inflammatory reaction goes through different phases where macrophages play an important role. Here, silver nanoparticles that are highly reactive molecules have been introduced into body and cells of rats at high dose, and Kim et al. (2008) showed infiltration of inflammatory cells in the liver of rats receiving silver nanoparticles for 28 days. In such a case, macrophages are no longer able to treat them and can release free radicals (reactive oxigen species/ROS such as $O_2^{\circ-}$, HO°, H_2O_2,...) in the body causing exacerbated inflammation. Thus, inflammatory mediators such as IL-6 and TNFα are released and involved in the initiation of the inflammation. The repeated-dose toxicity of AgNPs (42 nm) has been also investigated in mice by oral administration for 28 days (Park et al., 2010). By the administration of AgNPs (1.00 mg/kg), adverse impacts on liver and kidney were observed and cytokines including IL-6 were also increased by repeated oral administration, suggesting that repeated oral administration of nano-sized AgNPs may cause organ toxicity and inflammatory responses in mice. If the ROS production is too high so that the defence systems are overpassed, cells are exposed to an oxidative stress which maintains the inflammatory state. The oxidative machinery and inflammatory signaling are not only interrelated, but their impairment can lead to a higher risk of tissues damage, cardiovascular diseases and associated features as emphasized here by cardiac oxidative stress. Similarly, the expression of genes related to oxidative stress in the mouse brain (Rahman et al., 2009) showed that silver nanoparticles (25 nm) administered intraperitoneally produce neurotoxicity by generating free radicals leading to oxidative stress. However, some studies showed no toxic effect of AgNPs *in vivo*. Rats exposed for 28 days showed no change in body weight and hematology values (Kim et al., 2008). The method of preparation of NPs influence their toxicity. Indeed, the potential toxicity of the NPs depends on their surface characteristics (Donaldson et al., 2001).

However, several considerations must be added. Indeed, here, we used a standard diet, i.e. no nutritional imbalance was induced, and therefore the system is not likely to cause or enhance an oxidative stress or an high inflammatory condition under the action of NPs. To do this, a high-fat diet would have been used, which generally favors the induction of oxidative stress and inflammation (Décordé et al., 2009; Romain et al., 2012). This partly explains, for example, small changes in PON activity or pro-inflammatory cytokines, or no change in the activity of SOD and MDA level. The effects observed are attributable only to silver nanoparticles and not influenced by food. It must be kept in mind that oral route exposure may be important in many consumer products such as

toothpaste, reusable bottles, food packing material, nursing nipples, kitchen utensils, and toys (Chen and Schluesener, 2008; Edwards-Jones, 2009).

5. Conclusion

Finally, we demonstrated that *in vivo*, orally delivered silver nanoparticles induced deleterious effects that target liver and heart and led to oxidative stress and inflammation. Further studies are in progress to investigate whether AgNPs are absorbed as an entity or not.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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